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Words from the Editor-in-Chief

It gives me immense pleasure to present to you **Volume 39 of the Nepalese Veterinary Journal (NVJ)**. As the official publication of the Nepal Veterinary Association, NVJ continues to uphold its commitment to advancing veterinary science through the dissemination of high-quality, peer-reviewed research.

This issue showcases a diverse array of articles encompassing original research, case studies and short communications that reflect the growing scope and complexity of veterinary medicine in Nepal and beyond. Topics range from livestock health and epidemiology to molecular diagnostics and public health, highlighting the multidisciplinary nature of our profession.

The veterinary sector plays a critical role not only in animal health and welfare but also in food security, environmental sustainability, and One Health initiatives. The articles featured in this edition emphasize the importance of evidence-based approaches, innovation, and collaboration in tackling both emerging and endemic challenges.

I would like to express my sincere gratitude to all the authors for their valuable contributions, the peer reviewers for their time and expertise, and the editorial board for their continued dedication. Special thanks also go to the Nepal Veterinary Association for its unwavering support in maintaining the standards and relevance of the journal.

As we publish this 39th issue, I encourage all veterinary professionals, researchers, and students to actively engage with the content and contribute to future volumes. Your insights, observations, and experiences are vital to the continued growth of our profession.

Despite our best efforts, there may be inadvertent errors or oversights in this issue. For that, I extend my sincere apologies. We are continuously striving to improve, and I kindly invite constructive feedback from our readers to help us enhance the quality and impact of future issues.

Thank you for your continued support and engagement. We hope you find this issue informative and thought-provoking.

Editor-in-Chief
Narayan Paudyal, PhD

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Original Research

Reproductive Performance of Buffaloes at Two Different Locations in Inner Terai Region of Nepal

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ABSTRACT

The objectives of the present study were to understand the reproductive performance of buffaloes at two different locations in inner terai region of Nepal and to determine some factors affecting their reproductive performance. Murrah-cross buffaloes from Chitwan (n=200) and Surkhet (n=200) were used for this study. Data regarding parity, management practices, deworming history, various reproductive parameters, weaning period etc. were collected through face to face interview with the farm owners. Individual buffalo was assessed for body condition score (BCS) using 1-5 scale with 0.25 increment. Age at first estrus and age at first calving were similar between two locations. Overall, the age at first estrus and age at first calving were 31.16 ± 0.74 and 45.12 ± 0.72 months, respectively. However, the calving to conception interval (days), number of services per conception and inter-calving interval (days) were significantly lower (better) in Chitwan (193.58 ± 7.24 , 1.19 ± 0.02 and 504.43 ± 7.22 , respectively) as compared to that in Surkhet (209.25 ± 8.57 , 1.32 ± 0.02 and 522.64 ± 8.66 , respectively). The proportion of buffaloes that received concentrate feed and mineral mixture in addition to roughage was 80% in Chitwan and 46% in Surkhet. Likewise, the buffaloes in Chitwan had better ($P < 0.01$) BCS than in Surkhet. However, the days postpartum at weaning were fewer ($P < 0.01$) in Chitwan than in Surkhet. The number of services per conception was lower ($P < 0.01$) in the buffaloes that were dewormed within every six month as compared to that in buffaloes which were dewormed only occasionally or beyond six months interval. In conclusion, major reproductive performance parameters of Murrah-cross buffaloes were better in Chitwan than in Surkhet that might have been associated with better nutritional management in Chitwan as indicated by supplementation of concentrate feed and mineral mixture and the good BCS of the buffaloes. Finally, the reproductive performance of buffaloes was also affected by the frequency of deworming practice.

Keywords: Buffalo; Reproductive performance; BCS; Deworming; Nutritional management

INTRODUCTION

Buffalo is the most important livestock species in Nepalese livestock system. Buffalo in Nepal is used for multi-purposes - for milk, meat and manure production as well as for draft purpose. The total population of buffalo in Nepal is 5,257,591 and that of cattle is 7,458,885 in the year 2019/20 (MoALD, 2022). Despite being less in number than cattle, the buffalo provides far more contribution (57.2%) than cattle in terms of total annual milk production in the nation. Likewise, as compared to other species, the buffalo contributes the most (i.e. 36.1%) in terms of total meat production in the nation (MoALD, 2022). As the buffalo can excel under various climatic conditions and geographic regions ranging from tropical to temperate climate, buffalo has been rearing by the farmers of both hilly and Terai regions mostly at subsistence level. Almost all buffaloes in Nepal are riverine type (*Bubalus bubalis*) except a small population of swamp buffalo limited in wild form in Koshi Tappu Wildlife Reserves (Wahid and Roshina, 2011). Southern plain of the nation is the major buffalo production area having approximately 35% of the total buffalo population, with mostly the Indian Murrah breed or their crosses to local breeds (Neopane, 2006).

Buffalo is regarded as a slow/sluggish breeder since history because of delayed sexual maturity, long post-partum anestrus period, prolonged inter-calving intervals, weak estrus expression or silent heat, irregular ovulation, summer anestrus and higher incidence of embryonic and fetal deaths (Singh et al., 2000; Kumar et al., 2014; Devkota et al. 2023; Gautam et al. 2024). Such reproductive traits of buffaloes vary due to genetic and non-genetic factors which again are influenced by the geographical location, its climatic conditions and various management factors (Ramadan, 2018; Parmer et al., 2019; Paneru et al., 2021). Thus, it would be of great interest to know how the different geographical locations in inner terai region of Nepal would affect the reproductive performance of buffaloes. Although Chitwan and Surkhet both lie in the inner terai region of Nepal, their geographical and climatic variations [For example, Chitwan has more intense hot summer than Surkhet (<https://tckctck.org>)] as well as differences in nutritional, health and other management aspects may affect the overall reproductive performance of buffaloes. Furthermore, although there were some previous studies that documented the reproductive performance of indigenous breeds of buffaloes in Nepal (Pokharel et al., 1998; Amatya et al., 2000; Neopane et al., 2007; Pokharel et al., 2012; Shah et al., 2016; Paneru et al., 2021), to our knowledge, there was limited information regarding reproductive performance of Murrah-cross buffaloes in Nepalese context. Therefore, the objectives of the present study were to understand the reproductive performance of buffaloes at two different locations in inner terai region of Nepal and to determine some factors affecting their reproductive performance.

MATERIALS AND METHODS

Study area

This study was conducted in buffaloes of two different locations namely: plain areas of Chitwan and Surkhet districts (Fig. 1). Both locations lie in the inner terai region of Nepal under subtropical climatic condition. Although both locations have hot humid summer and cool dry winter, Surkhet is slightly cooler than Chitwan throughout the year (<https://tckctck.org>).

Chitwan district, located between 27°35' N and 84°30' E, lies in Bagmati Province, south central part of Nepal. Extended at an elevation from 110m to 850m m above sea level (<https://chitwannationalpark.gov.np>), Chitwan has annual average temperature 29.3°C that ranged from minimum 11.36°C in winter (January) to maximum 40.31°C in the summer (May). District headquarter Bharatpur typically receives about 252.9 mm of precipitation and has 182 rainy days annually (<https://tckctck.org/nepal/narayani/bharatpur>).



Fig. 1. Map of Nepal showing study area (Chitwan and Surkhet)

Surkhet district, located between 28°31' N and 81°46' E, lies in Karnali province, south western part of Nepal. Extended at an elevation from 198 m to 2347 m above sea level (Helvetas Nepal), Surkhet has annual average temperature 24.1°C that ranged from minimum 5.47°C in winter (January) to maximum 36.65°C in the summer (May). District headquarter Birendranagar typically receives about 186.6 mm of precipitation and has 153.6 rainy days annually (<https://tckctck.org/nepal/bheri/birendranagar>).

There were 39,818 buffalo rearing households with total 77,045 buffaloes in Chitwan district and 14,527 households with total 24,958 buffaloes in Surkhet (MoLD, 2017). Most of the buffaloes at plain areas of both districts were Murrah and their crossbred, although the exact blood level was not known.

Animals

Total 400 Murrah and their crossbred buffaloes (200 from each of Chitwan and Surkhet district) that were calved at least once were used for this study. All the buffaloes in the study were naturally bred with the buffalo bull kept in the community. The number of farms surveyed and the number of buffaloes studied have been shown in Table 1.

Table 1. Number of buffalo farms surveyed and number of buffaloes studied at two locations

Location	No. of buffalo farms surveyed		No. of buffaloes studied		
	Commercial farms	Household farms	From commercial farms	from household farms	Total
Chitwan	6	72	101	99	200
Surkhet	3	81	74	126	200

Data collection

A well-designed questionnaire was prepared and data regarding parity of the buffalo, management practices, deworming history, various reproductive parameters, calf sex, weaning period etc. were collected through face to face interview with the farm owners. Buffaloes from both the commercial farms and household farms were included in the study. Purposive sampling technique was used until the number of Murrah cross buffaloes that calved at least once reached 200 at each location with total of 400 animals.

Determination of body condition score (BCS)

Individual buffalo was visually assessed for BCS using 1-5 scale with 0.25 increment as described for Holstein cows (Edmonson et al., 1989).

Statistical analysis

Data entry was done in MS excel 2019 and statistical analyses were done using SPSS version 25. Age at first estrus, age at first calving, calving to conception interval, number of services per conception and inter-calving interval were calculated using descriptive statistical tools and expressed as mean $\pm$ SE. These reproductive parameters between two locations were compared using z-test. Parity of buffaloes, BCS and days at weaning between two locations were also compared using z-test. Effect of deworming practice on number of services per conception, and effect of calf sex on calving to conception interval were also analyzed using z-test.

RESULTS

Reproductive performance parameters of buffaloes

Various reproductive parameters of buffaloes at two locations have been shown in Table 2. Among major reproductive parameters, age at first estrus and age at first calving were similar at both locations. However, the calving to conception interval, number of services per conception and inter-calving interval were significantly lower in Chitwan than in Surkhet (Table 2).

Table 2. Major reproductive parameters of buffaloes at two different locations in subtropical region of Nepal

Reproductive parameters (mean±SE)	Overall	Location		P-value
		Chitwan	Surkhet	
Age at first estrus, months	31.16±0.74 (n=114)	31.13±0.84 (n=36)	31.07±0.65 (n=78)	0.87
Age at first calving, months	45.12±0.95 (n=114)	45.38±1.09 (n=36)	44.86±0.81 (n=78)	0.91
Calving to conception interval, days	206.35±8.69 (n=400)	192.74±7.32 (n=200)	219.96±10.06 (n=192)	0.029
Number of services per conception	1.25±0.20 (n=400)	1.19±0.02 (n=200)	1.32±0.39 (n=200)	0.009
Inter-calving interval, days	518.48±8.69 (n=400)	503.98 ±7.30 (n=200)	532.98±10.08 (n=200)	0.018

Practices of supplementing concentrate feed and minerals to the buffaloes at two locations

It was found that 80% of buffaloes in Chitwan were provided with concentrate feeds and mineral mixtures in addition to roughages, whereas in Surkhet only 46% buffaloes received concentrate feed and mineral mixtures (Fig. 2).

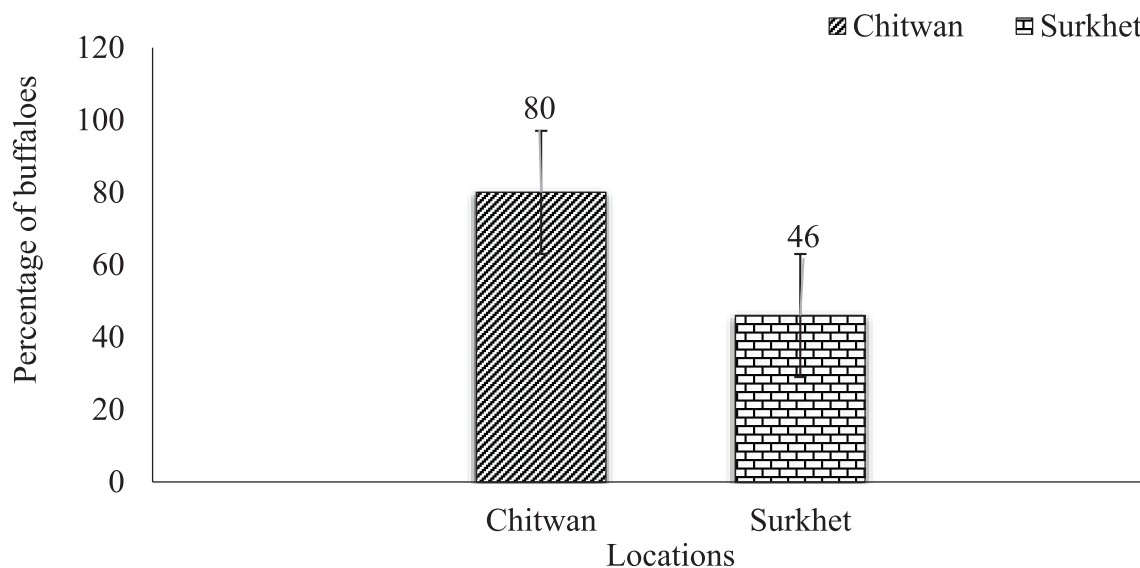


Fig. 2. Proportions of buffaloes receiving concentrate feed and mineral mixtures at two locations

Parity, BCS and days postpartum at weaning of buffaloes at two locations

Average (mean $\pm$ SE) parity, BCS and days postpartum (pp) at weaning of buffaloes at two locations have been shown in Table 3. It was found that the buffaloes in Chitwan were of significantly higher parities (not necessarily the age) than in Surkhet. Likewise, the buffaloes in Chitwan had significantly better BCS than in Surkhet. However, the days pp at weaning were significantly fewer in Chitwan than in Surkhet.

Table 3. Parity, BCS and days postpartum at weaning of buffaloes at two locations

Factors (Mean $\pm$ SE)	Locations		P-value
	Chitwan	Surkhet	
Parity	4.09 $\pm$ 0.12	3.40 $\pm$ 0.12	0.0001
BCS	3.11 $\pm$ 0.23	2.40 $\pm$ 0.27	0.0001
Days pp at weaning	91.03 $\pm$ 4.2	136 $\pm$ 6.04	0.0001

Effect of deworming practice on reproductive efficiency of buffaloes

Among 400 buffaloes, 45.8% were dewormed against internal parasites every six months or less, whereas 54.2% were dewormed only occasionally or beyond six months. It was found that number of services per conception was significantly higher in buffaloes that were dewormed only occasionally or beyond six months as compared to that in buffaloes dewormed within every six months (Table 4).

Table 4. Effect of deworming practice on number of services per conception in buffaloes

Deworming frequency	Number of services per conception	P-value
Within every six months (n=183)	1.12±0.02	0.001
More than six months (n=217)	1.28±0.04	

Effect of calf sex on the calving to conception interval

Information about the sex of calf was available only from 355 buffaloes. There was no any case of twinning. Among 355 buffaloes, the sex ratio (male:female) was 46:54. Calving to conception interval was not affected by the sex of the calf born (Table 5).

Table 5. Calving to conception interval in buffaloes with male versus female calves

No. of buffaloes with:	Calving to conception interval (Mean±SE)	P-value
Male calf (n=162)	211.69±11.54	0.44
Female calf (n=193)	200.84±8.60	

DISCUSSION

Since there was limited information regarding the reproductive performance of Murrah-cross buffaloes in southern plain of Nepal, this study tried to understand the same at two different locations (Chitwan and Surkhet) in inner terai region of Nepal. Phenotypically buffaloes included in this study at both locations were Murrah crossbred though the exact blood level was not known. Although both locations were in inner terai region having subtropical climatic conditions, Surkhet was slightly cooler than Chitwan (<https://tekctek.org>). Besides the obvious differences in various climatic conditions, the present study also tried to identify differences in some non-genetic factors between two locations that could have influenced the reproductive performances of buffaloes.

Among various reproductive performance parameters, the age at first estrus and age at first calving were similar at both locations. The overall age at first estrus of Murrah-cross buffalo in the present study (31 months) was lower than that of Nepalese indigenous breeds Lime [37.8 months reported by Paneru et al. (2021) and 51.6 months reported by Pokharel et al. (2012)] and Parkote [37.68 months reported by Paneru et al. (2021) and 51.8 months reported by Pokharel et al. (2012)]. Likewise, the age at first calving in the present study (45 months) was also lower than that of Lime (49.7 to 61.2 months) and Parkote (49.8 to 62.2 months) as reported by previous studies (Pokharel et al., 2012; Paneru et al., 2021). These findings suggest that the Murrah-cross buffaloes in southern plain of Nepal reach to puberty and calve at earlier age than the indigenous breeds of buffaloes. However, both the age at first estrus and age at first calving of Murrah-cross buffaloes in the present study were almost similar to that of Murrah breed in India (Gupta et al., 2012) and Bangladesh (Harun-Or-Rashid et al., 2019).

Calving to conception interval, number of services per conception and inter-calving interval were lower (i.e. better) in Chitwan than in Surkhet. The variation in calving to conception interval and thereby the inter-calving interval can exist in different locations because of differences in husbandry practices like lactation length, dry period, temperature humidity index, season of calving (Buglio et al., 2000). Better reproductive performance of buffaloes in Chitwan might be due to better nutritional management as supported by significantly better body condition score (BCS) in Chitwan (3.11) than that in Surkhet (2.40); and also the proportion of buffaloes getting daily concentrate feed and mineral supplement was higher in Chitwan (80%) than that in Surkhet (46%). Also, the weaning of calf in Chitwan was done at earlier days postpartum (91 d pp) than that in Surkhet (136 d pp). Weaning at earlier days postpartum might have resumed postpartum ovarian cyclicity earlier because it has been shown that continuous nursing during lactation prevents ovulation, which results in a prolonged period of postpartum anestrus and lowers reproductive efficiency (Montiel & Ahuja, 2005).

The overall inter-calving interval of buffaloes in the present study (518 days) was almost similar to the findings in Murrah buffaloes in India (514 days) (Gupta et al., 2012) and even shorter than that in Bangladesh (550 days) (Harun-Or-Rashid et al., 2019). Similarly, the number of services per conception at both locations (1.19 and 1.32, respectively) as well as among all buffaloes (1.25) in the present study was lower than that in Murrah buffaloes in Bangladesh (1.38) (Harun-Or-Rashid et al., 2019). These findings suggest that the Murrah-cross buffalo in the inner terai region of Nepal have comparable reproductive performance with that in neighbouring countries.

The present study also examined the effect of deworming practice and calf sex on reproductive performance of buffaloes. It was found that the frequency of deworming practice significantly affected the number of services per conception; the buffaloes that were dewormed within every six month required significantly less number of services per conception than those that were dewormed only occasionally or beyond six months. It was assumed that the birth of male calf, because of its large size, would cause more calving difficulties and ultimately lead to long calving to conception interval in dam. However, the sex of the calf born had no significant effect on the calving to conception interval, which was similar to the findings of previous study in Nili-Ravi buffaloes in Pakistan (Chaudhary et al., 1990).

CONCLUSION

Major reproductive performance parameters of Murrah-cross buffaloes were better in Chitwan than in Surkhet, although the age at first estrus and age at first calving were similar at both locations. Better reproductive performance of buffaloes in Chitwan might have been associated with better nutritional management as indicated by supplementation of concentrate feed and mineral mixtures and the good BCS of the buffaloes. Finally, the number of services per conception was significantly lower in the buffaloes that were dewormed within every six month as compared to that in buffaloes which were dewormed

only occasionally or beyond six months interval.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Amatya, N., Rasaili, D.P. and Rana, R.S. (2000). Evaluation of phenotypic and production characteristics of indigenous buffalo types in the western hills of Nepal. *Lumle Technical Paper* No. 2000/001.
2. Bughio, S., Shahani, A.K., Mirani, A.H., Moriani, A. A., Oad, F. C. and Bughio, B.A. (2000). Seasonal Trend of Calving and Subsequent Service Period in Buffaloes. *Pakistan Journal of Biological Sciences*, **3**(12): 2169-2170.
3. Chaudhry, M.A., Chohan, M.H., Pasha, T.N. (1990). Uterine involution and postpartum oestrus in primiparous Nili-Ravi buffaloes. *Pakistan Journal of Agricultural Research*, **11**(3): 183-191.
4. Devkota, B., Shah, S. and Gautam, G. (2023). Reproduction and fertility of buffaloes in Nepal. *Animals*, **13**: Article 1. <https://doi.org/10.3390/ani13010070>
5. Edmonson, A. J., Lean, I. L., Weaver, L. D., Farver, T. and Webster, G. (1989). A body condition scoring chart for Holstein dairy cows. *Journal of Dairy Science*, **72**: 68-78.
6. Gautam, G., Rokaya, S., Devkota, B. and Sapkota, S. (2024). Seasonality of reproduction in buffaloes in subtropical regions of southern Nepal. *Advances in Animal and Veterinary Sciences*, **12**(5): 895-901. <https://dx.doi.org/10.17582/journal.aavs/2024/12.5.895.901>
7. Gupta, J. P., Sachdeva, G., Gandhi, R.S. and Cahkaravarty, A.K. (2012). Non-genetic factors influencing growth and production performance in Murrah Buffaloes. *The Indian Journal of Dairy Science*, **65**: 239-241.
8. Harun-Or-Rashid, M., Sarkar, A.K., Hasan, M.M.I., Hasan, M. and Juyena, N.S. (2019). Productive, reproductive, and estrus characteristics of different breeds of buffalo cows in Bangladesh. *Journal of Advanced Veterinary and Animal Research*, **6**(4): 553-560. <https://doi.org/10.5455/javar.2019.f382>.
9. Helvetas Nepal, 2014. A brief profile of Surkhet District. Helvetas Nepal, Kathmandu.
10. <https://docsdrive.com/pdfs/ansinet/pjbs/2000/2169-2170.pdf>
11. Kumar, P.R., Singh, S.K., Kharche, S.D., Chethan, S.G., Behera, B.K., Shukla, S.N., Kumar, H. and Agarwal, S.K. (2014). Anestrus in cattle and buffalo: Indian perspective. *Advance Animal and Veterinary Science*, **14**; **2**(3): 124-38.
12. MoALD (2022). Statistical Information on Nepalese Agriculture 2020/21. Ministry of Agriculture and Livestock Development, Government of Nepal, Kathmandu, Nepal, 2022: pp. 3-4.
13. MoLD (2017). Livestock Statistics of Nepal. Government of Nepal, Ministry of Livestock Development Planning, Monitoring and Evaluation Division, Singhadurbar, Kathmandu, Nepal.
14. Montiel, F. and Ahuja, C. (2005). Body condition and suckling as factors influencing the duration of postpartum anestrus in cattle: A review. *Animal Reproduction Science*, **85**(1-2):1-26. <https://doi.org/10.1016/j.anireprosci.2003.11.001>.
15. Neopane, S.P. (2006). Characterization of indigenous animal genetic resources of Nepal. Proceedings

- of the 6th National Workshop on Livestock and Fisheries Research, 1-2 July 2004, NARC, Pp 1-11, Khumaltar, Lalitpur, Nepal.
16. Neopane, S.P., Gorkhali, N.A. and Pokharel, P.K. (2007). Indigenous buffalo of Nepal. Animal Breeding Division. National Animal Science Research Institute. Nepal Agricultural Research Council.
17. Paneru, U., Dhungana, K.P., Kanu, S. and Sharma, P. (2021). Genetic and non-genetic factors on productive and reproductive performance of indigenous buffalo. *Journal of Nepal Agricultural Research Council*, **7**: 83-91.
18. Parmar, G.A., Gupta, J.P., Chaudhari, J.D., Pandey, D.P., Prajapati, B.M., Sathwara, R.N. and Patel, P.A. (2019). Study of genetic and non genetic factors affecting age at first calving and wet average in Mehsana buffaloes. *Buffalo Bulletin*, **38 (1)**: 11-17.
19. Pokharel, P.K.; Kuwar, B.S., Shrestha, N.P., Neopane, S.P. and Shrestha, H.R. (1998). Identification, characterization and conservation strategy of Gaddi buffalo. Proceeding of the Fourth Global Conference of Domestic Animal Genetic Resources. Nepal Agricultural Research Council, Khumaltar, Lalitpur and Rare Breeds International, UK (edited by J NB Shrestha). Birendra International Convention Center, Baneshwar, Kathmandu, Nepal 17-21 August, 1998; Pp 77-80.
20. Pokharel, P., Neopane, S.P., Sapkota, S. and Kandel, R. (2012). *Nepalka Raithane Gharpaluwa Janawarharu* (in Nepali language). Animal Breeding Division, Nepal Agricultural Research Council, Khumaltar, Lalitpur, Nepal.
21. Ramadan, S.I. (2018). Effect of some genetic and non-genetic factors on productive and reproductive traits of Egyptian buffaloes. *Journal of Advanced Veterinary and Animal Research*, **5(4)**: 374–380.
22. Sah, A.K., Jha, P.K., Ali, M.S. and Pathak, L.R. (2013). Seasonal effect on calving pattern of Murrah crossbred buffaloes at National Buffalo Research Programme, Tarahara, Sunsari, Nepal. In: *9th National Proceedings of Livestock and Fisheries Research, Nepal*. Nepal Agricultural Research Council.
23. Shah, M.K., Hayashi, Y. and Kumagai, H. (2016). Productive and reproductive performance of indigenous Lime and Parkote buffaloes in the western hills of Nepal. *Lifescience Global*, **5**: 14-17.
24. Singh, J., Nanda, A.S. and Adams, G.P. (2000). The reproductive pattern and efficiency of female buffaloes. *Animal Reproduction Science*, **60–61**: 593–604. [https://doi.org/10.1016/S0378-4320\(00\)00109-3](https://doi.org/10.1016/S0378-4320(00)00109-3).
25. Wahid, H. and Rosnina, Y. (2011). Husbandry of Dairy Animals Buffalo: Asia. *Encyclopedia of Dairy Science*, 772–779. <https://doi.org/10.1016/B978-0-12-374407-4.00229-6>.

Pyometra in Bitch and its Associated Risk Factor in Nepal

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ABSTRACT

Pyometra is a life-threatening disease in canine and feline. Early recognition, diagnosis and appropriate intervention is required to avoid disastrous consequences. Pyometra is the accumulation of pus within the uterus of intact diestrual bitches. The complex aetiological factors of pyometra are hormonal imbalance, the virulence of the infecting bacteria, immunity of bitch, and the individual sensitivity to bacterial and inflammatory agents. Hormonal therapy, nulliparity and age of bitch are the associated risk factors. The irrational use of the synthetic progestin medroxyprogesterone acetate (MPA) for estrus prevention in the dog has provoked the incidence of pyometra in bitch. In pyometra (62-90%) cases, Escherichia coli was isolated predominantly. History of a variety of symptoms associated with both genital and extragenital lesions. Common symptoms seen are dehydration, polydipsia, polyuria, lethargy, abdominal pain, anorexia, vomiting. The best diagnostic tool is ultrasonographic imaging. The choice of treatment for the majority of obstetricians is ovariohysterectomy. A questionnaire was prepared on the goggle form. 32 responses were collected. The data was entered and analyzed in Excel as the data were descriptive. A higher incidence of pyometra in bitches was found in the age group of 3 years to 5 years. The open type of pyometra was estimated to have a higher incidence on pluriparous bitches. Mongrel breed was found with greater prevalence than other breeds. A higher incidence of pyometra in owned bitches was found which was due to the irrational use of MPA to prevent estrus in bitches. Avoiding misuse of hormones, nutritional management, and routine checkup will control the incidence of pyometra.

Keywords: Diestrus; Medroxyprogesterone acetate; Nulliparous; Ovariohysterectomy; Ultrasonography

INTRODUCTION

Pyometra, a critical uterine disorder affecting intact diestrual bitches, poses a significant threat to canine health and welfare. Characterized by the accumulation of pus within the uterus, this life-threatening condition demands timely recognition, accurate diagnosis,

and appropriate intervention to avoid disastrous consequences. In 1998, a dog census in Nepal was carried out by the National Zoonoses and Food Hygiene Research Center (NZFHRC), where the number of dogs populations in Nepal was estimated to be nearly 2 million which was 1 dog per 10 human population (Pantha et al., 2020). The population density of dog was 2,930 stray dogs/km² and the ratio of stray dogs to humans was 1:4.7 in Kathamandu in 1997 (Kato et al., 2003).

Common uterine diseases in intact dogs are cystic endometrial hyperplasia (CEH), mucometra, and pyometra. Pyometra is a life-threatening disease in canine and feline since early recognition, diagnosis and appropriate intervention is required to avoid disastrous consequences. Pyometra is the accumulation of pus within the uterus of intact diestrual bitches (Pretzer, 2008). The complex etiological factors of pyometra are hormonal imbalance, the virulence of the infecting bacteria, immunity of bitch, and the individual sensitivity to bacterial and inflammatory agents (Hagman, 2004). Hormonal therapy, nulliparity and age of bitch are the associated risk factors (Bousquet, 1997) whereas for contrast overt pseudopregnancy might be a protective factor (Fidler et al., 1966). The irrational use of the synthetic progestin medroxyprogesterone acetate (MPA) for estrus prevention in the dog has provoked the incidence of pyometra in bitch. Overproduction of growth hormone, suppression of plasma glucocorticoid levels, and the induction of mammary tumors are the results due to administration of MPA (Selman, Wolfswinkel, & Mol, 1996). Based on status of cervix, pyometra can be categorized as open-cervix or closed-cervix. To prevent bitch from worsening condition (septicemia and death) in closed pyometra, OVH must be done (Smith, 2006).

In pyometra (62-90%) cases, *Escherichia coli* was isolated predominantly (Coggan et al., 2008) During proestrus and estrus, *E. coli* gain entrance to the uterus as they are natural inhabitants of the vaginal (Watts, Wright, & Whithear, 1996). Other bacteria isolated are *Klebsiella* spp, *Staphylococcus* spp., *Streptococcus* spp., *Pseudomonas* spp., *Salmonella* spp. (Coggan et al., 2008). The progesterone-sensitized uterus is suitable not only for pregnancy but also for bacterial infection since progesterone stimulates endometrial gland growth and secretion, as well as cervical closure and suppression of myometrial contractions (Fransson & Ragle, 2003).

History of a variety of symptoms associated with both genital and extragenital lesions. In open pyometra, a purulent vaginal discharge is often present. Common symptoms seen are dehydration, polydipsia, polyuria, lethargy, abdominal pain, anorexia, vomiting or diarrhea, fever or hypothermia, abnormal color of the mucous membranes and elevated heart- and respiratory rates (Børresen, 1979). The clinical course of the disease varies from prolonged development of chronic uterine inflammation to endotoxic shock (Hardy & Osborne, 1974).

The most common findings in all the bitches affected with pyometra are normocytic and normochromic anemia, leukocytosis, a predominant absolute neutrophilia, lymphopenia, and monocytosis. Increased BUN, creatinine and hyperproteinemia are observed in

pyometra. Hematological and biochemical parameters are altered markedly in closed pyometra (Shah et al., 2017). Treatment can be done by the administration of third generation GnRH antagonist acyline @ 330 µg/ kg orally (single dose) with amoxicillin-clavulanate @ 12.5 mg/kg twice a day, orally for seven days (Batista et al., 2016). The best diagnostic tool is ultrasonographic imaging. An enlarged uterus is observed with hypoechoic tubular organ containing echogenic fluid (Patel et al., 2013) however, sometimes pus shows a slow, whirlpool-like pattern (Bigliardi et al., 2004). The choice of treatment for the majority of obstetricians is spaying. The most effective method over the conventional open method is Laparoscopic Assisted Ovariohysterectomy (LAOVH) for treatment of select cases of canine pyometra with careful case selection in order to improve the success rate (Becher-Deichsel et al., 2016).

With this in-depth analysis of pyometra in dogs, our study aims to provide veterinary practitioners and researchers with critical insights into the epidemiology, and treatment options for this complex and life-threatening condition, ultimately contributing to the improvement of canine health and welfare.

MATERIALS AND METHODOS

A questionnaire was prepared on the goggle form on July 15, 2021 and veterinarians, pet practitioners, veterinary officers from all over Nepal were asked to fill the form. 32 responses were collected. The respondents were private pet practitioners (veterinarians), private clinics and hospital, government veterinary officers from Veterinary Hospital and Livestock Service Expert Center (VHLSEC) and Central Referral Veterinary Hospital (CRVH), veterinary clinician from veterinary teaching hospital of TU, AFU and other institutes and veterinarians from INGOs and NGOs like HART, Animal Nepal etc. The data was entered in MS Excel and analyzed through excel.

RESULTS

Age group of bitches

Dogs in the age group of 3 years to 5 years were diagnosed with pyometra more frequently than dogs in other age groups. Dogs in the age group above 11 years weren't diagnosed with pyometra yet.

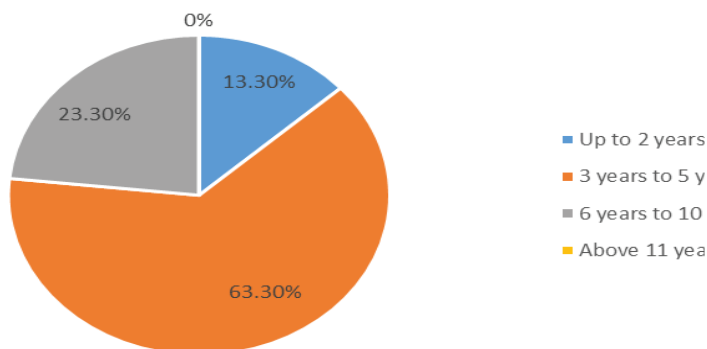


Fig. 1. Age group of dogs diagnosed with pyometra

Housing and parity of dog along with type of pyometra

Incidence of pyometra was high in the owned dog. Bitches in pluriparous had high incidence followed by primiparous and nulliparous. The open type of pyometra was found high than the closed type.

Table 1. Incidence of pyometra according to housing and parity of dog along with type of pyometra

Type of Pyometra	Percentage %	Housing of dog	Percentage %	Parity of Dog	Percentage %
Open	56.67	Owned	80	Nulliparous	20
Closed	43.33	Community	20	Primiparous	20
				Pluriparous	60

Factors that are associated with anamnesis of pyometra

Of the total cases, 86.67% of pyometra in bitches are due to injection of Medroxyprogesterone acetate followed by others (Nutritional, individual immunity, pseudopregnancy) and hormonal imbalance during pregnancy.

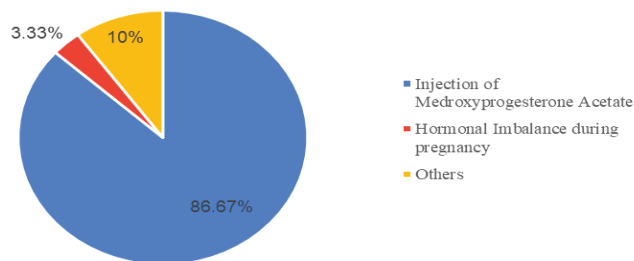


Fig. 2. Incidence of pyometra due to various factors

Breed of dog

Higher incidence (53.33%) of pyometra was found in Mongrel followed by Japanese Spitz (36.67%) and others (10%) which include Collies, Labrador, German shepherd, etc.

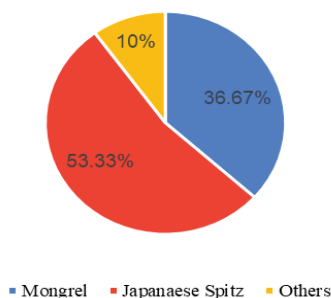


Fig. 3. Incidence of pyometra according to the breed of bitches

Treatment protocol in open and closed pyometra

In open pyometra 50% fluid found was purulent followed by mucopurulent (36.67%) and serosanguinous (13.33%). 56.67% of total cases of open pyometra, the treatment protocol preferred was pre-antibiotic, ovariohysterectomy (OVH), and post-antibiotic therapy while the same percentage of closed pyometra had the preference of OVH and post-antibiotic as a treatment protocol. Drugs preferred in pyometra were the cephalosporin group (53.33%) followed by the penicillin group (40%), the aminoglycosides (3.33%), and tetracycline (3.33%).

Table 2. Preference percentage of treatment in open and closed pyometra

Open Pyometra		Closed Pyometra	
Treatment Protocol	Preference	Treatment Protocol	Preference
Pre-antibiotic+ OVH	3.33%	Pre-antibiotic+ OVH	3.33%
OVH+ Post-Antibiotic	40%	OVH+ Post-Antibiotic	56.67%
Pre-antibiotic+ OVH+ Post-antibiotic	56.67%	Pre-antibiotic+ OVH+ Post-antibiotic	40%

DISCUSSION

In our study dogs of age group, 3 years to 5 years were diagnosed with pyometra more frequently than other age groups which are also similar with (Antonov et al., 2015) on which the age of bitch with pyometra varied from 1 to 17years (mean 7.55 ± 3.45 years). An incidence of 2% in bitches greater than 10 years of age was found (Pretzer, 2008). In a group of colony-raised Beagles, the incidence of pyometra was 15.2% of bitches >4 years of age (Smith, 2006). The age of the bitches diagnosed with pyometra ranges from young as 4 months of age to 16 years old (Baithalu, 2010). The reason behind the age of the dog is the use of MPA to get rid of the dog in estrus.

In our study, bitches in pluriparous had high incidence followed by primiparous and nulliparous. A higher incidence of pyometra is found in nulliparous bitches (Smith, 2006). There was an association between nulliparity and pyometra (Hagman, 2017). The reasons behind the high incidence of pyometra in the pluriparous dog are due to nutritional deficiency and improper care and management after several years of care taking.

Higher incidence (53.33%) of pyometra was found in Mongrel followed by Japanese Spitz (36.67%) and others (10%) which include Collies, Labrador, and German shepherd in our study. Breed susceptibility was observed with high risk include Rottweiler, Saint Bernard, Chow chow, Golden Retriever, Miniature Schnauzer, Irish Terrier, Airedale Terrier, Cavalier King Charles Spaniel, Rough Collie, and Bernese Mountain dog (Smith, 2006). Some breeds are predisposed for pyometra such as Retriever, Terrier, Saint Bernard, Rottweiler dog (Antonov et al., 2015).

CONCLUSION

In Nepal, a higher incidence of pyometra in bitches was found in the age group of 3 years to 5 years. The open type of pyometra was estimated to have a higher incidence on pluriparous bitches. Mongrel breed was found with greater prevalence than other breeds that is due to the low population of other breeds in comparison to mongrel. A higher incidence of pyometra in owned bitches was found which was due to the irrational use of MPA to prevent estrus in bitches. Avoiding misuse of hormones, nutritional management, and routine checkup will control the incidence of pyometra. Proper use of antimicrobial agents after antimicrobial sensitivity test will control drug resistance and also help in the good prognosis of bitch suffering from pyometra. Further research, dog census, and investigation are needed to improve the status and condition of the dog in Nepal.

Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Antonov, A. L., Atanasov, A. S., Fasulkov, I. R., Georgiev, P. I., Yotov, S., Karadaev, M., & Vasilev, N. (2015). Influence of some factors on the incidence of pyometra in the bitch. *Bulgarian Journal of Veterinary Medicine*, 18(4), 367–372.
2. Batista, P. R., Gobello, C., Rube, A., Corrada, Y. A., Tórtora, M., & Blanco, P. G. (2016). Uterine blood flow evaluation in bitches suffering from cystic endometrial hyperplasia (CEH) and CEH-pyometra complex. *Theriogenology*, 85(7), 1258–1261. <https://doi.org/10.1016/j.theriogenology.2015.11.016>
3. Bigliardi, E., Parmigiani, E., Cavirani, S., Luppi, A., Bonati, L., & Corradi, A. (2004). Ultrasonography and cystic hyperplasia–pyometra complex in the bitch. *Reproduction in Domestic Animals*, 39(3), 136–140. <https://doi.org/10.1111/j.1439-0531.2004.00502.x>
4. Bögel, K., & Joshi, D. D. (1990). Accessibility of dog populations for rabies control in Kathmandu Valley, Nepal. *Bulletin of the World Health Organization*, 68(5), 611–617.
5. Børresen, B. (1979). Pyometra in the dog. II.--A pathophysiological investigation. II. Anamnestic, clinical and reproductive aspects. *Nordisk Veterinaermedicin*, 31(6), 251–257.
6. Coggan, J. A., Melville, P. A., Oliveira, C. M. D., Faustino, M., Moreno, A. M., & Benites, N. R. (2008). Microbiological and histopathological aspects of canine pyometra. *Brazilian Journal of Microbiology*, 39(3), 477–483. <https://doi.org/10.1590/S1517-83822008000300020>
7. Fidler, I. J., Brodey, R. S., Howson, A. E., & Cohen, D. (1966). Relationship of estrous irregularity, pseudopregnancy, and pregnancy to canine pyometra. *Journal of the American Veterinary Medical Association*, 149(8), 1043–1046.
8. Gupta, A. K., Dhami, A. J., Patel, S. B., & Shah, R. G. (2013). Evaluation of clinical biochemistry of blood in bitches affected with pyometra. *Indian Journal of Animal Reproduction*, 34, 26–30.
9. Hardy, R. M., & Osborne, C. A. (1974). Canine pyometra: Pathophysiology, diagnosis and treatment of uterine and extra uterine lesions. *Journal of the American Animal Hospital Association*, 10(3), 245–268.
10. Kato, M., Yamamoto, H., Inukai, Y., & Kira, S. (2003). Survey of the stray dog population and the health education program on the prevention of dog bites and dog-acquired infections: A comparative study in Nepal and Okayama Prefecture, Japan. *Acta Medica Okayama*, 57(5),

261–266.

11. Kumari Baithalu, R., Maharana, B. R., Mishra, C., Sarangi, L., & Samal, L. (2010). Canine pyometra. *Veterinary World*, 3(7), 340–342.
12. Pantha, S., Subedi, D., Poudel, U., Subedi, S., Kaphle, K., & Dhakal, S. (2020). Review of rabies in Nepal. *One Health*, 10, 100155. <https://doi.org/10.1016/j.onehlt.2020.100155>
13. Pretzer, S. D. (2008). Clinical presentation of canine pyometra and mucometra: A review. *Theriogenology*, 70(3), 359–363. <https://doi.org/10.1016/j.theriogenology.2008.04.028>
14. Selman, P. J., Wolfswinkel, J., & Mol, J. A. (1996). Binding specificity of medroxyprogesterone acetate and proligestone for the progesterone and glucocorticoid receptor in the dog. *Steroids*, 61(3), 133–137. [https://doi.org/10.1016/0039-128X\(95\)00199-U](https://doi.org/10.1016/0039-128X(95)00199-U)
15. Shah, S. A., Sood, N. K., Wani, B. M., Rather, M. A., Beigh, A. B., & Amin, U. (2017). Haemato-biochemical studies in canine pyometra. *Journal of Pharmacognosy and Phytochemistry*, 6(4), 14–17.
16. Silva, E., Henriques, S., Brito, S., Ferreira-Dias, G., Lopes-da-Costa, L., & Mateus, L. (2012). Oestrous cycle-related changes in production of Toll-like receptors and prostaglandins in the canine endometrium. *Journal of Reproductive Immunology*, 96(1–2), 45–57. <https://doi.org/10.1016/j.jri.2012.08.003>
17. Smith, F. O. (2006). Canine pyometra. *Theriogenology*, 66(3), 610–612. <https://doi.org/10.1016/j.theriogenology.2006.04.014>
18. Watts, J. R., Wright, P. J., & Whithear, K. C. (1996). Uterine, cervical and vaginal microflora of the normal bitch throughout the reproductive cycle. *Journal of Small Animal Practice*, 37(2), 54–60. <https://doi.org/10.1111/j.1748-5827.1996.tb03061.x>

Prevalence of *Toxocara canis* Werner, 1782 in Pet and Community Dogs of Kathmandu Valley Based on Coprological Examination

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ABSTRACT

Dogs are known for being the definitive host of a zoonotic parasite Toxocara canis. To study the prevalence of Toxocara canis in pet and community dogs of Kathmandu Valley and to assess the associated risk factors a cross-sectional study was carried out from January to June 2023. 300 fecal samples were collected from 140 pet dogs and 160 community dogs of Kathmandu Valley per rectally. The Wisconsin centrifugation flotation method was used to examine the presence of T. canis eggs followed by the quantitative examination. A questionnaire survey was conducted among 140 dog owners. About 10.33 % of dogs (31/300) were positive for T. canis. Comparatively, the prevalence was higher in community dogs (61.29% versus 38.70% in pet dogs). The prevalence of the parasite in pet and community dogs was non-significant ($\chi^2=1.138$; $p>0.05$). The prevalence of T. canis was significant ($\chi^2 = 93.11$; $p \leq 0.05$) among age groups of dogs with the highest prevalence of 54.83% in puppies followed by 32.25% in young and 12.90 % in adults. Significantly, a higher prevalence of 87.09% ($\chi^2 = 31.95$ $p \leq 0.05$) was observed in non-dewormed dogs followed by 12.90% in dewormed dogs. A higher prevalence of 83.33% was observed in dogs manifesting gastrointestinal symptoms compared to asymptomatic dogs (16.67%). The study revealed the mean eggs per gram of 2484.21 in community dogs and 2100 in pet dogs. Out of 140 pet owners surveyed, 45% were aware of parasitic zoonoses while 55% were unaware. The study identified pets' deworming schedule, food habits, and defecation status as risk factors for parasitic infestation. Regular deworming treatments, strict enforcement of cleaning up of dog feces, and community dog population control seem to be the most recommendable means for reducing the risk of T. canis in dogs and humans.

Keywords: Prevalence; Risk factors; *Toxocara canis*; Wisconsin centrifugation flotation

INTRODUCTION

Dogs are associated with more than sixty zoonotic disease transmission including gastrointestinal parasites (GIP) that can cause serious public health concerns (Bastakoti et al., 2023; Satyal et al., 2013). Dogs are known for being the definitive host for several parasites. Out of many gastrointestinal parasites affecting dogs, *Toxocara canis* is the globally common parasite of dogs also observed in Nepal where the dog is the definitive host (DH) whereas, other mammals including the man are the paratenic host (PH) (Pathak et al., 2021). *T. canis* is one of the most widespread public health and economically important zoonotic parasitic infections humans share with dogs. This neglected disease has been shown through seroprevalence studies to be especially prevalent among children from socio-economically disadvantaged populations both in the tropics and sub-tropics and in industrialized nations (Macpherson, 2013). With *T. canis* ability to lay nearly 200 thousand eggs per day, it is expected that a single dog carrying at least six females, and one male adult nematode may shed millions of eggs that can heavily contaminate the environment. Hence, access to *T. canis* -infected dogs in parks, playgrounds, and recreational spaces has been identified as the main factor influencing the global prevalence of human toxocariasis (Conde et al., 2022).

A study conducted in Kathmandu Valley in 2020 estimated the overall prevalence of *T. canis* to be 13.64% (Lamsal et al., 2020). Centers for Disease Control and Prevention (CDC) have considered *T. canis* one of five neglected parasitic infections in the USA. CDC has targeted Toxocariasis as a priority for public health action. The impact of toxocariasis has been observed throughout the globe, with an estimated seroprevalence of 19% in a recent meta-analysis study. Previously it has been reported that at least 70 people in the United States are blind by toxocariasis and most of them are children (Wu and Bowman, 2022).

Although there have been several studies on *T. canis* in Nepal, the dogs remain vulnerable to the parasite. The dog population, both in the community and pets in Kathmandu valley, is steadily increasing yearly along with the greater rate of spread of various parasites like; *T. canis* parasite. The free-roaming dog litter on roads, children's parks, and playgrounds constitutes a potential risk of parasitic infection for human beings. The environmental contamination by *T. canis* in the valley has still not been studied. Similarly, several studies conducted in the past years have revealed the lack of knowledge regarding the zoonotic potential of *T. canis* among pet owners. So, the the present study was carried out to determine the prevalence of *T. canis* parasites in Kathmandu Valley based on quantitative examination and to understand the risk factors of *T. canis* infestations in dogs, considering the increasing trend of keeping pets among the households of Kathmandu valley.

MATERIALS AND METHODS

Study area and sample size

The study was conducted in the Kathmandu Valley considering three districts Kathmandu, Lalitpur, and Bhaktapur from January 2023 to June 2023. The study design was a cross-sectional study with purposive sampling. For the screening of *T. canis* in dogs, a total of 300 fecal samples were collected purposively. The sample size was calculated using the formula given by Daniel (2011).

Sample size (n) = $Z^2P(1-P)/d^2$

n = Sample size

Z = Statistic for the level of confidence (for the level of confidence of 95%, Z value is 1.96)

P = estimated true proportion 22.38%

d = Desired precision (d is considered 0.05 to produce good precision)

Sampling of dogs was done from the Veterinary Hospital and Livestock Service Expert Center, Lalitpur, and its sub-offices located at Kathmandu and Bhaktapur, Mobile Veterinary Hospital, Jawalakhel, Animal Nepal, Chovar, Kathmandu, Pasu Sewa Kendra, Sudal, Bhaktapur and 4-Care For Animals, Satdobato, Lalitpur.

Sampling technique

About 3-5 gm of fecal samples were collected per-rectally and kept in sterile zipper bags. The samples collected were then transported to the Veterinary Hospital and Livestock Expert Center, Lagankhel, Lalitpur for laboratory examination (Fig. 1). The samples were processed for examination on the collection day; otherwise, they were preserved in 10% formalin to prevent the development and hatching of parasitic eggs.

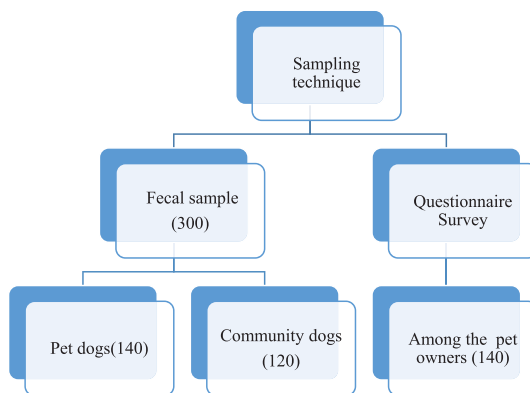


Fig. 1. Summary of the sampling method and sampled population from the Kathmandu Valley

Questionnaire

The semi-structured questionnaire for the survey was developed regarding general information on dog details, owner names, and deworming conditions. The survey was conducted among the owners of pet dogs from which the fecal samples were collected. Questions regarding the general information of dogs about the age, sex, breed, and deworming history of the dogs were asked to a member of each household.

Parasitological technique

The most commonly used method to recover parasitic eggs from feces is floatation techniques that rely on the difference in the specific gravity of eggs, fecal debris, and floatation solution. The method used for floatation was the Wisconsin Sugar Flotation technique (Martin, 2017).

For those samples with positive results, we separated them for the quantitative examination to find out Egg Per Gram (EPG) by using the Mc Master counting technique for determining the parasitic load in infected dogs (Bhatia et al., 2010).

Risk factors

The prevalence of infection was assorted in terms of age, sex, and deworming history. The age of dogs was estimated by the questionnaire survey with owners and observation of dentition in the three groups: puppy (0 to 6 months), young (6 months to 1 year), and adult (1 year above).

Data analysis

Data collected from each animal was coded and recorded in Microsoft Excel 2013 and was analyzed using IBM SPSS Statistics V25. Data was grouped age-wise as up to 6 months, from 6 months to 1 year, and above 1 year as puppies, young and adult respectively; sex-wise as male and female; type of dog as pet and community dog and deworming condition as regularly dewormed at 3 months and non-dewormed. The effect of sex, age, breed, and deworming pattern on prevalence was evaluated by the Chi-square test.

RESULTS

Prevalence of *T. canis* in dogs of Kathmandu Valley

The overall prevalence rate was 10.33% (31/300) in dogs of Kathmandu valley (Fig. 2). The prevalence rates of *T. canis* in community dogs and pet dogs were found to be 61.29% (19/31) and 38.70% (12/31) respectively (Table 1). There was no statistically significant difference between the *T. canis* positive and other non-positive cases in our study ($\chi^2 = 1.138$; $p \geq 0.05$).

Table 1. Prevalence of *T. canis* among the sampled population

Parameters	Positive %	Negative %	\Total
Pet Dogs	38.70% (12/31)	47.58% (128/269)	140
Community Dogs	61.29% (19/31)	52.41% (141/269)	160
Total	31	269	300

Out of 31 positive cases of toxocariasis in dogs, the prevalence of *T. canis* in puppies, young, and adult groups was found to be 54.83% (17/31), 32.25% (10/31), and 12.90% (4/31) respectively. The prevalence of zoonotic helminth *T. canis* was statistically significant among age groups of dogs ($\chi^2 = 93.11$; $p < 0.0001$). The prevalence of parasites was higher in male dogs (58.06%) than female dogs (41.93%). However, there was no significant difference in the prevalence of parasites among the male and female dogs ($\chi^2 = 0.46$; $p = 0.49$).

Fig. 2. *T. canis* egg (40x)

Prevalence of parasite based on deworming status of dogs

Significantly, a higher prevalence rate of *T. canis* was observed in dogs that were not dewormed (87.09%, 27/31) compared with dogs that are dewormed with suitable anthelmintic (12.90%, 4/31). The result showed a significant difference between the *T. canis* positivity among the dewormed and non-dewormed dogs ($\chi^2 = 31.95$; $p = 0.001$).

Knowledge of pet owners on deworming schedule

Questions were asked to the 140 pet owners about the frequency of deworming their pets. Among 140 respondents 12 (8.57 %) owners had practices of deworming at the four monthly intervals, 55 (39 %) at the six months interval, and 44 (31.42 %) annually whereas, about 29 (20 %) owners did not have any knowledge on the deworming schedule for their pet dogs. However, the highest prevalence of *T. canis* 58.33% ($n = 7$) was observed in the

pets whose owners were completely unaware of the deworming schedule and 41.67% (n = 5) in the pets who were dewormed once a year.

Owner's knowledge of parasitic zoonoses

Among 140 pet owners only 63 (45 %) owners knew about parasitic zoonoses, and the remaining 77 (55 %) owners did not know about parasitic zoonoses.

Evaluation of risk factors

Prevalence of *T. canis* egg based on defecation status of dogs

A higher prevalence of 14.81% (4/27) was observed in dogs defecating indoors and a lower prevalence of *T. canis* 7.07% (8/113) was observed among the dogs defecating outdoors. However, the differences in results were not statistically significant ($\chi^2 = 1.66$, p-value = 0.18).

Prevalence based on food habits of pet dogs

The prevalence of the parasite was 33.33% (4/12) had been fed dog foods (pedigree, commercially available dog foods in the market). Significantly, a lower prevalence of 41.67% (5/12) and 25% (3/12) was observed who were fed homemade foods and leftovers respectively. However, the result is non-significant ($\chi^2 = 16.84$, p-value = 0.0002).

Prevalence based on GI symptoms manifested by dogs

In a total of 140 samples, the prevalence of *T. canis* was relatively more common in dogs with gastrointestinal symptoms than those that were asymptomatic (83.33%; 10/12) vs. 16.67%; 2/12)). There was no significant association between the prevalence of the parasite and the symptoms manifested by the dogs ($\chi^2 = 1.55$, p-value = 0.21).

Prevalence of parasites based on the presence of coprophagic behavior in pets

The prevalence of *T. canis* among dogs with coprophagic behavior was observed to be 8.33% (1/12) and the prevalence of the parasite in dogs who did not have coprophagic behavior was observed to be 91.67% (11/12). There was no significant difference between the prevalence of parasites and the coprophagic behavior of pets ($\chi^2 = 0.52$; p-value = 0.46).

Comparative Egg Per Gram (EPG)

The quantitative examination of 31 positive fecal samples revealed a mean EPG value of 2335.48. However, the higher EPG value was observed in community dogs (2484.21) and 2100 in pet dogs of Kathmandu Valley (Table 2).

Table 2. Comparison of EPG value among *T. canis* positive samples

Dogs	<i>T. canis</i> positive (Number)	Mean EPG (Counts)	Minimum EPG (Counts)	Maximum EPG (Counts)
Pet dogs	12	2100.00	400.00	3000.00
Community	19	2484.21	350.00	3200.00
Total	31	2335.48	350.00	3200.00

DISCUSSION

The outcomes of this research have provided insights into the prevalence of the common yet neglected zoonotic helminth *T. canis* among the pet and community dogs of Kathmandu valley, Nepal. In the present study, 10.33% of dogs were positive for *T. canis* infestation in the Kathmandu valley, Nepal. The result is comparatively lower than the findings in Kathmandu valley in 2019 which revealed a 13.64% overall prevalence of *T. canis* (Lamsal et al., 2019). The result of this study is lower than the prevalence observed in Kathmandu Valley by Satyal, 2013 revealed 19.52% prevalence (Satyal, 2013) and 37% in the Rupandehi district of Nepal (Yadav et al., 2017) however, higher than the findings in research conducted in different regions globally (Hackett and Lappin, 2003; Nolan and Smith, 1995). Toxocariasis was comparatively higher than the reports (Minnar, 2001; Traub, 2013) but almost similar to the previous research findings (Yadav, 2017; Rahaman, 1973; Sariego, 2012). Those disparities in the prevalence rate of *T. canis* arose due to variable geography, owners' awareness, and government policy regarding animal health welfare.

In the present study, community dogs had a significantly higher prevalence rate of 61.29% compared with pet dogs which was only 38.70%; like the findings of (Lamsal et al., 2019) and Surgan, 1980. Higher prevalence in the community dogs might be associated with free exposure to the eggs in the environment, repeated access to infection, poor health conditions with malnutrition, paratenic infection due to their widespread and random distribution, lack of deworming, scavenging habit and unrestricted fouling of environment, dogs' excretion as well as vertical transmission via placenta or trans mammary route to the puppies.

A recent study mentioned the higher prevalence of *T. canis* in puppies (below 6 months of age) (54.83%) followed by 32.25% in young and 12.90% in adults. Results seem to be consistent with the findings of Conde, 2022 & Lamsal et al., 2019. This might be due to the underdeveloped immune system of puppies, and prenatal and trans-mammary transmission of *T. canis* infestations in young dogs (Hendrix, 1986). Although most of the research studies have divided the age of dogs into puppies, young and adult, the universal fact is that puppies have a greater frequency of ascarid worm- *T. canis* than young and adults (Sukupayo and Tamang, 2023).

Significantly higher prevalence of *Toxocara. canis* infestations in non-dewormed dogs (87.09%) than in dewormed dogs (12.90 %) ($p < 0.05$) which shows the effectiveness of anthelmintic usage in dogs.

It was evident from this study that most owners are not aware of the zoonotic potential of the parasites carried by their dogs, or their mode of transmission to humans. This lack of knowledge seems to be the main reason for the apparent negligence of the owners in deworming their dogs (Katagiri, 2008).

In this study, the quantitative evaluation of the parasitic burden has also been estimated. The mean eggs per gram of feces in the positive sample was estimated to be 2335.48. However, the highest EPG value was observed in community dogs (2484.21) followed by 2100 mean EPG value in pet dogs and 925.00 in the positive fecal samples collected from public places of Kathmandu valley. The result is consistent with the study conducted in Southern Poland which estimated the EPG value of 500 to 3600 *T. canis* eggs from the rural areas (Studzinska et al., 2017). This affects the degree of pollution with the parasite, which is also crucial for human health. It may be concluded that a high number of EPG in community dogs is due to no access to anthelmintics treatments, poor nutritional status, and weak immune response.

The present study has shown several risk factors associated with the prevalence and transmission of *T. canis* in dogs and humans. The specific localization of areas with *T. canis* eggs could provide information to guide strategies to reduce the infection burden in dogs and promote serological screening of the human population for *Toxocara* in Nepal.

CONCLUSION

T. canis, being highly prolific and resistant, their infestations must be controlled because of its prevalence, and its veterinary and public health importance due to the risk of transmission in humans. The overall prevalence rate was 10.33% in dogs of Kathmandu valley. The prevalence rate of *T. canis* in community dogs was 61.29% and pet dogs was 38.70 %. Based on the outcome of the present study deworming of dogs with effective anthelmintics should be enforced based on periodic mass treatment in endemic areas especially in community dogs. Similarly, knowledge of pet owners on hygienic managemental practices is essential to reduce the parasitic burden on pets. School education for awareness among children is highly recommended.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Awale, S. (2022, April 21). Kathmandu street-smart dogs. *Nepali Times*. <https://www.nepalitimes.com/here-now/kathmandu-s-street-smart-dogs>
2. Bastakoti, R., Pathak, C. R., & Pandey, A. (2023). Prevalence of zoonotic parasites of street dogs and its threats in Madi Valley, Chitwan, Nepal. *Indian Journal of Veterinary Public Health*, 9(3), 47.
3. Bhatia, B., Pathak, K., & Juuyal, P. (2010). *Textbook of veterinary parasitology* (3rd ed., pp. 149–158). Kalyani Publishers.
4. Bowman, D. (2020). The anatomy of the third-stage larva of *T. canis* and *Toxocara cati*. *Advances in Parasitology*, 109, 1–24. <https://doi.org/10.1016/bs.apar.2020.03.002>
5. Centers for Disease Control and Prevention. (2019). The life cycle of *T. canis*. <https://www.cdc.gov/parasites/toxocariasis/biology.html>
6. Conde, M. D. P., Portugaliza, H. P., & Lanada, E. B. (2022). Prevalence of *T. canis* infection in dogs and *Toxocara* egg environmental contamination in Baybay City, Leyte, Philippines. *Journal of Parasitic Diseases*, 46(4), 1021–1027. <https://doi.org/10.1007/s12639-022-01525-y>
7. Daniel, J. (2011). *Sampling essentials: Practical guidelines for making sampling choices* (p. 295). Sage Publications.
8. Díaz-Reganon, D., Agulla, B., & Piya, B. (2020). Stray dogs in Nepal have a high prevalence of vector-borne pathogens: A molecular survey. *Parasites & Vectors*, 13, 174. <https://doi.org/10.1186/s13071-020-04057-7>
9. Giri, D. R. (2010). Screening of zoonotic helminths in canines of Kathmandu district brought to Central Veterinary Hospital, Tripureshwor. [Internship report, IAAS, Tribhuvan University].
10. Hackett, T., & Lappin, M. (2003). Prevalence of enteric pathogens in dogs of north-central Colorado. *Journal of the American Animal Hospital Association*, 39(1), 52–56. <https://doi.org/10.5326/0390052>
11. Lamsal, S., & Prajapati, S. (2020). Study on prevalence of *T. canis* in stray and pet dogs of Kathmandu Valley, Nepal. *Acta Scientific Medical Sciences*, 4(2), 2–5.
12. Macpherson, C. N. (2013). The epidemiology and public health importance of toxocariasis: A zoonosis of global importance. *International Journal for Parasitology*, 43(12–13), 999–1008. <https://doi.org/10.1016/j.ijpara.2013.07.004>
13. Martin, J. L. (2017, April). The fecal egg count reduction test using the Wisconsin sugar flotation technique. *Kansas State Veterinary Diagnostic Laboratory*. https://ksvdl.org/resources/news/diagnostic_insights_for_technicians/april2017/fecal.html
14. Rahaman, M. H. (1973). Incidence of some helminth parasites of zoonotic significance in street dogs in some districts of Bangladesh. *Bangladesh Veterinary Journal*, 7, 14–16.
15. Minnaar, W. N. (2001). Helminths in dogs belonging to people in a resource-limited urban community in Gauteng, South Africa. *Onderstepoort Journal of Veterinary Research*, 68, 111–117.

16. Nolan, T. J., & Smith, G. (1995). Time series analysis of the prevalence of endoparasitic infections in cats and dogs presented to a veterinary teaching hospital. *Veterinary Parasitology*, 59(2), 87–96. [https://doi.org/10.1016/0304-4017\(94\)00742-U](https://doi.org/10.1016/0304-4017(94)00742-U)
17. Pathak, C., Pandey, G., Shrestha, S., Ranjit, E., Rana, H., & Thapa, S. (2021). In vitro cultivation of *T. canis* larva for extraction of excretory-secretory (Tces) antigen. In S. P. Shrestha (Ed.), *Proceedings of 12th National Workshop on Livestock and Fisheries Research in Nepal* (p. 42). Nepal Agricultural Research Council.
18. Sariago, I., & Kourí, K. (2012). Toxocariasis in Cuba: A literature review. *PLoS Neglected Tropical Diseases*, 6(2), e1382. <https://doi.org/10.1371/journal.pntd.0001382>
19. Satyal, R. C., Manandhar, S., Dhakal, S., Mahato, B. R., Chaulagain, S., Ghimire, L., & Pandeya, Y. R. (2013). Prevalence of gastrointestinal zoonotic helminths in dogs of Kathmandu, Nepal. *International Journal of Infection and Microbiology*, 2(3), 91–94. <https://doi.org/10.3126/ijim.v2i3.8211>
20. Studzińska, M. B., Demkowska-Kutrzepa, M., Borecka, A., Meisner, M., Tomczuk, K., Roczeń-Karczmarz, M., Kłapeć, T., Abbass, Z., & Cholewa, A. (2017). Variations in the rate of infestations of dogs with zoonotic nematodes and the contamination of soil in different environments. *International Journal of Environmental Research and Public Health*, 14(9), 1003. <https://doi.org/10.3390/ijerph14091003>
21. Sukupayo, P. R., & Tamang, S. (2023). Prevalence of zoonotic gastrointestinal helminth parasite among dogs in Suryabinayak, Nepal. *Veterinary Medicine International*, 2023, 3624593. <https://doi.org/10.1155/2023/3624593>
22. Traub, R. J. (2002). The role of dogs in transmission of gastrointestinal parasites in a remote tea-growing community in northeastern India. *American Journal of Tropical Medicine and Hygiene*, 67, 539–545. <https://doi.org/10.4269/ajtmh.2002.67.539>
23. Wu, T. K., & Bowman, D. D. (2022). *T. canis*. *Trends in Parasitology*, 38(8), 709–710. <https://doi.org/10.1016/j.pt.2022.01.002>
24. Yadav, K. K., & Shrestha, B. (2017). Prevalence of zoonotic gastrointestinal helminth parasites in pet and stray dogs of Rupandehi District, Nepal. *Microbiology & Infectious Diseases*, 1(2), 1–7. <https://doi.org/10.33425/2639-9458.1014>

Aqueous Extract of Tuber Root of *Asparagus racemosus* Augments the Wound Healing in Sparague-Dawley Rat

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ABSTRACT

Asparagus racemosus (AR) is an herbal medicinal plant exhibiting the antioxidant and immunomodulatory properties. Despite having these properties, the wound healing ability of AR is not fully elucidated. The present study was aimed to investigate the wound healing activity of aqueous extract of AR tuber root on excision wound in Sparague-Dawley rats. A total of 24 rats were randomly selected and allocated equally in AR-100, AR-500 and Positive control (P-Ctrl) and Negative control (N-Ctrl) groups. The excisional wounds were created in dorsal cervical region in all rats. The rats of AR-100, AR-500 and P-Ctrl groups received oral administration of aqueous extract of AR tuber root at a rate of 100 mg/kg, 500 mg/kg and Doxycycline hyclate 30 mg/kg, respectively. However, normal drinking water was provided to rats of N-Ctrl group. The incisional biopsies were taken to assess the inflammatory progress such as cell infiltrations, neovascularization and collagen synthesis histologically on day 3, 7, 14 and 25. Inflammation was severe ($P < 0.05$) in rats treated with both doses of AR on day 3, 7 and 14 but mild moderate ($P < 0.05$) on day 25. Neovascularization scores did not vary among the groups on day 3, 7 and 14. However, rats treated with AR-500 mg/kg b.wt. resulted in less vascularization ($P < 0.05$) as compared to both control rats on day 25. The AR-100 and 500 mg/kg augmented the collagen synthesis ($P < 0.05$) at all observational time point. In conclusion, both doses of aqueous extract of AR tuber root promoted the wound healing, however, the effectiveness of AR with 500 mg/kg b.wt. seemed superior for excisional wound healing in rats. Further research is imperative to explore the molecular mechanism of the active ingredients of *A. racemosus* in different wound models.

Keywords: *Asparagus racemosus*; Wound healing; Histopathology; Collagen; Rat

INTRODUCTION

Wound is the damage or destruction to an organ's normal anatomical structure and function (Robson, 2001). Wound healing is the intricate process that consists of several overlapping phases, including hemostasis, inflammation, proliferation, and remodeling (Fernández-Guarino et al., 2023). Several factors can interfere with one or more phases of healing, resulting in impaired wound healing (Anderson & Hamm, 2014).

Despite the significant advances of the pharmaceutical industries, the drugs directly promoting the wound healing process is still limited (Akkol et al., 2011). Additionally, availability of oral medications that improve the wound healing is not intervened (Levine, 2017). Antibiotics as well as non-steroidal anti-inflammatory drugs are often used presently for wound healing. However, these therapies are expensive and exhibit the numerous undesirable side effects (Mcknight et al., 2000; Shenoy et al., 2011). Hence, there is a great need for the development of nontoxic and effective wound healing replacements.

Notably, the plant extracts can induce wound healing and tissue regeneration through multiple interrelated mechanisms. These extracts are abundantly available, inexpensive and cause minimal side effects, making them a potential alternative therapeutics for the wound healing (Maver et al., 2018). So far, several herbal preparations have been devised for their uses as wound healers (Alam et al., 2011; Kartini et al., 2021). The details about the traditional uses of herbal medicines for wound healing in different animal models have been elucidated recently (Dhakal et al., 2022). The *Asparagus racemosus* is widely distributed in Nepal and other geographical domains of world (Hasan et al., 2016; Alok et al., 2013). The flowers, fruits and roots of *A. racemosus* possess the medicinal value and its roots have been traditionally used to treat the wounds (Saini et al., 2016; Ajitha & Sharma, 2017). Javaid et al. (2022) have described that this herbal medicine shows the antibacterial, galactagogue, neuroprotective, antiulcer, anti-inflammatory, immunomodulatory, antioxidant and thereby the wound-healing properties. Specifically, Kodancha et al. (2011) intervened that the wound healing abilities of *A. racemosus* occurred due to the presence of antioxidants. Their study used the various dosages of aqueous extract of tuber roots and reported the remarkable skin breaking strength, wound contraction rate and improvement of epithelialization time in both incisional and excisional wound models of rats. So far, no research has investigated its wound healing effects at the cellular or histopathological level. Therefore, the present study was designed to evaluate the wound healing activity of the aqueous extract of tuber root of *A. racemosus* (test extract) at various doses and its underlying effects on cellular events at different phases of wound healing on excision wound model in rats.

MATERIALS AND METHODS

Collection and identification of plants

A. racemosus was collected between June to July, 2023 in its whole (adult stage) from the Kafle Community Forest of Lalitpur district and was identified by National Herbarium in Godavari, Lalitpur, Nepal (505/079-080).

Preparation aqueous extract of tuber root of *Asparagus racemosus*

The fresh clean roots were crushed by using electric blender and boiled in 5-liter distilled water for 2 hrs. After that, the aqueous suspension was filtered into a different beaker using Whatman No.1 filter paper. Then, extract was dried in a water bath for 24 hrs. Then, aqueous extract was obtained. The obtained extract was stored at 4°C for further use.

Acute toxicity study

Initially, 15 rats were divided evenly into three groups. The extract was administered orally at single dose of 100, 500 and 1000 mg/kg b.wt. to each group respectively. All of the rats were observed for general behavior, abnormalities associated with central nervous system, cardiovascular system and digestive system and mortality at hourly interval for next 24 hours and thereafter for a total of 14 days.

Experimental rats

A total of 24 adult Sprague Dawley rats (male 12; female 12) was used for the experiment. The rats were purchased from Animal House Section of Natural Products Research Laboratory, Thapathali Kathmandu. They were kept in ambient temperature in standard-sized plastic cage and maintained at the facility at Veterinary Pharmacology Laboratory, Agriculture and Forestry University. All the rats were fed with pelleted feed and ad libitum potable water. The study followed the laboratory animal welfare guidelines and experiment was approved by ethical committee of Nepal Veterinary Council (Permission No. 295/2080-81).

Study design

The research was performed as completely randomized blinded double control design. The study used apparently healthy Sparague-Dawley rats (n=24) of both sexes. The rats were allocated randomly in Negative control (N-Ctrl), *Asparagus racemosus* extract (AR-100), AR-500 and Positive control (P-Ctrl) groups. Each group contained six rats. All the rats were anaesthetized with intraperitoneal injection of using ketamine-HCl (50 mg/kg, KMINE, National Health care Pvt. Ltd. Bara, Nepal). Thereafter, the rats' dorsal fur was trimmed and shaved with a blade. The surgical area was scrubbed with chlorhexidine and finally painted with 70% alcohol. The location of wound was ascertained at dorsal thoracic region and marked properly 1 cm away from the spinal column. 500 mm² full thickness circular excisional wounds were created carefully with the help of toothed

forceps, scalpel blade and scissors. The day at which wound is created was considered as Day 0. The entire wound was kept open. The study was carried out at Pharmacology laboratory, Faculty of Animal Science, Veterinary Science and Fisheries, Agriculture and Forestry University, Rampur, Chitwan, Nepal.

The treatment protocol used in this study has been outlined below.

- **Negative control (N-Ctrl) group:** Rats were non-medicated but availed distilled water,
- ***Asparagus racemosus* aqueous extract (AR-100) group:** Rats received aqueous extract of tuber root of *Asparagus racemosus* @100 mg/kg b.wt. orally once daily for 25 days
- ***Asparagus racemosus* aqueous extract (AR-500):** Rats were given aqueous extract of tuber root of *Asparagus racemosus* @500 mg/kg b.wt. orally once daily for 25 days
- **Positive control (P-Ctrl) group:** Rats were medicated with Doxycycline Hyclate tab @ 30 mg/kg b.wt. orally once a daily for 25 days

The scheme of the experiment is represented in Fig. 1.

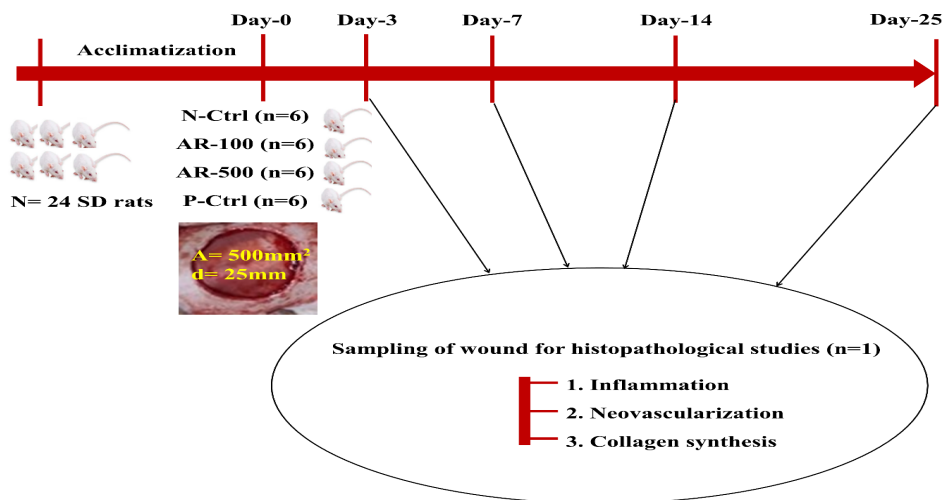


Fig. 1. Scheme of the experiment

Histopathological studies

One rat representing each group was anaesthetized with ketamine @ 50 mg/kg b.wt. intraperitoneally. A full-thickness skin incisional biopsy was taken from the center of wound along with its edge on day 3, 7, 14 and 25. Tissue samples were fixed in 10% neutral buffer formalin till processing. Then, routine procedures for processing, paraffin embedding tissues (dehydration, clearing and wax infiltration) and Hematoxylin-Eosin (H & E) staining was performed following the previous standard protocol (de Moura

Estevão et al., 2019; Shah et al., 2016. The morphological analyses were performed on granulation tissue. Six fields per slice were evaluated to include the borders and the center of the wound. For each parameter, six slices of a sample tissue from each rat were analyzed (de Moura Estevão et al., 2019). The qualitative histological studies were performed by analyzing the H& E stained tissues with the 40× objective of microscopic image analyzer (Euromex, Holland) and then by using a specific score for inflammation, vascularization and extracellular matrix deposition/collagen synthesis as described below in Table 1-3, respectively (de Moura Estevão et al., 2019).

Table 1. Morphological score of inflammation in H& E-stained histological section of excisional rat's wounds

Score	Parameter
0	Absence of inflammatory cell
1	Presence of few inflammatory cells
2	Presence of many inflammatory cells
3	Exaggerated inflammatory cellularity

Table 2. Morphological scores of vascularization in H&E-stained histological sections of excisional rats wound

Score	Parameter
0	Normal vascularization
1	Discrete vascular formation
2	Moderate vascular formation
3	High vascular formation

Table 3. Morphological scores of extracellular matrix deposition in H&E- stained histological sections of excisional rats' wounds

Score	Parameter
0	Whole extracellular matrix in normal skin
1	incomplete presence of extracellular matrix (identified by many fibroblasts and thin collagen fiber)
2	presence of extracellular matrix in the whole wound area (identified by many fibroblasts and thin collagen fiber)
3	presence of extracellular matrix in the whole wound area (identified by few fibroblasts and thick collagen fiber)

Statistical Analysis

The data are presented as mean±SD. Statistical analyses were performed using the SPSS information system for windows (SPSS V 16, SPSS Institute Inc. USA). The score of histopathological examination was analyzed using Kruskal–Wallis ANOVA followed by the rank-based Mann–Whitney *U*-test. The P-values <0.05 were considered significant.

RESULTS

The present investigation was performed in two phases. The acute toxic effects of the *A. racemosus* were intervened with its graded doses in first phase. Based on the findings of first phase, the 100 and 500 mg/kg aqueous extract of tuber root of *A. racemosus* was selected for studying its underlying effects on cellular events at different phases of wound healing on excision wound model in rats.

Toxic effects of plants

The oral dosages of 100, 500, and 1000 mg/kg b.wt. of test extract in rats exhibited no toxicological signs on the central nervous system, cardiovascular system, digestive system or general behavior. In present study, the dosages 100 mg/kg b.wt. and 500 mg/kg b.wt. were chosen for further research on wound healing.

Gross healing of wound

The comparative gross healing progress of wounds between the treatment groups on day 3, 7, 14 and 25 is shown in Fig. 2. The wounds of treatment group AR-100, AR-500 and P-Ctrl appeared to heal faster than the wound of N-Ctrl group on day 25.

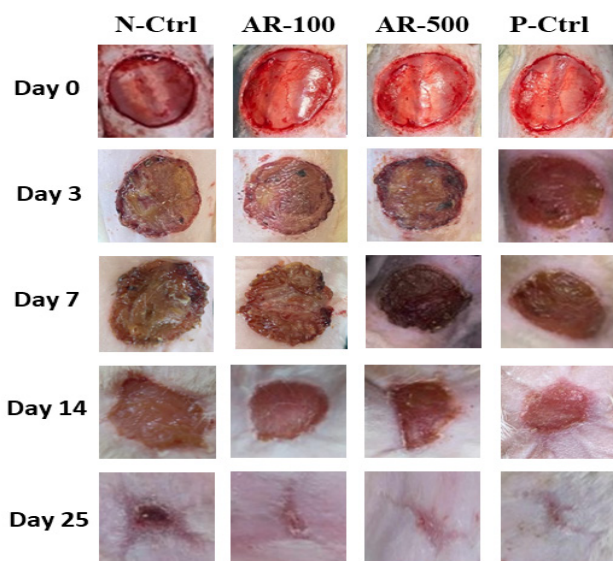


Fig.2. Gross photographic images of wound in different treatment groups

Histopathological analysis of wound

Inflammation

The severity of inflammation varied among the groups at different time points. As compared with N-Ctrl, inflammation score was higher ($P < 0.01$; 0.05) in AR-100 and AR-500 groups, however the inflammation scores of AR-100, AR-500 and N-Ctrl group

measured higher ($P < 0.01$; 0.05) than that of P-Ctrl group on day 3, 7 and 14. Interestingly, the inflammation scores of AR-100, AR-500 and P-Ctrl groups decreased inadvertently as compared to N-Ctrl group on day 25 as shown in Fig. 3.

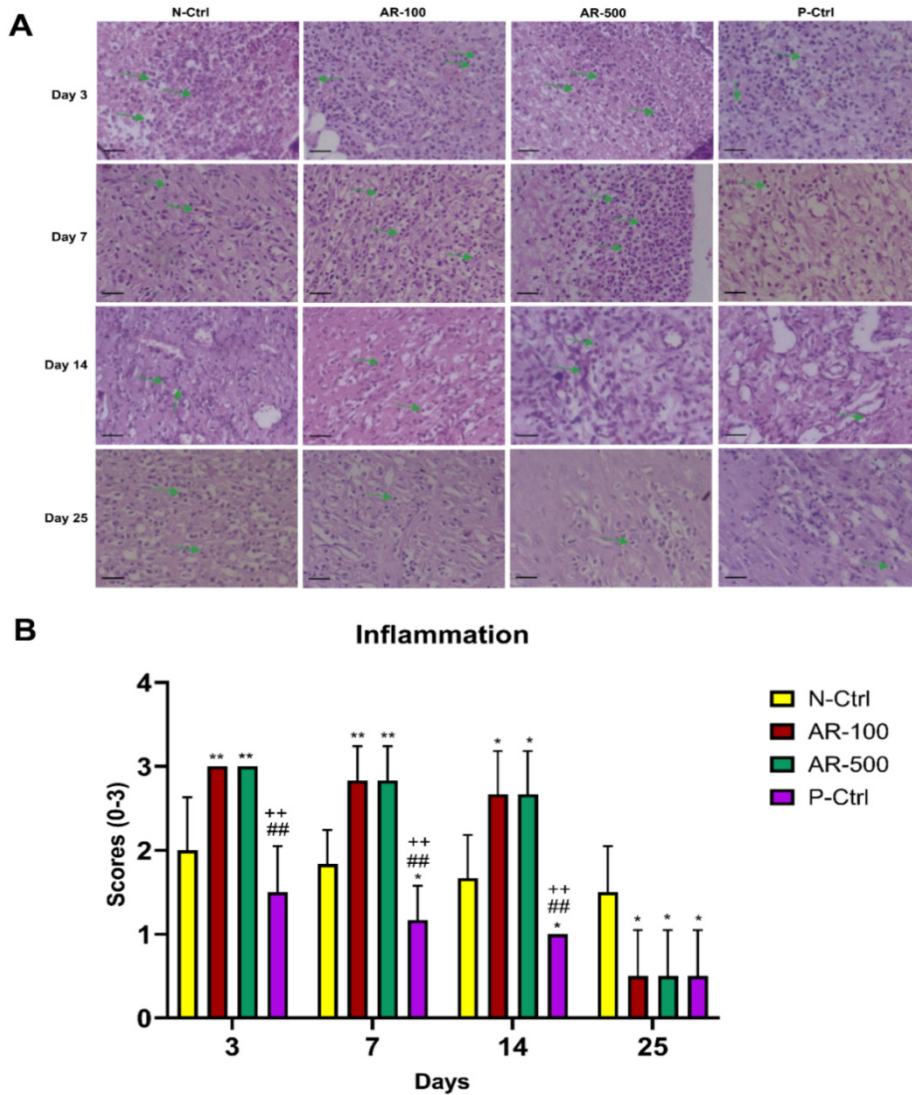


Fig. 3. Histopathological view of wound healing. A. Photomicrographs showing inflammatory cells infiltration in different treatment groups at magnification 40X (green arrow represents the inflammatory cells). Scale bar represents 100 μ m. B. Inflammation score in different treatment groups

$**P < 0.01$; $*P < 0.05$ AR-100/AR-500/P-Ctrl versus N-Ctrl; $##P < 0.01$ AR-500/P-Ctrl versus AR-100; $++P < 0.01$ AR-500 versus P-Ctrl

Vascularization rates

As shown in Fig. 4, the vascularization scores did not vary among the groups on day 3, 7 and 14. However, the neovascularization was lower ($P<0.05$) in rats of AR-500 group than those of both P-Ctrl and N-Ctrl groups on day 25.

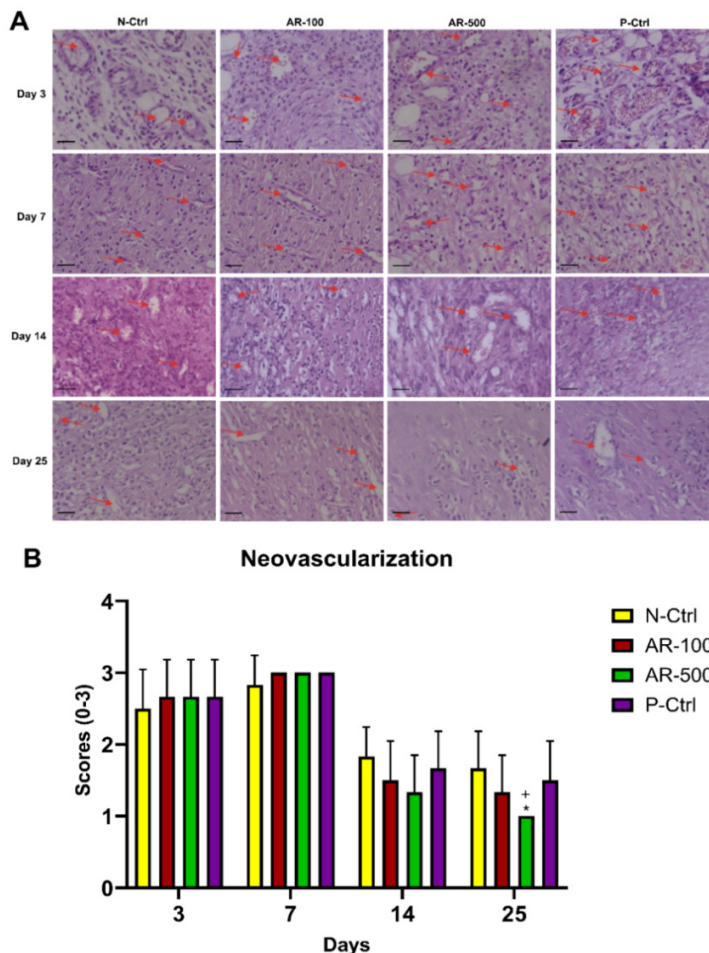


Fig. 4. Histopathological view of wound healing. A. Photomicrographs showing vascularization in different treatment groups at magnification 40X (Red arrow represents the blood vessel). Scale bar represents 100 μ m. B. Vascularization scores in different treatment groups.

* $P<0.05$ AR-100/AR-500/P-Ctrl versus N-Ctrl; + $P<0.05$ AR-500 versus P-Ctrl

Extracellular matrix deposition/collagen synthesis

The rats of AR-100 and AR-500 groups exhibited higher rates of extracellular matrix deposition or collagen synthesis ($P<0.01$; 0.05) as compared to rats of N-Ctrl group on day 3. The extracellular matrix deposition or collagen synthesis rates were higher ($P<0.01$) in rats of AR-100, AR-500 and P-Ctrl groups than the rats of N-Ctrl group on day 7, 14 and 25 as shown in Fig. 5.

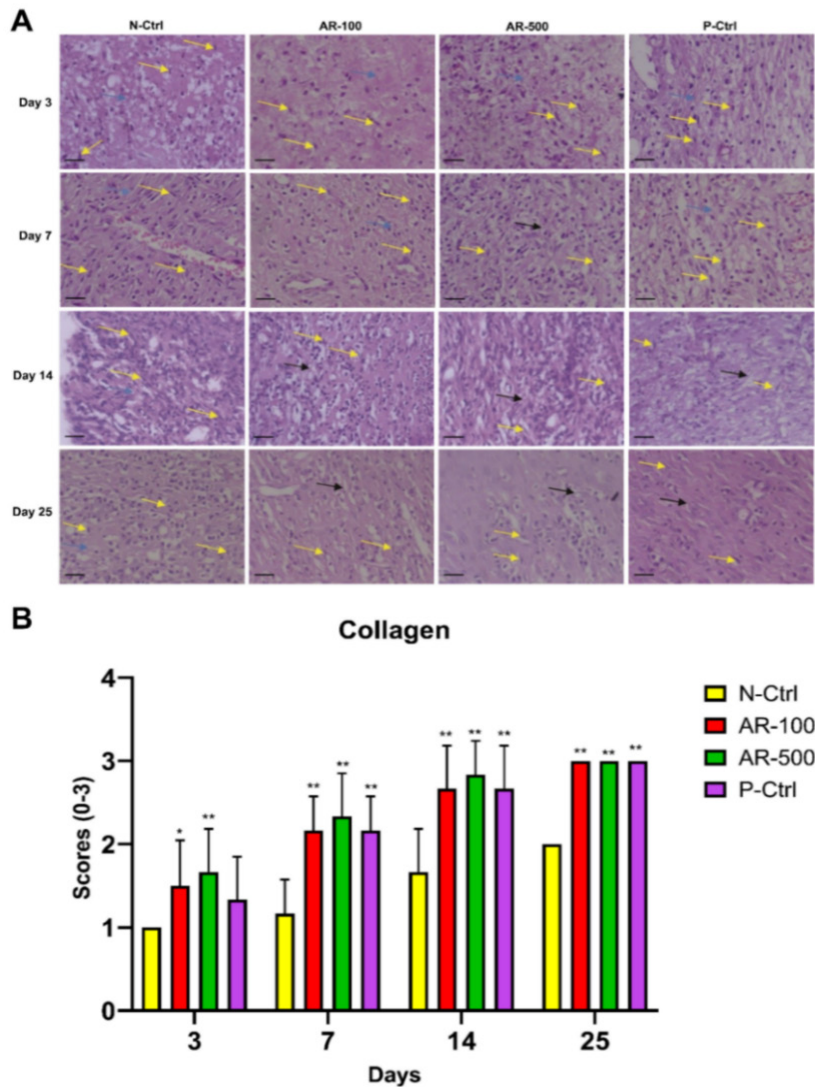


Fig. 5. Histopathological view of wound healing. A. Photomicrographs showing Extracellular matrix deposition/collagen synthesis in different treatment groups at magnification 40X (yellow arrow represents the fibroblast; pink arrow represents thin collagen fiber and black arrow represent the thick collagen fiber). Scale bar represents 100 μ m B. Extracellular matrix deposition/collagen synthesis score in different treatment groups

** $P < 0.01$; * $P < 0.05$ AR-100/AR-500/P-Ctrl versus N-Ctrl

DISCUSSION

Wound is a break in the integrity of tissue due to external as well as internal injuries. An intricate but integrated series of molecular and cellular events occurs during the healing

of wound (Dhakal et al., 2022). The use of herbal medicines seems efficacious presently for the complication free wound healing. The oral administration of test extract did not show any toxic effects at different gradient doses (100, 500, 1000 mg/kg b.wt.). The results of this study are consistent with findings of previous study (Kodancha et al., 2011), which showed no acute toxicity of test extract up to 8000 mg/kg b.wt. administered orally. This study demonstrated the beneficial action of AR root extract on excision wound healing at different dosages in Sprague Dawley Rats. The inflammatory events, neovascularization and extracellular matrix deposition/collagen synthesis were assessed in all groups histopathologically.

Inflammation score was increased remarkably in AR-100 and AR-500 groups than that of N-Ctrl group. However, inflammation score in AR-100 and AR-500 groups were similar on day 3,7,14. This is due to the immunomodulators and immunostimulants present in the test extract, that produces leukocytosis, neutrophilia, while simultaneously increasing phagocytic activity of macrophage and polymorphs (Muruganandan et al., 2001; Sharma et al., 2013; Thakur et al., 2012). Neutrophils initiate healing at the injury site by directly affecting angiogenesis and cell proliferation (Phillipson & Kubes, 2019). Also, the immunomodulators and immunostimulants present in the test extract causes monocytosis, after which monocytes rapidly develops into macrophages (Sindrilaru & Scharffetter-Kochanek, 2013). These macrophage phagocytose dead neutrophil as well as infectious materials and dead cellular material for cleaning of wound (Frykberg & Banks, 2015). The test extract causes proliferation of lymphocytes and increase phagocytosis of foreign material and tissue debris (Arora et al., 2014). Inflammation scores of AR-100 and AR-500 and P-Ctrl groups decreased inadvertently as compared to N-Ctrl group on day 25. This reduced inflammation is due to the anti-inflammatory effect produced by test extract by inhibiting the expression of inflammatory mediators such as tumor necrosis factor - α (TNF- α) to promote wound healing (Gang et al., 2024). In addition to that, this decreased inflammation score is may be due to reduced total leucocyte, lymphocyte and monocyte count caused by accelerated epithelization but excessive scarring in wound remodeling stage (Wang et al., 2019).

Although the neovascularization rates were not remarkably varied among the groups on day 3 to 14, but the vascularization score was decreased significantly in AR-500 in comparison with the N-Ctrl group as well as P-Ctrl group on day 25 which indicates the wound was healed perfectly. This could be because as the collagen builds up in the granulation tissue to generate scars, the blood vessel density reduces (Tonnesen et al., 2000).

Extracellular matrix deposition/collagen synthesis score was significantly increased in AR-100 and AR-500 group as comparison with the N-Ctrl group on day 3, 7, 14 and 25. This is because the test extract raises the level of interleukin-1 and tumor necrosis factor which stimulates the fibroblast activity and increase collagen synthesis (Kodancha et al., 2011).

CONCLUSION

The aqueous extract of root of *A. racemosus* seems safe because its oral dose up to 1000 mg/kg bwt didn't result in any acute toxic effects on CNS and other major body systems. Inflammation was severe in rats treated with AR on day 3, 7 and 14 but moderately mild on day 25. Vascularization rate was similar in all rats on day 3 to 14. However, the rats treated with AR-500 mg/kg b.wt. resulted in less vascularization on day 25 which indicates the wound was healed perfectly. The both doses of AR augmented the collagen synthesis throughout the study period. So, it is concluded that both doses of test extract promote the wound healing. However, the effectiveness of 500 mg/kg b.wt. dose rate is relatively higher.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Ajitha, S., & Sharma, D. N. (2017). A comprehensive review of the pharmacological actions of *Asparagus racemosus*. *American Journal of Pharmatech Research*, 7 (February), 1–20.
2. Akkol, E. K., Süntar, I., Koca, U., & Keleş, H. (2011). Wound healing activity of *Rubus sanctus* Schreber (Rosaceae): Preclinical study in animal models. *Evidence-Based Complementary and Alternative Medicine*, 2011. <https://doi.org/10.1093/ecam/nep137>
3. Alam, G., Singh, M. P., & Singh, A. (2011). Wound healing potential of some medicinal plants. *International Journal of Pharmaceutical Sciences Review and Research*, 9(1), 136–145. <https://doi.org/10.47583/ijpsrr.2022.v77i01.017>
4. Alok, S., Jain, S. K., Verma, A., Kumar, M., Mahor, A., & Sabharwal, M. (2013). Plant profile, phytochemistry and pharmacology of *Asparagus racemosus* (Shatavari): A review. *Asian Pacific Journal of Tropical Disease*, 3(3), 242–251. [https://doi.org/10.1016/S2222-1808\(13\)60049-3](https://doi.org/10.1016/S2222-1808(13)60049-3)
5. Anderson, K., & Hamm, R. L. (2014). Factors that impair wound healing. *Journal of the American College of Clinical Wound Specialists*, 4(4), 84–91. <https://doi.org/10.1016/j.jccw.2014.03.001>
6. Arora, S., Kapila, S., Singh, R. B., Pandey, M. M., Rastogi, S., Rawat, A. K. S., & Katara, A. (2014). Immunomodulatory and antioxidative potential of milk fortified with *Asparagus racemosus* (Shatavari). *Journal of Medicinal Plants Studies*, 2(6), 13–19.
7. de Moura Estevão, L. R., Cassini-Vieira, P., Leite, A. G. B., de Carvalho Bulhões, A. A. V., da Silva Barcelos, L., & Evêncio-Neto, J. (2019). Morphological evaluation of wound healing events in the excisional wound healing model in rats. *Bio-Protocol*, 9(13), 1–12. <https://doi.org/10.21769/BioProtoc.3285>
8. Fernández-Guarino, M., Hernández-Bule, M. L., & Bacci, S. (2023). Cellular and molecular processes in wound healing. *Biomedicines*, 11(9). <https://doi.org/10.3390/biomedicines11092526>
9. Frykberg, R. G., & Banks, J. (2015). Challenges in the treatment of chronic wounds. *Advances in Wound Care*, 4(9), 1–23. <https://doi.org/10.1089/wound.2015.0635>
10. Gang, R., Okello, D., & Kang, Y. (2024). Medicinal plants used for cutaneous wound healing in Uganda; ethnomedicinal reports and pharmacological evidences. *Heliyon*, 10(9), e29717. <https://doi.org/10.1016/j.heliyon.2024.e29717>

11. Hasan, N., Ahmad, N., Zohrameena, S., Khalid, M., & Akhtar, J. (2016). *Asparagus racemosus*: For medicinal uses & pharmacological actions. *International Journal of Advanced Research*, 4(3), 259–267.
12. Dhakal, I., Tiwary, A. K., KC, S., Thakur, B., Regmi, B., & Shah, M. K. (2022). Exploration on traditional uses of herbal medicines for wound healing in different animal models. *International Journal of Zoology and Animal Biology*, 5(1), 1–10. <https://doi.org/10.23880/izab-16000349>
13. Javaid, K., Hussain, S., Syed, S. K., Ahmad, I., Massey, S., Asghar, A., Amjad, M., Rukhsar, A., & Hafeez, M. (2022). Phytochemistry, medicinal and nutritional importance of *Asparagus racemosus*. *Postępy Biologii Komórki*, 49(3), 175–192. <https://doi.org/10.59674/pbk2>
14. Kartini, K., Wati, N., Gustav, R., Wahyuni, R., Anggada, Y. F., Hidayani, R., Raharjo, A., Islamie, R., & Putra, S. E. D. (2021). Wound healing effects of *Plantago major* extract and its chemical compounds in hyperglycemic rats. *Food Bioscience*, 41, 100937. <https://doi.org/10.1016/j.fbio.2021.100937>
15. Kodancha, S., Ravindra, S., Subir, S., Sahana, & Deepa. (2011). Wound healing profile of *Asparagus racemosus* (Liliaceae) wild. *Current Pharma Research*, 2(1), 111–114.
16. Levine, J. M. (2017). The effect of oral medication on wound healing. *Advances in Skin and Wound Care*, 30(3), 137–142.
17. Maver, T., Kurečić, M., Smrke, D. M., Kleinschek, K. S., & Maver, U. (2018). Plant-derived medicines with potential use in wound treatment. *Herbal Med*, 10.
18. McKnight, W., Asfaha, S., Wallace, J. L., & Muscara, M. N. (2000). Wound collagen deposition in rats: Effects of an NO-NSAID and a selective COX-2 inhibitor. *Canadian Journal of Physiology and Pharmacology*, 78(8), 681–686.
19. Muruganandan, S., Garg, H. G., Lal, J., Chandra, S., & Kumar, D. (2001). Studies on the immunostimulant and antihepatotoxic activities of *Asparagus racemosus* root extract. *Journal of Ethnopharmacology*, 77(1), 67–70.
20. Phillipson, M., & Kubes, P. (2019). The healing power of neutrophils. *Trends in Immunology*, 40(7), 635–647. <https://doi.org/10.1016/j.it.2019.05.001>
21. Robson, M. C. (2001). Wound healing: Biologic features and approaches to maximum healing trajectories. *Current Problems in Surgery*, 38(2), 72–141.
22. Shah, M. K., Wan, J., Janyaro, H., Tahir, A. H., Cui, L., & Ding, M. X. (2016). Visceral hypersensitivity is provoked by 2,4,6-trinitrobenzene sulfonic acid-induced ileitis in rats. *Frontiers in Pharmacology*, 7(JUL), 1–9. <https://doi.org/10.3389/fphar.2016.00214>
23. Sharma, U., Kumar, N., Singh, B., Munshi, R. K., & Bhalerao, S. (2013). Immunomodulatory active steroidal saponins from *Asparagus racemosus*. *Medicinal Chemistry Research*, 22(2), 573–579. <https://doi.org/10.1007/s00044-012-0048-4>
24. Shenoy, R. R., Sudheendra, A. T., Nayak, P. G., Paul, P., Kutty, N. G., & Rao, C. M. (2011). Normal and delayed wound healing is improved by sesamol, an active constituent of *Sesamum indicum* (L.) in albino rats. *Journal of Ethnopharmacology*, 133(2), 608–612. <https://doi.org/10.1016/j.jep.2010.10.045>
25. Sindrilaru, A., & Scharffetter-Kochanek, K. (2013). Disclosure of the culprits: Macrophages—Versatile regulators of wound healing. *Advances in Wound Care*, 2(7), 357–368. <https://doi.org/10.1089/wound.2012.0407>
26. Thakur, M., Connellan, P., Deseo, M. A., Morris, C., Praznik, W., Loeppert, R., & Dixit, V. K. (2012). Characterization and in vitro immunomodulatory screening of fructo-oligosaccharides of *Asparagus racemosus* Willd. *International Journal of Biological Macromolecules*, 50(1), 77–81. <https://doi.org/10.1016/j.ijbiomac.2011.09.027>
27. Tonnesen, M. G., Feng, X., & Clark, R. A. F. (2000). Angiogenesis in wound healing. *Journal of Investigative Dermatology Symposium Proceedings*, 5(1), 40–46.
28. Wang, X., Balaji, S., Steen, E. H., Li, H., Rae, M. M., Blum, A. J., Miao, Q., Butte, M. J., Bollyky, P. L., & Keswani, S. G. (2019). T lymphocytes attenuate dermal scarring by regulating inflammation, neovascularization, and extracellular matrix remodeling. *Advances in Wound Care*, 8(11), 527–537. <https://doi.org/10.1089/wound.2019.0981>

Prevalence of Gastrointestinal Nematodes in Dogs of Kathmandu Valley, Nepal

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ABSTRACT

*The nature of the close association between dogs and their owners could increase the chances of cross-transmission of zoonotic parasites among them. This study aims to determine the general prevalence, epidemiology, and risk factors associated with gastrointestinal nematode parasites (GINPs) in pet and community dogs in Kathmandu Valley, Nepal. In the purposive randomized study, a total of (N = 300) fecal samples were collected per rectally from the dogs [pet (n = 140) and community (n = 160)] of different locations within the valley during January-June 2022. The collected samples were kept in a zip-lock plastic bag maintained cold and were examined within 2 hours of collection at the Veterinary Hospital and Livestock Service Expert Center in Lagankhel, Lalitpur. Thus, the overall prevalence of GINPs in dogs was 29.34% (88/300). Out of 88 positive samples; *Ancylostoma caninum*, *Toxocara canis*, and mixed infections were found in 73.86%, 35.22%, and 9.09% respectively. The prevalence of GINPs in pet dogs and community dogs were 27.14%, and 31.25% respectively ($p > 0.05$). The prevalence rate of GINPs between different age groups was 73.07% (19/26), 17.33% (26/150), and 34.67% (43/124) in puppy, young, and adult respectively ($p < 0.05$). About 23.98% (53/221) dewormed and 44.30% (35/79) non-dewormed dogs were positive for GINPs respectively ($p < 0.05$). These risk factors show statistical differences suggesting that there might be a considerably high prevalence of GINP infections in the dogs of Kathmandu valley. A high prevalence rate increases susceptibility for owners, unaware of the risk of zoonotic parasitic infections from their pets.*

Keywords: Hookworms; Kathmandu Valley; Risk factors; Roundworms

INTRODUCTION

The domestic dogs (*Canis familiaris*), the first domesticated mammal around 15000 years ago from wolves (Morey, 2006) and have coexisted with men all around the world. In the Nepalese context, dogs are kept as companion animals, for security purposes, religious rituals, hunting, etc. Dogs greatly improve their owners' physical, social, and mental health, especially in youngsters and the elderly, by promoting regular exercise, improving their social connections, lowering stress, and offering emotional support.

Being companion animals there is a close association between dogs and their owners. It is considered that gastrointestinal parasitism is one of the most common health problems among all dogs (Pathak *et al.*, 2021). Generally, dog types have been categorized as street dogs (community dogs) and pet dogs (Bhatta *et al.*, 2018). Dogs are those that are sub-clinically infected with different gastrointestinal nematode parasites (GINPs) even though they seem to be healthy. However, in heavy infection or dogs with immunocompromised and malnourished conditions anemia and protein loss may be manifested (Coati *et al.*, 2003). Moreover, competition for nutrients and tissue damage during feeding and migration could cause severe clinical signs such as anorexia, anemia, diarrhea, and edema associated with poor growth and development, mortality in young, aged, and immuno-suppressed dogs (Iddrissi *et al.*, 2022).

Dogs are associated with more than 60 zoonotic diseases among which helminth parasites can pose serious health concerns worldwide and have a significant economic impact from the veterinary standpoint (Safarov *et al.*, 2022). Community dogs mostly reside near public places where a huge flow of human population such as temples, and gardens in search of food and shelter. Community dogs harbor a wide range of helminth species even without clinical manifestations could become the major source of helminth parasites for both pet dogs and humans. The prevalence of parasites considerably varies according to regions, diagnostic techniques employed, and types of dogs. Among cestodes *Taenia hydatigena*, and nematodes; *Toxocara* spp. and *Ancylostoma* spp. are of great health importance to humans, among several zoonotic parasites that infect dogs (Bastakoti *et al.*, 2023; McManus, 2006).

Along with the urbanization and improvement in the household economic status in major cities of Nepal like Kathmandu, people are more interested in having pets and are frequently found walking dogs around the street. This could create the chance of transmission of parasites between community dogs and pet dogs. The trend of importing foreign dog breeds may carry new strains of parasites that could be zoonotic and can be transferred to dogs (personal pets and community), humans, or both. Therefore, the main objective of this study is to determine the prevalence, epidemiology, and risk factors associated with gastrointestinal nematode parasites (GINPs) in dogs of Kathmandu valley, Nepal.

MATERIALS AND METHODS

Study site and design

A cross-sectional study with purposive sampling was conducted during January-June 2022 in the Kathmandu Valley which consists of three districts; Kathmandu (27°41'38.76" North latitude and 85°22'39" East longitude), Lalitpur (27°32'53.88 North latitude and 85°20'15" East longitude) and Bhaktapur (27°40'31.44" North latitude and 85°25'55.56" East longitude), lies in Bagmati zone of Nepal. Sample collection in dogs was done from Veterinary Hospital and Livestock Service Expert Center (Lalitpur), Sneha's Care, United Nation Park, Jwagal, Lalitpur.

Sample size

For the screening of nematodes in dogs, a total of three hundred (N = 300) fecal samples were collected purposively from pet dogs (n = 140) and community dogs (n = 160). The total sample consists of the different age groups: puppy (n = 26), young (n = 150), and adult (n = 124) where male (n = 191) and female (n = 109). Age groups categorized dogs as puppies (0-9 months), young (10 months- 3 years), and adults (3 years and above).

The sample size calculated was 267.

The sample size was calculated using the formula given by Daniel (2011).

$$\text{Sample size}(n) = Z^2P(1-P)/d^2$$

n = Sample size

Z = Statistic for the level of confidence (for the level of confidence of 95%, Z value is 1.96)

P = estimated true proportion 22.38%

d = Desired precision (d is considered 0.05 to produce good precision)

Fecal sample collection, processing, and identification of parasites

Around 3-5 grams of fecal samples were taken per-rectally from dogs and stored in well-labeled sterile zip-lock plastic bags before being sent to the laboratory at the Veterinary Hospital and Livestock Service Expert Center in Lagankhel, Lalitpur. Within 2hrs of collection, the samples were examined qualitatively by using the Wisconsin sugar flotation technique and quantitatively with the help of the McMaster counting technique (Ito, 1981).

A semi-structured questionnaire survey was developed regarding general information on dog details, owner name, and deworming condition. The survey was conducted among the owners of household dogs from which the fecal samples were collected. Questions regarding the general information of dogs about the age, sex, breed, and deworming history of the dogs were asked to a member of each household.

Data analysis

Data collected from each animal was coded and recorded in Microsoft Excel 2013 and was analyzed using IBM SPSS Statistics V20. Data would be grouped age-wise as Puppy (0-9 months), Young (10 months – 3 years), and Adult (3 years and above); sex-wise as male and female; type of dog as pet and community dog and deworming condition as regularly dewormed at 3 months and non-dewormed. The effect of sex, age, breed, and deworming pattern on prevalence was evaluated by the Chi-square test.

RESULTS

Overall prevalence of nematode parasites

Out of 300 fecal samples of dogs, 88 were positive with at least one of the parasites so the overall prevalence was 29.34%.

Intestinal nematode species isolated

Among 88 positive samples; *Ancylostoma caninum*, *Toxocara canis*, and mixed infections were found in 73.86%, 35.22%, and 9.09% respectively (Table 1).

Table 1. Prevalence of individual nematode

S.N.	Parasitic infections	Frequency of parasites
1	<i>Ancylostoma caninum</i>	65/88(73.86%)
2	<i>Toxocara canis</i>	31/88(35.22%)
3	Mixed infection	8/88(9.09%)

Prevalence of gastrointestinal nematode species

The prevalence of nematodes in pet dogs and community dogs was found to be 27.14% (38/140) and 31.25% (50/160) respectively (Fig. 1).

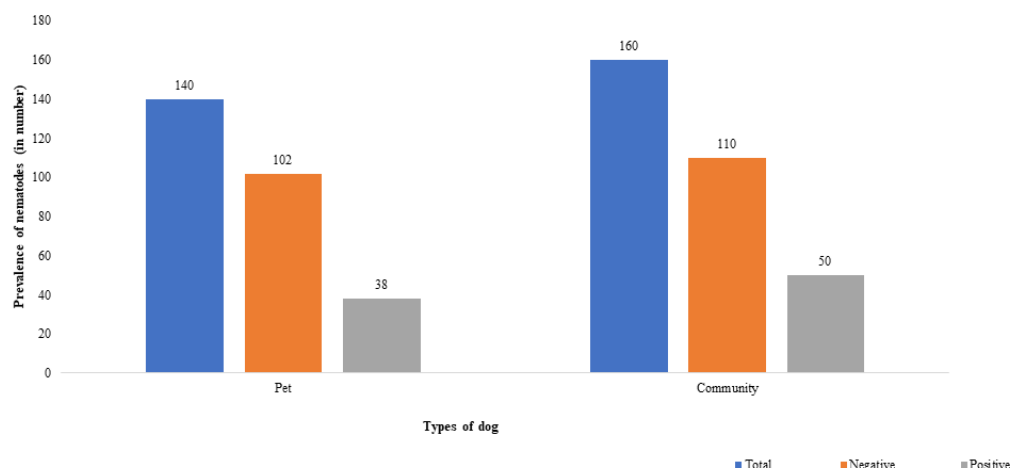


Fig. 1. Prevalence of nematode parasite based on types of dogs.

The result showed that the prevalence of nematode parasites was non-significant ($\chi^2 = 0.068$; $p = 0.436$, $df = 1$) for different types of dogs. It implies that pets and community dogs are equally susceptible to parasitic infection.

Age-wise prevalence of nematode parasites

Considering age-wise prevalence, 19 (73.07%) puppy dogs out of 26 were found positive; 26 (17.33%) young dogs out of 150 were found positive and 43 (34.67%) adult dogs out of 124 were found positive for GINPs (Fig. 2). The result showed that the prevalence of nematode parasites was significant ($\chi^2 = 36.129$; $p = 0.001$) among age groups of dogs. It implies that age is the determining factor for nematode infection.

Sex-wise prevalence of nematode parasites

Among male fecal samples, 56/191 (29.31%) were positive whereas among females, 32/109 (29.35%) were positive for the presence of intestinal nematode species. The

prevalence rate of GINPs was non-significant ($\chi^2 = 0.001$; $p = 0.994$, $df = 1$) in males and females. So, the sex of dogs is not a determining or discriminating factor for parasitic infection.

Deworming status-wise prevalence of nematodes

Out of total dogs, the prevalence of nematode parasites in dogs that were regularly dewormed within 3 months was 23.98% (53/221), and in dogs that were not dewormed was 44.30% (35/79). The prevalence of parasites among dewormed and non-dewormed dogs was significantly different ($\chi^2 = 11.594$; $p = 0.001$, $df = 1$). It implies that deworming status affects parasitic infection.

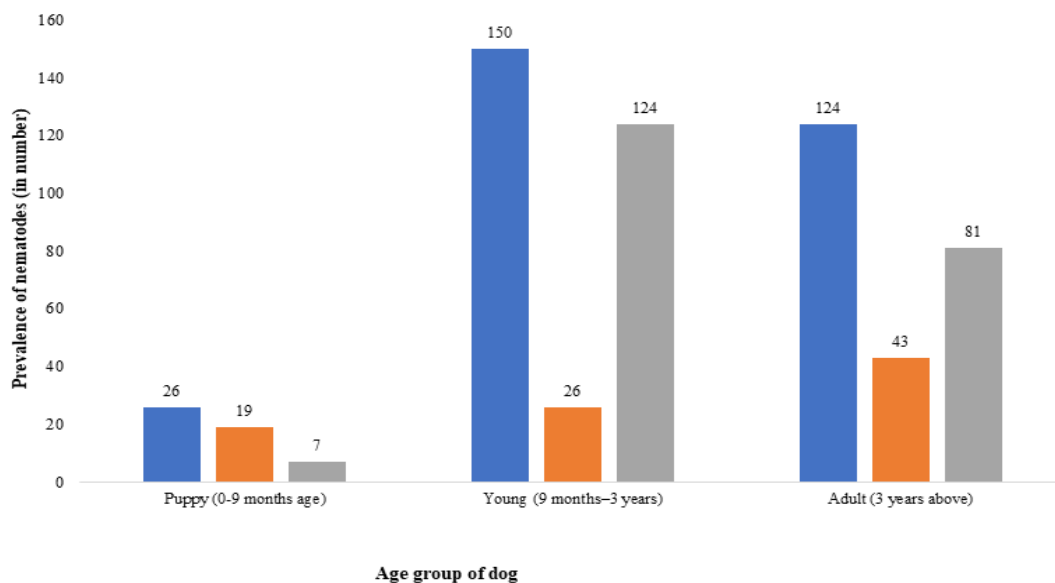


Fig. 2. Age-wise prevalence of nematode parasites.

Breed-wise prevalence of nematodes

The prevalence of nematodes in pure breed, cross breed, and local breed was found to be 29.57% (21/71), 26.67% (20/75), and 30.51% (47/154) respectively. The prevalence of nematodes for different breeds was non-significant ($\chi^2 = 0.364$; $p = 0.834$). It implies that breed is not the responsible factor for parasitic infections.

Egg Per Gram and egg size of gastrointestinal nematode parasites

The mean EPG of nematode parasites in dogs was found to be 2259. The highest count was 6100 and the lowest count was 250. The Average size of eggs of *Toxocara canis* and *Ancylostoma caninum* is found to be (82.8 μm by 70 μm) (Fig. 3) and (55.3 μm by 69 μm) (Fig. 4) respectively.

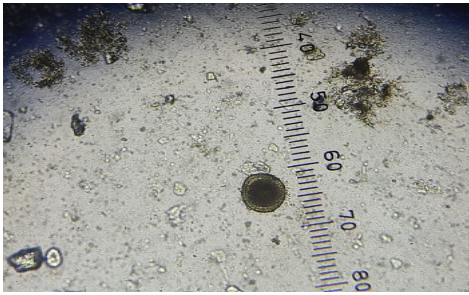


Fig. 3. Micrometry of egg of *Toxocara* spp.

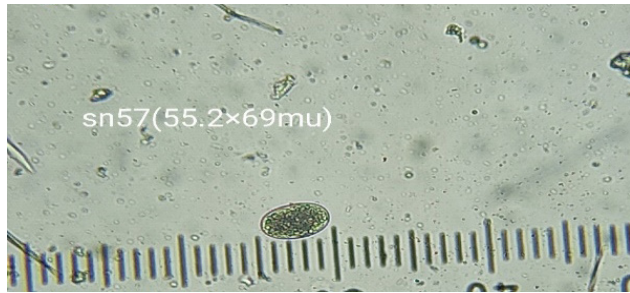


Fig. 4. Micrometry of egg of *Ancylostoma* spp.

DISCUSSION

In this study, out of 300 fecal samples, the overall prevalence of gastrointestinal nematodes in dogs of Kathmandu valley was 88 (29.34%), which is similar to the findings of Satyal et al (2013). It shows that the helminth parasitic problem persists in the canines of Kathmandu valley.

The prevalence of common parasites observed in the present study was *Ancylostoma caninum* (n = 65, 73.86%) and *Toxocara canis* (n = 31, 35.22%). The predominant parasites encountered in the current study were hookworm species and *T. canis* which supports the findings by Swai et al (2010). The higher prevalence of hookworm species and *Toxocara canis* may be due to the possibility of puppies being infected by their dam by trans-mammary or transplacental routes. In addition, thousands of EPG counts for *T. canis*, and millions of shedding of hookworm eggs for weeks in puppies could be the reasons for high environmental contamination (Swai et al., 2010). The higher frequency of hookworms observed in this study suggests that this condition could be more widely distributed than was previously assumed by Heukelbach and Feldmeier (2008).

The prevalence of nematodes helminth parasites was significantly lower ($p < 0.05$) in pet dogs (27.14%) than in community dogs (31.25%). Prevalence is higher in community dogs than pet dogs which may be due to higher sample size or more exposure of those dogs to the environment, poor nutritional status which leads to poor immune system. The overall prevalence of nematodes in dogs was statistically higher ($p < 0.05$) in puppies (73.07%) than in young (17.33%) and adult dogs (34.67%). This is similar to the findings of Jones et al (2011). This might be due to underdeveloped immunity, with transplacental and lactogenic modes of transmission, and susceptibility to other viral infections in young dogs (Swai et al., 2010; Jones et al., 2011). *Toxocara* spp. and *Ancylostoma* spp. are the most frequently diagnosed GINPs in puppies because of their nature of transplacental and trans-mammary transmission (Ramirez et al., 2004). These transmission factors, coupled with inadequate information by dog keepers on the risks of disease transmission and poor level of hygiene have resulted in an increased risk of exposure to zoonosis (Mirzaei & Fooladi, 2012).

The prevalence of intestinal nematodes was significantly lower ($p < 0.05$) in regularly dewormed dogs (23.98%) than in those dogs that were not dewormed or not regularly

dewormed (44.30%). It shows that deworming affects helminth occurrence (Mirzaei & Fooladi, 2012; Zewdu et al., 2010). This means that regular deworming of pets is necessary to minimize the effect of gastrointestinal helminth parasites for the better health condition of the pets.

CONCLUSION

This study confirmed that there is still a considerably high prevalence of nematode parasite infection in the dogs of the Kathmandu Valley. A significant overall frequency of 29.34% is found in the study on gastrointestinal nematode parasites (GINPs) in dogs in the Kathmandu Valley, Nepal. Comparatively in the community dogs, the prevalence rate of GINPs was higher than the pet dogs and notable variations according to age, with puppies exhibiting the greatest infection incidence. Prevalence rates were also impacted by the past deworming experience, with non-dewormed dogs showing a substantially greater prevalence than dewormed dogs. The results underline the danger of zoonotic transmission and stress the necessity of routine deworming, raising public awareness, and taking preventive steps to shield dogs and their owners from GINPs.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Abo-Shehada, M. N. (1989). Prevalence of *Toxocara* ova in some schools and public grounds in northern and central Jordan. *Annals of Tropical Medicine & Parasitology*, 83(1), 73–75.
2. Bastakoti, R., Pathak, C. R., & Pandey, A. (2023). Prevalence of zoonotic parasites of street dogs and its threats in Madi Valley, Chitwan, Nepal. *Indian Journal of Veterinary Public Health*, 9(3), 47.
3. Bhatia, B., Pathak, K., & Juyal, P. (2010). *Textbook of veterinary parasitology* (3rd ed.). Kalyani Publishers.
4. Bhatta, B. R., Kaphle, K., & Yadav, K. K. (2018). Situation of livestock, production, and its products in Nepal. *Archives of Veterinary Science and Medicine*, 1(1), 1–8.
5. Birchard, S. J., & Sherding, R. G. (2005). *Saunders manual of small animal practice* (3rd ed.). Elsevier Inc.
6. Bugg, R. J., Robertson, I. D., Elliot, A. D., & Thompson, R. C. A. (1999). Gastrointestinal parasites of urban dogs in Perth, Western Australia. *The Veterinary Journal*, 157(3), 295–301.
7. Coati, N., Hellmann, K., Mencke, N., & Epe, C. (2003). Recent investigation on the prevalence of gastrointestinal nematodes in cats from France and Germany. *Parasitology Research*, 90(Suppl 1),

S146–S147.

8. Daniel, J. (2011). *Sampling essentials: Practical guidelines for making sampling choices*. Sage Publications.
9. Heukelbach, J., & Feldmeier, H. (2008). Epidemiological and clinical characteristics of hookworm-related cutaneous larva migrans. *The Lancet Infectious Diseases*, 8(5), 302–309.
10. Idrissi, H., Khatat, S. E. H., Duchateau, L., Kachani, M., Azrib, R., & Sahibi, H. (2022). Canine cardiopulmonary nematodes in Morocco: Prevalence of *Dirofilaria immitis* and report of the first autochthonous infection with *Angiostrongylus vasorum*. *Moroccan Journal of Agricultural Sciences*, 3(2), 117–123.
11. Ito, S. (1981). Modified Wisconsin sugar centrifugal flotation technique for nematode eggs in bovine feces. [Journal name not provided].
12. Jones, O., Kebede, N., Kassa, T., Tilahun, G., & Macias, C. (2011). Prevalence of dog gastrointestinal parasites and risk perception of zoonotic infection by dog owners in Wondo Genet, Southern Ethiopia. *Journal of Public Health and Epidemiology*, 3(11), 550–555.
13. Kochanowski, M., Dabrowska, J., Karamon, J., Cencek, T., & Osinski, Z. (2013). Analysis of the accuracy and precision of the McMaster method in the detection of the eggs of *Toxocara* and *Trichuris* species (Nematoda) in dog feces. *Folia Parasitologica*, 60(3), 264.
14. McManus, D. P. (2006). Molecular discrimination of taeniid cestodes. *Parasitology International*, 55, S31–S37.
15. Mirzaei, M., & Fooladi, M. (2012). Prevalence of intestinal helminths in owned dogs in Kerman city, Iran. *Asian Pacific Journal of Tropical Medicine*, 5(9), 735–737.
16. Morey, D. F. (2006). Burying key evidence: The social bond between dogs and people. *Journal of Archaeological Science*, 33(2), 158–175.
17. Pathak, C., Pandey, G., Shrestha, S., Ranjit, E., Rana, H., & Thapa, S. (2021). In vitro cultivation of *Toxocara canis* larva for extraction of excretory-secretory (TCES) antigen. In S. P. Shrestha (Ed.), *Proceedings of 12th National Workshop on Livestock and Fisheries Research in Nepal, 3–4 March 2021* (p. 42).
18. Ramírez-Barrios, R. A., Barboza-Mena, G., Muñoz, J., Angulo-Cubillan, F., Hernández, E., González, F., & Escalona, F. (2004). Prevalence of intestinal parasites in dogs under veterinary care in Maracaibo, Venezuela. *Veterinary Parasitology*, 121(1–2), 11–20.
19. Rana, H. B., & Manandhar, K. D. (2016). Studies on the prevalence of zoonotic important protozoan parasite *Sarcocystis* sp. in Nepal. *Journal of Advanced Academic Research*, 3(2), 147–149.
20. Robertson, I. D., & Thompson, R. C. (2002). Enteric parasitic zoonoses of domesticated dogs and cats. *Microbes and Infection*, 4(8), 867–873.
21. Safarov, A., Mihalca, A. D., Park, G. M., Akramova, F., Ionică, A. M., Abidinabiev, O., & Azimov, D. (2022). A survey of helminths of dogs in rural and urban areas of Uzbekistan and the zoonotic risk to human population. *Pathogens*, 11(10), 1085.
22. Satyal, R. C., Manandhar, S., Dhakal, S., Mahato, B. R., Chaulagain, S., Ghimire, L., & Pandeya, Y. R. (2013). Prevalence of gastrointestinal zoonotic helminths in dogs of Kathmandu, Nepal. *International Journal of Infection and Microbiology*, 2(3), 91–94.
23. Swai, E. S., Kaaya, E. J., Mshanga, D. A., & Mbise, E. W. (2010). A survey on gastrointestinal parasites of non-descript dogs in and around Arusha Municipality, Tanzania. *International Journal of Animal and Veterinary Advances*, 2(3), 63–67.
24. Thompson, R. C. A. (2015). Neglected zoonotic helminths: *Hymenolepis nana*, *Echinococcus canadensis*, and *Ancylostoma ceylanicum*. *Clinical Microbiology and Infection*, 21(5), 426–432.
25. Zewdu, E., Semahegn, Y., & Mekibib, B. (2010). Prevalence of helminth parasites of dogs and owners' awareness about zoonotic parasites in Ambo town, central Ethiopia. *Ethiopian Veterinary Journal*, 14(2), 17–30.

Use of Feed Additives as Alternatives to Antibiotics in Broiler Production

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ABSTRACT

*This work aimed to look for alternatives to the use of antibiotics in broiler production was carried with 360 Cobb-500 broiler chickens. Research was arranged in completely randomized design with 6 treatments and 3 replications keeping 20 birds per unit on different isocaloric and isonitrogenous diets for starter, grower and finisher. The inclusion of 0.25gm/kg Colistin sulphate (T2), 600 ppm Clove oil (T3), 0.25gm/kg probiotics (T4), 0.5% Organic acid T5, 0.4% Chutro (*Berberis aristata*) (T6) in basal diet (T1) were used. Feed consumption, feed conversion ratio, body weight gain, carcass characteristics and serum profiles were investigated with economic analysis. Data were analyzed by M-Stat-C 1996 (version 1.3) and mean comparison was performed by DMRT_{0.05} and LSD_{0.05}. The highest ($p<0.01$) cumulative feed consumption was recorded in T4 and the lowest in T6. The weekly cumulative mean weight was similar among the treatments. The average feed conversion ratio was found superior ($p<0.05$) in T6 and poor on T1 and T4. The highest dressing percentage was found in T4 and lowest (74.9 %) in T1. The economic analysis showed that the BC ratio was highest in T6. Serum globulin was highest in *B. aristata* based diet, glucose in T3 and calcium in T4. Serum Phosphorus content was significant among the treatments and basal diet showed highest phosphorus content. Thus, we can conclude that the *B. aristata*, organic acid, probiotic, clove oil can replace the antibiotics in broiler feed and *B. aristata* based diet performed best from economic point of view.*

Keywords: *Berberis aristata*; Probiotics; Clove oil; Antibiotics; Poultry

INTRODUCTION

The use of antibiotics in broiler production has long been a cornerstone of modern poultry farming, aimed at enhancing growth performance, feed conversion efficiency and preventing infectious diseases for more than 60 years at sub-therapeutic doses (Castanon, 2007). The effect of antibiotics on improving performance was first reported by Moore *et al.* (1946) exhibited increased growth responses of birds fed streptomycin. Many experiments were conducted later in the early 1950s in chicken (Groschke & Evans, 1950). In-feed antibiotic (IFA) use soon became a common and well-established practice in the animal industry and rose with the intensification of livestock production. In a review conducted by Rosen (1995), it was concluded that the inclusion of antibiotics in the diets gave a positive response 72% of the time. It was also proposed that the net effect of using IFA in the poultry industry was a 3–5% increase in growth and feed conversion efficiency (Choct, 2001). Despite the well-demonstrated beneficial effects of IFA in improving the growth rate, reducing mortality and increasing resistance to disease challenges, their use was also known to be associated with some disadvantages and challenges. Concerns existed that the use of IFA leads to development of antimicrobial resistance (Endtz *et al.*, 1990), posing a potential threat to human health (WHO, 2012), necessitating a paradigm shift towards the exploration of alternative strategies. Among these alternatives, feed additives have garnered considerable attention for their potential to promote growth, improve gut health, and modulate the immune system in broiler chickens (Ayalew *et al.*, 2022; Rahman *et al.*, 2022).

The rampant use of antibiotics in animal production, especially in poultry farming, has surged to the incidence of antibiotic-resistant bacteria, downgrading the effectiveness of antibiotics in veterinary and medical field (Muteeb *et al.*, 2023). Furthermore, the issue of antibiotic residues in animal products and the environment has gathered focus regarding food and environmental safety (Arsène *et al.*, 2022). As a result, there has been a growing consensus among stakeholders, including consumers, producers, and regulatory authorities, to reduce the reliance on antibiotics in animal agriculture (Innes *et al.*, 2021).

Despite these debates on the role of IFA use in conferring antimicrobial resistance to human pathogens, the European Union issued a ban on the approval for antibiotics as growth promoters since January 2006 on precautionary grounds (Dibner & Richards, 2005). The decline in the use of antibiotic growth promoters (AGPs) in the future seems inevitable, and the practice of using antimicrobials may prove economically impractical because of market limitations and export restrictions (Dibner & Richards, 2005). In view of the increasing concerns over AGP use, the quest for novel alternate replacements to mitigate antibiotic use in animal agriculture has grown over the years. In the past two decades, a great deal of research has focused on the development of antibiotic alternatives to maintain or improve poultry health and performance. Alternatively, the use of organic acids (Tiwari *et al.*, 2017) and plant derived products having the medicinal properties have been known to replace the of hazardous side effects of the antibiotics. The use of sorbic acid and citric acid (Islam *et al.*, 2012) and the use of formic acid (Garcia *et al.*, 2007) or the organic acids salts (Pelicano *et al.*, 2005) is gaining popularity. An ideal alternative should have

the same beneficial effects of AGP, ensure optimum animal performance, and increase nutrient availability (Huyghebaert *et al.*, 2011). Considering the proposed mechanism of action of AGPs (microbiome and immune-modulating activities), a practical alternative should possess both of these properties in addition to having a positive impact on feed conversion and/or growth (Huyghebaert *et al.*, 2011; Seal *et al.*, 2013). Several classes of alternatives have been proposed and tested in poultry production, including probiotics, prebiotics, synbiotics, organic acids, enzymes, phytogenics and metals. Novel alternatives such as hyperimmune egg yolk IgY, antimicrobial peptides (AMP), bacteriophages, and clay have come into existence in recent years.

Despite the increasing knowledge in favour of the use of feed additives in broiler farming, there exists a wider gap and challenges that need to be fine-tuned. These include the need for standardized methodologies for evaluating efficacy of feed additives, optimizing dosage regimens for different production systems, and assessing their long-term impact on animal health and product quality. So, this research is focused on the enhancing knowledge of antibiotic alternatives in broiler feed to improve performances and productivity while reducing reliance on antibiotics.

MATERIALS AND METHODS

Experimental design

The 360 Cobb-500-day-old broiler chicks were enrolled in completely randomized Design with 6 treatments and 3 replications each (Table 1) in 18 cages (1m x 2m) having 20 birds each.

Table 1. Treatment allocations for use of antibiotics alternative in poultry production

Dietary treatments	
Basal diet (BD)	T1
BD + antibiotic (Colistin Sulphate 0.25gm/kg diet)	T2
BD + clove oil (600 ppm)	T3
BD + probiotic (0.25g/kg)	T4
BD + Organic acid (0.5% in diet)	T5
BD + <i>B. aristata</i> (0.4% in diet)	T6
BD- basal diet	

Formulation of experimental diets

Three types of isonitrogenous and isocaloric ration, starter (7-14 days), Grower (15-28 days) and finisher (28-49 days) used in this work were formulated on the basis of nutrients requirements of Cobb 500 (Cobb 500 Manual, 2018). Nutrient requirement of broiler in different stage is presented in Table 2. The broiler starter pellet diet (B0) was offered for 1 week for the adjustment period.

Table 2. Nutrient requirement of broiler (Cobb 500 manual guide, 2018)

	Starter	Grower	Finisher
ME, Kcal/Kg	2975	3025	3100
Crude protein %	21-22	19-20	18-19
Calcium %	0.9	0.84	0.76
Phosphorus (Available)	1.22	1.12	1.02
Digestible Lysine	0.46	0.45	0.42
Digestible Methionine	0.91	0.85	0.80
Digestible Met+Cys %	1.28	1.18	1.07
Digestible Arginine %	0.20	0.18	0.18
Digestible Tryptophan %	0.83	0.73	0.66
Digestible Threonine %	0.77	0.72	0.67
Digestible Isoleucine%	0.89	0.85	0.76
Digestible Valine %	1.00	1.00	1.00
Linoleic acid %	1.22	1.12	1.02

Source: Nutrient requirement Cobb 500, 2018

Preparation of broiler house and rearing

Floors, interior of walls and roof were scrubbed and leftover litters were removed from brooder house and washed with clean water. Rice husk was spread over the floor space as bedding materials. Lime was also mixed with husk in ratio of 500 gm on 100 sq. feet area. Birds were fed twice daily at 8 A.M and at 4 P.M and watered ad libitum. Vaccination was performed as per the recommended schedule.

Observation and parameters recorded

Dry matter, crude protein, crude fibre, ether extract and ash were of experimental feeds and feces analyzed according to Association of Official Analytical Chemist (AOAC, 2000) at Animal Nutritional Laboratory, AFU, Rampur, Chitwan. Blood collection was performed from wing vein from a bird from each replication at the end of the experiment and serum was harvested. The serum glucose, Calcium, Phosphorus, Cholesterol, protein and Albumin was analyzed by standard procedures.

The initial and weekly body weight of the birds was recorded and weight gain was obtained t for each week. The weekly feed conversion ratio (FCR) of the birds in each pen was calculated.

Weekly body weight gain= Final weight – initial weight of birds

$$FCR = \frac{\text{Weight gain (gm)}}{\text{Feed intake (gm)}}$$

At the end, digestion trial was conducted to determine the digestibility of nutrients. Three birds were selected randomly from each treatment and were fasted for 24 hours. Then,

birds were transferred in the cages and were fed *ad libitum* for 3 days. Feed refusals and faeces excreted were recorded. Fecal materials were collected for 3 days, composited, dried, ground and analyzed as stated in AOAC (2000). The digestibility coefficient was calculated as

$$\text{Digestibility Coefficient} = \left(\text{Feed consumed (Kg)} - \frac{\text{Feces voided (kg)}}{\text{feed consumed}} \right) * 100$$

Economic analysis

The economics of broiler production was calculated at the end of experiment. Gross expenditure was calculated by the sum up of cost of chicks, medicines, vaccines, feeds and labors. The gross income was obtained by selling the birds. Net income was calculated as follows.

$$\text{Net income per bird (Rs)} = \text{Gross income (Rs)} - \frac{\text{Gross expenditure (Rs)}}{\text{Number of live birds}} * 100$$

$$\text{Income per bird (Rs)} = \frac{\text{Gross income (Rs)}}{\text{Number of live birds}} * 100$$

$$\text{Benefit Cost Ratio} = \frac{\text{Total income (Rs)}}{\text{Total Gross expenditure (Rs)}}$$

Statistical analysis

Data were worked out for One way analysis of variance at 5% level of significance by MSTAT-C version 1.5. Means separation were carried out by Least Significant Difference ($\text{LSD}_{p<0.05}$) and Duncan's Multiple Range Test ($\text{DMRT}_{p<0.05}$).

RESULTS

Weekly cumulative feed consumption

The mean weekly cumulative feed consumption of Cobb-500 broiler on different diets is presented in Table 3. In the 2nd week, there was difference ($p<0.05$) among the treatments. However, the highest ($p<0.01$) feed consumption was observed in T4 (871.50 gm) which was different from T1, T2 and T6 during the 2nd week of the study. The lowest weekly cumulative feed consumption was observed in T2 (784.23 gm) which is similar with T6. There were significant ($p<0.01$) differences among the treatments in the 2nd, 3rd, 4th, 5th and 6th weeks of research, T4 were found to have significantly higher weekly cumulative feed consumption. Interestingly, T1 and T5 group of broilers consumed comparable amount of feed with that of T4 whereas T6 ate consistently lower feed right from the beginning.

Table 3. Weekly cumulative mean feed consumption (g/bird/week) of broiler on different diets.

Treatment	Weekly cumulative mean feed consumption (gm)					
	1 st	2 nd	3 rd	4 th	5 th	6 th
Basal diet/T ₁	306.62	819.57 ^{bc}	1646.95 ^b	2621.33 ^b	3860.62 ^b	5180.97 ^a
BD + (Colistin 0.25gm/kg diet)/T ₂	305.88	784.23 ^d	1562.39 ^c	2405.52 ^c	3605.56 ^c	4886.59 ^{bc}
BD + clove oil (600 ppm)/T ₃	304.91	846.47 ^{ab}	1714.24 ^{ab}	2609.24 ^b	3622.88 ^c	4712.99 ^c
BD + probiotic (0.25g/kg)/T ₄	315.65	871.50 ^a	1760.37 ^a	2867.50 ^a	4021.21 ^a	5255.97 ^a
BD + Organic acid (0.5%)/T ₅	305.83	849.33 ^a	1663.18 ^b	2592.10 ^b	3788.16 ^b	5065.27 ^{ab}
BD + <i>B. aristata</i> (0.4%)/T ₆	308.50	803.28 ^{cd}	1552.90 ^c	2419.26 ^c	3319.39 ^d	4296.00 ^d
Probability	0.219	0.0002	0.0002	0.0001	0.0001	0.0001
CV %	1.74	1.92	2.4	2.83	2.37	2.43
SEM	3.09	9.2	22.87	42.23	50.62	68.76

Means in the column with different superscripts (^{abc}) differ significantly. ^{ns}not significantly different, *significant at 5% ($p<0.05$) and **significant at 1% ($p<0.01$)

Mean cumulative body weight

Weekly cumulative live weight (gm) of broiler on different diets is presented in Table 4. Accordingly initial live weight of broiler in each treatment was on an average (40 gm; $p>0.05$). The weight gain of the broiler during study showed an increasing trend and was different ($p<0.01$) among the treatments. Significantly higher live weight was recorded in T4 (146.00 gm) and was comparable with T2 and T5 with poorest gain in T3 which was similar with T1 and T6 during 1st week. Similarly, the cumulative weight was different ($p<0.05$) in 2nd, 3rd and 5th week where T5 (2nd and 3rd week) and T3 (5th week) showed the significant weight gain ($p<0.01$). In contrast, the cumulative weight gain was indifferent in 4th and 6th week but T5 gained higher weight at the finishing age.

Table 4. Mean cumulative weekly weight (gm) per bird of broiler on different diets.

Treatment	Weekly cumulative mean weight (gm)					
	1 st	2 nd	3 rd	4 th	5 th	6 th
T1	135.00 ^b	336.60 ^{bcd}	625.40 ^{abc}	993.80	1300.20 ^c	1905.00
T2	145.20 ^a	353.80 ^{ab}	632.00 ^{ab}	1081.20	1544.80 ^a	2148.75
T3	129.90 ^b	347.10 ^{abc}	605.00 ^{bc}	1058.70	1549.80 ^a	1958.75
T4	146.00 ^a	327.60 ^{cd}	642.20 ^a	1054.10	1494.50 ^{ab}	1940.31
T5	144.00 ^a	358.20 ^a	636.20 ^{ab}	1094.60	1425.20 ^b	2168.75

T6	135.60 ^b	325.60 ^d	598.80 ^c	1149.00	1515.70 ^{ab}	2242.19
Probability	0.001	0.014	0.048	0.230	0.001	0.303
CV %	2.54	3.21	2.75	6.53	3.93	10.24
SEM	2.04	6.32	9.89	40.38	33.35	121.79

Means in the column with different superscripts differ significantly. ^{ns}not significantly different, *significant at 5% ($p<0.05$) and **significant at 1% ($p<0.01$)

Feed conversion ratio

Average weekly feed conversion ratio (FCR) of Cobb-500 on different diets is shown in Table 5. There was difference ($p<0.01$; $p<0.05$) in FCR among treatments during the complete experimental period. In the first week, T1, T3 and T6 broilers showed promising FCR ($p<0.01$) than others, while T2, T3 and T5 was significant during the 2nd week and T2 and T6 in the 3rd week. Similarly, FCR was better in T2 and T6 in 4th week, T2, T3 and T6 in 5th week and T2 and T6 in finishing age.

Table 5. Mean feed conversion ratio of broiler on different diets at Pokhara, Kaski.

Treatment	Mean feed conversion ratio					
	1 st	2 nd	3 rd	4 th	5 th	6 th
T1	2.27 ^a	2.44 ^{bc}	2.63 ^{abc}	2.64 ^{ab}	2.98 ^a	2.72 ^a
T2	2.11 ^b	2.22 ^d	2.47 ^c	2.23 ^{cd}	2.34 ^c	2.27 ^{ab}
T3	2.34 ^a	2.44 ^{bc}	2.83 ^a	2.47 ^{abc}	2.34 ^c	2.41 ^{ab}
T4	2.16 ^b	2.66 ^a	2.75 ^{ab}	2.72 ^a	2.69 ^b	2.72 ^a
T5	2.13 ^b	2.37 ^c	2.62 ^{abc}	2.40 ^{bc}	2.67 ^b	2.43 ^{ab}
T6	2.28 ^a	2.47 ^b	2.59 ^{bc}	2.11 ^d	2.01 ^c	1.96 ^b
Probability	0.0003	0.001	0.03	0.0021	0.0003	0.037
CV %	2.21	1.60	4.26	6.07	5.95	11.26
SEM	0.03	0.02	0.07	0.09	0.09	0.16

Means in the column with different superscripts differ significantly, ^{ns}not significantly different, * significant at 5% ($p<0.05$) and ** significant at 1% ($p<0.01$)

Economics of Cobb-500 poultry raised with six different treatments

The economic analysis of Cobb-500 birds managed under 6 different treatments is given in Table 6. The total expenditure was highest in the bird fed with T3 diet (Rs. 514.45), followed by the bird fed with T4 (Rs. 509.58), T1 (Rs. 504.30), T5 (Rs. 499.58), T2 (Rs. 497.14) and T6 (Rs. 456.67) respectively. However, the B:C ratio was highest in T6 (1.33) and followed by T5 (1.17) and T2 (1.17), T3(1.03) and T4 (1.03) and T1 (1.02) respectively. From the economic point of view, *B. aristata* based diet performed best.

Table 6. Economic analysis of Cobb 500 broiler on different diets at Pokhara, Kaski.

Commodity	T1	T2	T3	T4	T5	T6
Expenditure (Rs.)						
Chick cost	105	105	105	105	105	105
Feed cost	289.17	282.01	299.32	294.45	284.45	241.54
Equipment	27	27	27	27	27	27
Labor	70.36	70.36	70.36	70.36	70.36	70.36
Medicine & Vaccination	12.77	12.77	12.77	12.77	12.77	12.77
Total cost	504.30	497.14	514.45	509.58	499.58	456.67
Income (Rs.)						
Live weight	514.35	580.16	528.86	523.88	585.56	605.39
Net profit	10.05	83.02	14.41	14.30	85.98	148.72
BC Ratio	1.02	1.17	1.03	1.03	1.17	1.33

Blood parameters

The effect of different treatments on serum profile is presented in Table 7. The serum phosphorus concentration was significantly higher ($p<0.05$) in T1 followed by T5 and the least in T3. Likewise, the serum albumin globulin ratio showed significant difference ($p<0.05$) with the highest albumen globulin ratio in T4 and lowest in T1 diet group suggesting the better immunity in the birds receiving T4 and T6 diets.

Table 7. Serum profile of broiler on different diets at Pokhara, Kaski.

Treatment	Glucose (mg/dl)	Calcium (mg/dl)	Phosphorus (mg/dl)	Cholesterol (mg/dl)	Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A:G
T1	315.00	14.90	5.65 ^a	73.00	2.62 ^a	1.46 ^{ab}	1.15 ^{ab}	1.26 ^b
T2	314.50	13.50	3.60 ^c	34.50	1.95 ^b	1.09 ^b	0.87 ^{ab}	1.39 ^b
T3	318.50	13.35	2.65 ^d	63.00	2.28 ^{ab}	1.31 ^{ab}	0.98 ^{ab}	1.41 ^b
T4	275.00	15.40	3.20 ^{cd}	73.50	2.15 ^{ab}	1.75 ^a	0.71 ^b	2.46 ^a
T5	282.00	13.05	4.65 ^b	54.00	2.56 ^a	1.25 ^{ab}	1.32 ^a	1.32 ^{ab}
T6	270.00	10.45	3.95 ^{bc}	64.00	1.97 ^b	1.33 ^{ab}	10.64 ^{ab}	2.17 ^b
Probability	>0.05	0.3077	0.001	0.318	0.056	0.229	0.1989	0.046
CV %	15.16	19.23	11.08	36.35	12.82	22.35	48.7	65.56
SE	25.9	1.49	0.25	12.66	0.17	0.18	0.25	0.93

Means in the column with different superscripts differ significantly, ^{ns}not significantly different, * significant at 5% ($p<0.05$) and ** significant at 1% ($p<0.01$)

DISCUSSION

The highest cumulative mean feed consumption was found in broiler kept on BD + probiotic (0.25g/kg). The results obtained in this study is similar with Selle *et al.* (2004), Paul *et al.* (2007) and Koley & Samanta (2012), who reported that supplementation of formic acid salts improved the feed consumption compared to control group. The results obtained are in contrary to the findings of Waldroup *et al.* (1995), Vale *et al.* (2004)

and Sukandhiya *et al.* (2017), who found reduced or no significance influence on feed consumption. In comparison to the control and antibiotics diet, clove oil containing diet had lowest feed consumption coincides with the findings of Mohammadi *et al.* (2014) which might be due to the active component eugenol which stimulates the growth and proliferation of *Lactobacillus* that changes the villi height and crypt depth of the small intestine.

Broiler fed with BD + Colistin sulphate (0.25gm/kg diet), BD + organic acid (0.5%), BD + clove oil (600ppm) and BD + *B. aristata* (0.4%) had higher mean weekly cumulative weight over BD only and BD + organic acid. In agreement with present study, increased in body weight by clove oil supplementation alone at different levels in broilers was reported by Dalkilic & Guler (2009), Mukhtar (2011) and Mehr *et al.* (2014). However, studies on combination of sodium diformate and clove oil were scanty, but combination of other organic acids and essential oils were found. A significant increase in body weight gain of broilers was reported due to combination of an essential oil and an organic acid by Alcicek *et al.* (2004), Ertas *et al.* (2005) and Bozkurt *et al.* (2012).

In view of supplementation of *B. aristata*, our findings coincide with ARS Annual report 2072; the better FCR might be due to the antimicrobial and antioxidant properties of the active component- berberine alkaloid. Similarly, there is improved FCR and weight gain than control diet in organic acid containing diet which might be due to the effect on pH level of the upper GI tract (Samanta *et al.*, 2008).

Overall, the lowest FCR was observed in broiler fed with BD + Colistin sulphate followed by BD + *B. aristata* and BD only. The results obtained in this study fall in line with the findings of Paul *et al.* (2007), Al-Kassi & Mohssen (2009), Ghazalah *et al.* (2011), Luckstadt *et al.* (2014), Pohl *et al.* (2012) and Venkatasubramani *et al.* (2014), who recorded improvement in FCR with inclusion of formic acid salts as feed supplement in broilers diet compared to control. On contrary, Isabel & Santos (2009) and Sukandhiya *et al.* (2017) reported significantly poor FCR for birds receiving feed containing formic acid. In present study, clove oil supplementation significantly improved feed efficiency as compared to control group. The results are in well accordance with Dalkilic & Guler (2009), Agostini *et al.* (2012) and Mehr *et al.* (2014). A significant improvement in feed efficiency in broilers on adding essential oil and organic acid was reported by Bozkurt *et al.* (2012), Giannenas *et al.* (2014) and Ragaa *et al.* (2016).

We observed non-significant effect of treatment diets in the serum glucose concentration but found the least in T6 and the highest in T3. This study did not specifically provide exact data on how clove oil promoted higher glucose concentration. This finding of serum glucose is against the finding of Mohammadi *et al.* (2014). Similar variations were seen in the cases in serum mineral, calcium, phosphorus. Among the alternatives, organic acid containing diet had highest serum phosphorus might be due to the increase nutrient digestibility by elevating protein and DM retention, improving mineral absorption and Phosphorus utilization (Nehzad *et al.*, 2011). The serum globulin level was highest (10.64 g/dl) in T6 and lowest in T4 suggesting enhanced immunity of the birds receiving T6 diet.

The serum protein content was observed higher in BD + organic acid (0.5%) and BD only. The results are in corroboration with Panda *et al.* (2009), Ghazalah *et al.* (2011) and Ramigani *et al.* (2015), who found significant increase in serum total protein on supplementation of formic acid. The results are in accordance with findings of Tariq *et al.* (2014), who reported serum protein, albumin, globulin; albumin-globulin ratio was not affected due to clove oil supplementation. Tollba *et al.* (2010) revealed that essential oil and organic acid supplementation increased plasma total protein as well as albumin and globulin, which was in harmony with present study. Findings of present study are supported by Panda *et al.* (2009), Ghazalah *et al.* (2011), Koley & Samanta (2012) and Ramigani *et al.* (2015), who reported increased in serum calcium and phosphorus level due to organic acid supplementation. This study is in line to Kalia *et al.* (2017) who states mortality decrease in probiotic diet at high altitude.

CONCLUSION

The weekly cumulative feed consumption showed remarkable trends from the 2nd to the 6th weeks, with clove oil-containing diet exhibiting the lowest consumption, potentially attributed to eugenol's influence on gut microbiota. Cumulative body weight demonstrated significant differences among treatments throughout the study period, with highest weight in T4 during the 1st week. FCR varied significantly among treatments across weeks, with T2 showing better FCR during the 1st week and T6 exhibiting superior performance during the 6th week. Economic analysis favored *B. aristate* based diet from a profitability standpoint. Serum parameters highlighted significant differences in phosphorus levels among treatments, with T6 showing the highest serum globulin, potentially indicating improved immunity. Overall, the study provides valuable insights on the efficiency of different dietary interventions on the growth, health, and economic performance of Cobb-500 broilers.

Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. A.O.A.C. (2000). *Official methods of analysis* (13th ed.). Association of Official Analytical Chemists.
2. Agostini, P. S., Sola-Oriol, D., Nofrías, M., Barroeta, A. C., Gasa, J., & Manzanilla, E. G. (2012). Role of in-feed clove supplementation on growth performance, intestinal microbiology, and morphology in broiler chicken. *Livestock Science*, 147(1–3), 113–118.
3. Alcicek, A., Bozkurt, M., & Çabuk, M. (2004). The effect of a mixture of herbal essential oils, an organic acid or a probiotic on broiler performance. *South African Journal of Animal Science*, 34(4), 217–222.
4. Al-Kassi, A. G., & Mohssen, M. A. (2009). Comparative study between single organic acid effect and synergistic organic acid effect on broiler performance. *Pakistan Journal of Nutrition*, 8(6), 896–899.
5. Arsène, M. M. J., Davares, A. K. L., Viktorovna, P. I., Andreevna, S. L., Sarra, S., Khelifi, I., & Sergueievna, D. M. (2022). The public health issue of antibiotic residues in food and feed: Causes, consequences, and potential solutions. *Veterinary World*, 15(3), 662–671. <https://doi.org/10.14202/vetworld.2022.662-671>

6. Ayalew, H., Zhang, H., Wang, J., Wu, S., Qiu, K., Qi, G., Tekeste, A., Wassie, T., & Chanie, D. (2022). Potential feed additives as antibiotic alternatives in broiler production. *Frontiers in Veterinary Science*, 9, 916473. <https://doi.org/10.3389/fvets.2022.916473>
7. Bozkurt, M., Küçükyılmaz, K., Pamukçu, M., Çabuk, M., Alçiçek, A., & Çatli, A. U. (2012). Long-term effects of dietary supplementation with an essential oil mixture on the growth and laying performance of two-layer strains. *Italian Journal of Animal Science*, 11, e5.
8. Castanon, J. I. R. (2007). History of the use of antibiotic as growth promoters in European poultry feeds. *Poultry Science*, 86, 2466–2471.
9. Choct, M. (2001). Alternatives to in-feed antibiotics in monogastric animal industry. *American Soybean Association Technical Bulletin*, 30, 1–6.
10. Cobb-Vantress. (2018). *Cobb 500 broiler management guide*. <https://www.cobb-vantress.com>
11. Dibner, J. J., & Richards, J. D. (2005). Antibiotic growth promoters in agriculture: History and mode of action. *Poultry Science*, 84, 634–643.
12. Endtz, H. P., Mouton, R. P., Vander Reyden, T., Ruijs, G. J., Biever, M., & van Klingeren, B. (1990). Fluoroquinolone resistance in *Campylobacter* spp. isolated from human stools and poultry products. *The Lancet*, 335, 787.
13. Ertas, O. N., Guler, T., Çiftçi, M., Dalkılıç, B., & Simsek, U. G. (2005). The effect of an essential oil mix derived from oregano, clove and anise on broiler performance. *International Journal of Poultry Science*, 4(11), 879–884.
14. Ghazalah, A. A., Atta, A. M., Elkloub, K., Moustafa, M. E., & Shata, R. F. (2011). Effect of dietary supplementation of organic acids on performance, nutrients digestibility and health of broiler chicks. *International Journal of Poultry Science*, 10(3), 176–184. <https://doi.org/10.3923/ijps.2011.176.184>
15. Giannenas, I., Papaneophytou, C. P., Tsalie, E., Pappas, I., Triantafyllou, E., Tontis, D., & Kontopidis, G. A. (2014). Dietary supplementation of benzoic acid and essential oil compounds affects buffering capacity of the feeds, performance of turkey poults and their antioxidant status, pH in the digestive tract, intestinal microbiota and morphology. *Asian-Australasian Journal of Animal Sciences*, 27(2), 225–236. <https://doi.org/10.5713/ajas.2013.13376>
16. Groschke, A. C., & Evans, R. J. (1950). Effects of antibiotics, synthetic vitamins, vitamin B12 & an APF supplement on chick growth. *Poultry Science*, 29, 616–618.
17. Huyghebaert, G., Ducatelle, R., & Van Immerseel, F. (2011). An update on alternatives to antimicrobial growth promoters for broilers. *The Veterinary Journal*, 187, 182–188.
18. Innes, G. K., Markos, A., Dalton, K. R., Gould, C. A., Nachman, K. E., Fanzo, J., Barnhill, A., Frattaroli, S., & Davis, M. F. (2021). How animal agriculture stakeholders define, perceive, and are impacted by antimicrobial resistance: Challenging the Wellcome Trust's Reframing Resistance principles. *Agriculture and Human Values*, 38(4), 893–909. <https://doi.org/10.1007/s10460-021-10197-y>
19. Islam, K. M., Schaeublin, H., Wenk, C., Wanner, M., & Liesegang, A. (2012). Effect of dietary citric acid on the performance and mineral metabolism of broiler. *Journal of Animal Physiology and Animal Nutrition*, 96(5), 808–817. <https://doi.org/10.1111/j.1439-0396.2011.01225.x>
20. Kalia, S., Bharati, V. K., Gogoi, D., Giri, A., & Kumar, B. (2017). Studies on the growth performance of different broiler strains at high altitude and evaluation of probiotic effect on their survivability. *Scientific Reports*, 7, 46074. <https://doi.org/10.1038/srep46074>
21. Koley, S., & Samanta, G. (2012). Laying performances, gut health and blood profile of Khaki Campbell ducks supplemented with dietary organic acids. *Indian Journal of Poultry Science*, 47(1), 67–74.
22. Mehr, M. A., Hassanabdi, A., & Nassiri, M. K. (2014). Supplementation of clove essential oil and probiotic to the broiler's on performance, carcass and blood components. *Journal of Applied Animal Science*, 4(1), 117–122.
23. Mohammadi, Z., Ghazanfari, S., & Moradi, M. A. (2014). Effect of supplementing clove essential oil to the diet on microflora population, intestinal morphology, blood parameters and performance of broilers. *European Poultry Science*, 78.
24. Moore, P. R., Evenson, A., Luckey, T. D., McCoy, E., Elvehjem, C. A., & Hart, E. B. (1946). Use of sulfasuxidine, streptothricin and streptomycin in nutritional studies with the chick. *Journal of Biological Chemistry*, 165, 437–441.
25. Mukhtar, M. A. (2011). The effect of dietary clove oil on broiler performance. *Australian Journal of Basic and Applied Sciences*, 5(7), 49–51.
26. Muteeb, G., Rehman, M. T., Shahwan, M., & Aatif, M. (2023). Origin of antibiotics and antibiotic resistance, and their impacts on drug development: A narrative review. *Pharmaceuticals (Basel)*, 16(11), 1615. <https://doi.org/10.3390/ph16111615>

27. Panda, A. K., Raju, M. V. L. N., Rao, S. V., Sunder, G. S., & Reddy, M. R. (2009). Effect of dietary addition of organic acid on performance, serum biochemical parameters and carcass traits of broiler chicken. *Indian Journal of Animal Nutrition*, 26(2), 186–190.
28. Paul, S. K., Halder, G., Mondal, M. K., & Samanta, G. (2007). Effect of organic acid salt on the performance and gut health of broiler chicken. *The Journal of Poultry Science*, 44(4), 389–395.
29. Pelicano, E. R. L., Souza, P. A., Souza, H. B. A., Figueiredo, D. F., Boiago, M. M., Carvalho, S. R., & Bordon, V. F. (2005). Intestinal mucosa development in broiler chicken fed natural growth promoters. *Brazilian Journal of Poultry Science*, 7, 221–229.
30. Pohl, S. A., Caldwell, D. J., Lee, J. T., Coppedge, J. R., Dunn-Horrocks, S. L., Stringfellow, K. D., Jessen, K., & Farnell, M. B. (2012). Effects of dietary calcium formate inclusion on broiler performance, skeletal development, and gut maturation. *Journal of Applied Poultry Research*, 21(2), 311–317.
31. Ragaa, N. M., Korany, R. M., & Mohamed, F. (2016). Effect of thyme and/or formic acid dietary supplementation on broiler performance and immunity. *Agriculture and Agricultural Science Procedia*, 10, 270–279.
32. Rahman, M. R. T., Fliss, I., & Biron, E. (2022). Insights in the development and uses of alternatives to antibiotic growth promoters in poultry and swine production. *Antibiotics*, 11, 766. <https://doi.org/10.3390/antibiotics11060766>
33. Ramigani, V. R., Ramana, J. V., Srinivasa, R. D., Shakila, S., & Suresh, J. (2015). Effect of dietary supplementation of organic acid combinations on gut pH and *E. coli* count of intestinal contents in broilers. *Indian Journal of Poultry Science*, 50(1), 24–27.
34. Rosen, G. D. (1995). Antibacterials in poultry and pig nutrition. In *Biotechnology in animal feeds and animal feeding* (pp. 143–172).
35. Samanta, S., Haldar, S., & Ghosh, T. K. (2008). Production and carcass traits in broiler chickens given diets supplemented with inorganic trivalent chromium and an organic acid blend. *British Poultry Science*, 49, 155–163.
36. Seal, B. S., Lillehoj, H. S., Donovan, D. M., & Gay, C. G. (2013). Alternatives to antibiotics: A symposium on the challenges and solutions for animal production. *Animal Health Research Reviews*, 14, 78–87.
37. Selle, P. H., Huang, K. H., & Muir, W. I. (2004). Effects of potassium diformate inclusion in broiler diets on growth performance and nutrient utilization. In *Proceedings of the 16th Australian Poultry Science Symposium* (pp. 55–58).
38. Sukandhiya, K., Mani, K., Rajendran, K., Sangli Vikram Kumar, K., Prabhakaran, U., & Sasidhar, T. (2017). Influence of dietary supplementation of sodium diformate on the intestinal histo-morphology of broilers in environmentally controlled housing system. *International Journal of Chemical Studies*, 5(6), 842–843.
39. Tariq, H., Raman Rao, P. V., Mondal, B. C., & Malla, B. A. (2014). Effect of Aloe vera (*Aloe barbadensis*) and clove (*Syzygium aromaticum*) supplementation on immune status, haematological and serum biochemical parameters in Japanese quails. *Indian Journal of Animal Nutrition*, 31(3), 293–296.
40. Tiwari, P., Ahmad, K., & Hassan Baig, M. (2017). *Gymnema sylvestre* for diabetes: From traditional herb to future therapeutic. *Current Pharmaceutical Design*, 23(11), 1667–1676.
41. Tollba, A. A. H., Shabaan, S. A. M., & Abdelmageed, M. A. A. (2010). Effects of using aromatic herbal extract and blended with organic acids on productive and physiological performance of poultry. *Egyptian Poultry Science*, 30(1), 229–248.
42. Vale, M. M. D., Menten, J. F. M., Morais, S. C. D. D., & Brainer, M. M. D. A. (2004). Mixture of formic and propionic acid as additives in broiler feeds. *Scientia Agricola*, 61, 371–375.
43. Venkatasubramani, R., Vasanthakumar, P., Chandrasekaran, D., Rajendran, D., & Purushothaman, M. R. (2014). Performance of broilers fed formic and propionic acid supplemented diets. *Animal Nutrition and Feed Technology*, 14(1), 81–90.
44. Waldroup, A., Kaniawati, S., & Mauromoustakos, A. (1995). Performance characteristics and microbiological aspects of broilers fed diets supplemented with organic acids. *Journal of Food Protection*, 58(5), 482–489.
45. World Health Organization. (2012). *The evolving threat of antimicrobial resistance: Options for action*. <https://www.who.int/publications/i/item/9789241503181>

Assessment of Antibiotic Residue in Chicken Meat Sampled from Retail Meat Shop of Kaski, Nepal

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ABSTRACT

A cross-sectional study was conducted to assess the antibiotic residue in chicken meat from retail meat shops in the Kaski district of Nepal. A total of 148 (74 Muscle and 74 liver) samples were collected and analyzed using a rapid test kit. All the procedure was followed as per the guideline provided by the manufacturer. The test revealed that 1.35 % and 24.32 % of muscle samples were positive for Group A (Tetracycline residue) and Group B (Sulphonamides, Aminoglycosides, and Macrolides residues) respectively. Similarly, 18.92 % of livers contain Group A residue and 54 % have Group B residue. The multidrug residue (containing both Groups) was found in one muscle and 13 liver samples. The positive samples were further evaluated using the quantitative method of ELISA. The results showed no sample above the maximum residual limit (MRL) for tetracycline (200 µg/kg) with mean and maximum residue concentrations in liver samples as 1.23 ± 0.44 µg/kg and 3.48 µg/kg respectively. For sulphonamides, three liver samples exceeded the MRL (100 µg/kg) with the highest residue of 137.68 µg/kg. In both tests, antibiotic residue detected in the liver was significantly higher ($P \leq 0.05$) than in the muscle. As none of the muscle samples crossed the MRL value, we can conclude that the meat from the retail meat shops in the Kaski district is safe from the perspective of tested antibiotic residue. However, antibiotic residue even at lower than the MRL indicates farmers are using antibiotics without following withdrawal period. Related stakeholders should be aware and guidelines for prudent use of antibiotics in poultry should be developed.

Keywords: Antibiotic; Chicken meat; Kaski; Nepal; Residue

INTRODUCTION

Chicken meat stands as one of the top meat commodities produced and consumed in Nepal. According to livestock statistics of Nepal 2019/20, chicken alone contributes 45 % of total meat produced in Nepal (DLS, 2021). Chicken meat is the cheapest and healthiest meat among the different meat consumed worldwide. Poultry farming is an integral part of the Nepalese economy contributing 4 % of the National Gross Domestic Product (FAO, 2014). It has less religious taboo compared to buff and pork. Per capita consumption of meat increases every year with the increasing purchasing capacity of people. The poultry population in Nepal has almost doubled in the last decade (Yadav et al., 2021).

On the other hand, the poultry industry of Nepal is facing several challenges to sustain. The outbreak of economically important diseases throughout the country is one of the top constrain hindering the growth of this sector (Osti et al., 2017). During disease outbreaks, farmers use many kinds of antibiotics to minimize the mortality of birds. Besides, antibiotics are used for preventing disease and as a growth promoter. Antibiotics are the synthetic or semisynthetic substances used to treat various bacterial infections. Poultry farmers use antibiotics to treat bacterial infection such as colibacillosis, salmonellosis, necrotic enteritis, fowl cholera, etc. In poultry, the most common route of administration of antibiotics in poultry is oral through drinking water. After absorption of antibiotics from the intestine, most of them are distributed throughout the body in different concentrations and eliminated from the body after some time. If withdrawal period is not followed, the residue of these antibiotics remains in chicken tissue. Therefore, consuming meat contaminated with antibiotic residue has adverse effects like hypersensitivity, carcinogenicity, mutagenicity, etc. on human health. Moreover, antibiotic residue is also linked with antimicrobial resistance. sulphonamides and tetracycline are the two major antibiotics frequently used in broiler farming of Nepal (Pantha et al., 2019).

The issue of antibiotic residue in chicken tissue has been getting more attention recently as consumers are more aware of food safety. Besides, antibiotic resistance is considered a global public health threat. So, it is essential to monitor and examine antibiotic residue in marketed meat. CODEX, FAO is an international organization setting standards in food safety which has also set maximum residue levels for antibiotic residue in chicken meat. Veterinary Standard and Drug Regulatory Laboratory have also published standard MRL for different antibiotic residues in chicken tissue. The MRL value for sulfonamide in chicken meat is 100 µg/kg and that of tetracycline is 200 µg/kg (VSDLR, 2017). Several researchers studied antibiotic residues in chicken meat marketed in different parts of Nepal. However, most of them are limited to qualitative studies. Therefore, this study is designated to quantify antibiotics in chicken meat marketed in retail meat shops of Kaski district. The findings highlight the safety of meat for human consumption.

Various methods are available for qualitative and quantitative detection of antibiotic residue. In this study, a commercially available rapid test kit was used to screen antibiotic residue. ELISA was used to quantify the antibiotic residue in positive samples after rapid test. ELISA test was adopted because it has high sensitivity and time saving. Result obtained from the ELISA test was compared with the MRL value to determine whether the meat is safe for human consumption or not. This study is designed for screening and quantification of antibiotic residues in chicken meat (liver/muscle) collected from the retail meat shops of Kaski district of Nepal.

Antibiotics are widely used in chicken for the purpose of treatment, prevention and also as a growth promoter. Under/over dosing of antibiotic, failure to follow withdrawal period, misdiagnosis, overuse of antibiotic in viral disease, off-label use of antibiotic and counter sale of antibiotic leads to contamination of chicken meat product with antibiotic residue. These residues have determinant hazards on human health. Moreover, it is proven that antibiotic residue in meat is also linked with the emergence of antibiotic resistance (Patra et al., 2019). So, it is essential to assess and examine meat of chicken origin from a public health perspective.

Kaski district of Nepal is one of the major districts with 3068254 heads of fowl (DLS, 2021). Nepal being a member of the World Health Organization (WHO), the World Animal Health Organization (OIE), the Food and Agriculture Organization (FAO), and the World Trade Organization (WTO), it has an obligation to respect the standards and rules on MRL set by CODEX, FAO. The previous study on antibiotic residue in marketed chicken meat from different regions of Nepal showed a higher prevalence of various antibiotic residues including tetracycline and sulphonamides in chicken meat. However, most of the studies were limited to qualitative tests. Very few studies have been done to identify the residue status of chicken meat in the Kaski district.

Many consumers have a perception that the broiler birds are raised feeding high amount of antibiotics and hormones and the residues of these antibiotics are causing a significant health impact in human. The outcomes of this research will provide a clear-cut understanding of whether the marketed chicken meat is safe for human consumption or not from the viewpoint of antibiotic residue contamination. This research will also be fruitful in raising the importance of one health concept by emphasizing human health through food safety.

MATERIALS AND METHODS

The sample for this study was collected from Kaski district of Gandaki Province, Nepal. The district has an area of 2,017 square km, with Pokhara as its district headquarters. The district consists of one metropolitan city and four rural municipalities (MoFA, 2018). Pokhara is considered as the tourism capital of Nepal and the capital city of Gandaki

Province. According to poultry entrepreneur's forum of the Kaski district, it has around 690 registered chicken meat shops. The number of meat shops for sample collection was calculated using Raosoft software (cited in: <http://www.raosoft.com/samplesize.html>) considering the 14.23 % prevalence of antibiotic residue in chicken meat in the Kaski district (Gumpo et al., 2020). Shops were selected based on simple random sampling method using MS excel. One sample either liver or muscle was collected randomly from each meat shop. A total of 148 meat samples (74 breast muscle samples and 74 liver samples) were collected from 148 retail meat shops of Kaski District of Nepal during the month of February 2021. About 60 grams of meat and liver sample were collected aseptically in a sterile sample container, labeled, and transported to the laboratory in an ice pack box (4°C). Samples were stored at -20 degrees centigrade until analysis. Samples were analyzed in Veterinary Standard and Drug Regulatory Laboratory, Budanilkantha, Kathmandu, Nepal. After the rapid screening, each sample was stored at -84 degree centigrade for further analysis. Sample positive in rapid screening were subjected to quantification using ELISA kits. Chi-square test and Fisher exact was performed using Open Epi tool version 3.0. The concentrations obtained from ELISA test result analysis were compared with MRL set by CODEX, FAO and VSDLR, Nepal.

Rapid screening for antibiotic residue

Each sample was screened first by using a rapid test kit obtained from Asian Medic Co., Ltd. The protocol of the rapid test was followed as per the "Manual of test kit for determination of Drug residues in Meat" provided by the kit manufacturer. The test kit used for this study obtained from Asian Medic Co., Ltd, has 93 % accuracy, 78.9 % sensitivity, and 96.7 % specificity. First of all, each sample was thawed out of the refrigerator for 4 hours. Skin, external fat and fascia were removed from the muscle sample, cut into small pieces and minced in an electronic tissue blender for 3 minutes. Five gram of sample was kept in two centrifuge tube of 50 ML capacity, one for test for Group A and another for Group B antibiotics. 5 ml Extraction solution A provided in kit was added in one centrifuge tube and 5 ml extraction solution B was kept in another centrifuge tube. The rest of the procedure was similar for both group of antibiotics. Extraction solution A was used to determine group A antibiotics i.e. for the Tetracycline group and solution B was used for determination of group B antibiotics i.e. Macrolide, Aminoglycoside and Sulfonamide group. Each tube was then shaken vigorously using vortex mixture for 10 minutes. Then the tubes were placed in water bath and heated slowly until reaching 60 C and maintained for 5 minutes. Each test tube was removed from water bath and cool down. After that, each sample were centrifuged at 3500 rpm for 15 minutes and supernatant was removed and placed in EP tube. pH of supernatant was adjusted to 6.5 for Group A tube and 7 for Group B tube with addition of drop of 1 N NaOH.

Four drops of the supernatant obtained from using different extraction solution were added to tube provided in the kit. Four drops of the negative control sample provided in the kit

were also added in one tube. All the tubes were incubated at 64 degrees centigrade for more than 2.45 hours in the water bath until the color of the medium in negative control tube completely changed from purple to yellow. Colour changes in the sample tube were also observed and compared with negative control and classified as positive and negative based on the colour change. Changes in color from purple to yellow indicate negative result for drug residue of particular group. The presence of purple colour indicates the presence of drug residue. Data was recorded and entered in MS excel and analyzed.

ELISA test for antibiotic residue

The samples positive in the rapid test were subjected for further analysis using ELISA kit of Sulphonamides and Tetracycline from Elabscience, USA. This is a competitive enzyme immunoassay for quantitative analysis of Tetracycline, Mincocycline, Chlortetracycline and Oxytetracycline in milk, honey and meat. Procedure for assay was followed as per the manufacturer's guidelines. This kit has sensitivity of 0.03 ppb and detection limit of 12 ppb. Cross reactivity of kit with Tetracycline, Chlortetracycline is 100% while that of Oxytetracycline is 51% and Doxycycline is 8.5%.

First of all, reconstitution buffer and wash buffer were diluted with deionized buffer. The extraction solution was prepared by mixing diluted HCL and methanol anhydrous. The sample was prepared by removing fat, skin and fascia followed by homogenization. A homogenized sample of about 1 gram was taken in a 50 ml centrifuge tube and 2 ml of extraction solution was added. After vortexing for 5 minutes, the mixture was centrifuged at 4000 RPM for 10 minutes at room temperature. Next, 50 µl of supernatant was taken in another centrifuge tube and mixed with 950 µl reconstitution buffer. 50 µl was taken for analysis.

Standard solution of different concentrations was prepared. 96 well micro titer plate provided by the company was taken and each well was numbered in order. Each sample and standards are tested in duplicate. First, 50 µl of standard and sample liquid were added in well. Each well was then filled with 50 µl of antibody working solution and covered with a plate sealer. The plate was oscillated for 5 seconds and incubated at 37 C for 30 minutes. The seal and liquid in each well were removed. Wash buffer of 300 µl was added and washed 5 times. The plate was inverted and pated against thick clean absorbent/tissue paper. After this, 100 µl of HPR conjugate was added to each well and mixed with 50 µl of substrate reagent A and B followed by washing. The sample was oscillated for 5 seconds and mixed thoroughly. Then the plate was incubated at 37 C for 30 min in shading for colour development. Stop solution (50 µl) was added to each well and oscillated for mixing. For optical density measurement (OD) the plate was installed in a micro plate reader set at 450 nm (reference wavelength 630 nm).

This kit has a sensitivity of 0.05 ppb and detection limit of 0.5 ppb. The sample was prepared by removing fat, skin and fascia followed by homogenization. A homogenized

sample of about 3 grams was taken in 50 ml centrifuge tube and 3 ml of 0.02 M PB Buffer (Solution 2) were added and oscillated. Then 4mL of Ethyl acetate and 2 ml of Acetonitrile was added. After vortexing for 10 minutes, the mixture was centrifuged at 4000 rpm for 10 minutes at room temperature. 2 ml of supernatant was taken in another centrifuge tube and dry at 50-60°C in water bath. The residue obtained was dissolved with 1 ml of N-hexane, 1mL of Reconstitution Buffer (Solution 4) was added and oscillated for 1 min to mix fully. After this, centrifuge the mixture for 5 min at 4000 rpm. N-hexane upper layer was discarded and 50µL of the lower layer was taken for analysis.

Standard liquid of different concentrations was prepared. 96 well micro titer plate was taken and each well was numbered in order. Each sample and standards are tested in duplicate. 50 µl of standard and sample liquid were added in well. Each well was then filled with 50 µl of HPR conjugate followed by 50 µl of antibody working solution and oscillated gently for 5 seconds. Then the plate was covered with plate sealer and incubated at 25°C for 45 minutes in shading light. The seal and liquid in each well were removed. 300 µl of wash buffer (Solution 5) was added and washed for 5 times. The plate was inverted and pated against thick clean absorbent/tissue paper. After this, 50 µl of substrate reagent A was added to each well and mixed with 50 µl of substrate reagent B followed by oscillation for 5 seconds. Then the plate was incubated at 25°C for 15 minutes in shading light for colour development. Stop solution (50 µl) was added to each well and oscillated for mixing. To measure optical density (OD), the plate was installed in micro plate reader set at 450 nm. Reading was done within 10 min after adding the stop solution.

RESULTS

Result of rapid test

Out of 148 muscle and liver samples analyzed 72 samples were found to contain atleast one type of antibiotic residue. Out of 72, none of the muscle samples were found to have only group A antibiotic residue, 18 had group B residue and one sample had both group A and group B. Out of 74 liver samples analyzed, 14 liver samples were positive for group A residue, 40 liver samples are positive for group B residue and 13 liver samples were positive for both group A and group B residue as shown in Table 1 and 2.

Table 1. Antibiotic residue for Group A (Tetracycline)

Sample	Positive	Negative	Total
Muscle	1(1.35 %)	73 (98. 65 %)	74
Liver	14 (18.92 %)	60 (81.08 %)	74
Grand total	15 (10.14 %)	133 (89.86 %)	148

All 148 sample were subjected to a rapid test to detect tetracycline residue. Among 74 muscle sample tested, only 1 (1.35 %) sample was postive for tetracycline residue. However, 14 (18.92 %) liver samples were positive for tetracycline residue. In total, 15

(10.14 %) sample contained tetracycline residue. The group A residue in liver was found in a significantly higher number of samples compared to muscle samples ($P < 0.01$). The odd ratio was found to be 0.05 for group A.

Table 2. Residue for Group B (Macrolid, Aminoglycoside & Sulphonamides)

Sample	Positive	Negative	Total
Muscle	18 (24.32 %)	56 (75.68%)	74
Liver	40 (54 %)	34 (45.95%)	74
Grand total	58 (39.19 %)	90 (60.81 %)	148

Out of 74 muscle and 74 liver sample subjected to rapid screening, 18 muscle (24.32%) and 40 (54 %) liver samples contained antibiotic residue of Macrolids, Aminoglycosides and Sulphonamides. The group A residue was found in a significantly higher number of liver samples than muscle samples ($P < 0.01$). The odds ratio for group B was 0.27.

Out of 74 muscle samples analyzed, only one muscle sample had multidrug residue (containing both group A and B). No muscle samples have only group A residue and 17 (22.97 %) of the muscle samples were positive for only group B residue. Similarly, 13 (17.57 %) liver samples have multidrug residue, only one liver sample had only group A residue and 44 (59.46%) of liver samples were positive for group B residue. Altogether, out of 148 samples, 9.46 % of the samples have multidrug residue and 30 samples have either group A or group B residue, as shown in Table 3.

Table 3. Sample containing multi drug residue (Both Group A and Group B)

Sample	Multidrug residue	Only group A residue	Only group B residue	Single residue (A+B)	Negative	Total
Muscle	1 (1.35 %)	0	17 (22.97%)	17 (22.97%)	56 (75.67%)	74
Liver	13 (17.57%)	1	27 (36.48%)	28 (37.84%)	33 (44.59%)	74
Grand total	14 (9.46 %)	1	44 (59.46%)	45 (30.40%)	89 (60.13%)	148

ELISA result

The samples positive in the rapid test were further evaluated using the ELISA test. Using curve expert software, a standard curve was created by plotting the OD value of each standard on X- axis against concentration on the Y- axis and a semi logarithmic plot was drawn. There is a negative correlation between the OD value of samples and the concentration of antibiotic residue. The average absorbance value was added to the standard curve to get the corresponding concentration. The concentration in the samples can be calculated by comparing the OD of the samples to the standard curve. Finally, the obtained concentration value was multiplied by the dilution factor.

The result obtained from ELISA test is summarized in Table 4. ELISA test for tetracycline revealed no sample crossing MRL level. The residue level of tetracycline in one muscle sample was 1.84 µg/kg. Mean residue concentration and maximum residue concentration of tetracycline in the liver sample were found as 1.2267 ± 0.438 µg/kg and 3.48 µg/kg respectively.

On ELISA test for sulphonamides, only three liver sample exceeded the maximum residue level (100 µg/kg) with maximum residue of 126.52 µg/kg. All the muscle samples tested for sulphonamides residue have concentration below MRL. The mean sulphonamides residue in the chicken liver sample was higher (28.84 ± 10.13 µg/kg) than in the muscle sample (7.25 ± 3.04 µg/kg). On the student t-test, the concentration of sulphonamides residue in liver was significantly higher than in the muscle sample ($P < 0.01$).

Table 4. Summary of results obtained from ELISA test

Antibiotic	Tissue	Total no of sample	Mean residue (µg/kg)	Maximum residue	MRL value (µg/kg)	No. of sample above MRL
Sulphonamides (µg/kg)	Muscle	18	13.72 ± 14.3	50.77	100	Nil
	Liver	40	35.05 ± 35.14	137.68	100	3
Tetracycline (µg/kg)	Muscle	1	1.84	1.84	200	Nil
	Liver	14	1.23 ± 0.44	3.48	600	Nil

DISCUSSION

These day antimicrobial resistant is considered as a global public threat. Multi-drug resistant bacteria were detected in chicken carcass marketed in retail meat shops in Nepal (Nelson et al., 2020). It has already been proven that antibiotic residue, even at lower concentration is one of the prominent factor contributing to the emergence of antibiotic residue. Several researcher have conducted studies on antibiotic residue of chicken meat in different region of Nepal. From results obtained from the rapid test, 24.32% of muscle samples and 55.40% of the liver samples were found to contain antibiotic residue. Out of 148 samples assessed, 39.86 % samples were contaminated with antibiotic residue which is higher than the residue detected (24.66%) by Maharajan et al., 2020 in Kathmandu valley and lower than the residue detected (57%) by Thapa, 2021 in Dharan.

Raut et al., (2017) found a higher prevalence i.e. 39% of muscle samples and 71 % of the liver sample positive for a residue due to one more group (Penicillin group) being tested. However, in Kathmandu valley Maharjan et al. (2020) detected 10.66 % muscles and 40 % liver samples were positive. Similarly, another study by Sapkota et al. (2019) in Kathmandu valley detected antibiotic residue in 16.67% of muscle and 10% the liver using a Microbial Inhibition Test. In addition, Pantha et al. (2019) study on Kathmandu valley recorded 30.81 % of breast muscles having antibiotic residue. The difference could

be due to the sensitivity of the test kit used, sampling area, sample size, etc considered for the test.

Out of 148 samples tested in this study, 10.14% samples were positive for group A (Tetracycline) on the rapid test. In detail, 1.35% and 18.92% of liver samples were positive for group Maharjan et al., 2020 found a little bit higher i.e. 5.33% of muscle and 22.66% liver samples positive for tetracycline in Kathmandu valley while Gumpo et al. (2020) found higher i.e. 50 % of muscle sample positive for Tetracycline residue. Similarly, 58% sample was positive for group B (aminoglycosides, macrolid and sulphonamides). In detail, 24.32 % of the muscles sample and 54 % of liver samples were positive for group B residue. Among 148 samples analyzed, 9.46% of samples (1.35% muscle and 17% of liver) have multidrug residue and 30.40% have either group A or group B residue. This finding is in agreement with the finding of Koirala et al., 2021 as they detected one fourth of the farmers using more than two antibiotics at a time.

ELISA is used in this study to quantify the residue of selected antibiotic as an immunoassay method that provides excellent sensitivity and selectivity to determine antibiotic residues in meat samples (Ramtla et al., 2017). The mean concentration of tetracycline residue in liver sample of this study (1.22 ppb) is close to the concentration (1.44 ppb) obtained by Gumpo et al., 2020. Ramtla et al. (2017) conducted a study on evaluating of antibiotic residues in raw meat using ELISA, considering 150 samples from retail meat shop in Africa. They found the mean residual concentration of sulphonamides in the muscle samples as 47.5 ± 6.5 ppb and that in liver sample is 73.4 ± 12.56 ppb with none of sample crossing MRL. In comparison, the mean residue concentration of sulphonamides in this study was lower both in muscle (13.72 ± 14.3 ppb) and in liver samples (35.05 ± 35.14 ppb). Similarly, they found higher mean concentration of tetracycline (62.5 ± 23 ppb for muscle and 125.5 ± 22.5 ppb for liver) compared to 1.83 ppb in muscle and 1.23 ppb in liver sample in present study. The difference could be due to difference in the geographical region, antibiotic use practices, level of farmer awareness and withdrawal period observed.

The antibiotic residue detected in all muscle sample analyzed was below the MRL which agreed with the study done by Gumpo et al., 2020 and Ramtala et al., 2017. Significantly higher ($P < 0.05$) number of liver sample was found to be contaminated with antibiotic compared to muscle samples. This finding is supported by (Maharjan et al., 2020; Gumpo et al., 2020 & Thapa, 2021). Similarly antibiotic concentration detected was significantly higher ($P < 0.05$) in liver sample than in muscle samples. This finding also is in agreement with the finding of Cheong et al. (2010) & Shabazi et al. (2015).

The literature available regarding the prevalence of antibiotic residue in chicken meat samples indicates higher rates of antibiotic residues. Variation in the recorded antibiotic test may be due to the difference in sample size, method used, region, and other factors. A significantly higher number of liver sample contain significantly higher concentration

of antibiotic residue tested. The highest detection level could be due to primary roles of the liver in the metabolism and detoxification of drugs.

In summary, tetracycline residue was detected in less number of muscle and liver samples compared to sulphonamide residue. Similarly, the mean concentration of Tetracycline was lower compared to that of sulphonamide. This could be due to wider use of sulphonamides than tetracycline in broiler farming. An ELISA is an inexpensive, time-saving method sufficient to monitor antibiotic residue in large number of samples. However, this study would be more confirmatory with a minimal false positive result if quantification of the antibiotic residue using sophisticated and standard confirmatory methods like HPLC and LCMS had been done. The concerned government authorities need to monitor the residues regularly in the chicken meat as paravets and farmers frequently administer antibiotics without following the withdrawal period. Therefore, to avoid unwanted effects, it is essential to make related stakeholders aware of the proper use of veterinary drugs. Therefore, guidelines for antimicrobial use in livestock and poultry should be developed and implemented soon.

CONCLUSION

This study clearly indicated that the marketed chicken meat does not contain antibiotic residue above the MRL. However, 4.05% of liver samples in this study were found to have antibiotic residue above MRL, which indicates the risk of consumption of liver from the perspective of antibiotic residue. Though the antibiotics residue detected were below MRL, higher prevalence (40%) of antibiotic residue in marketed meat primarily in liver tissue (55%) indicates the intensive use of antibiotics in broiler farming. So, there is need of proper regulation and development of guideline for prudent use of antibiotic in poultry.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Abou-Raya, S. H. (2013). Effect of ordinary cooking procedures on tetracycline residues in chicken meat. *Journal of Food and Drug Analysis*, 21(1), 80–86.
2. Aman, I. M., Ahmed, H. F., Mostafa, N. Y., Kitada, Y., & Kar, G. (2017). Detection of tetracycline veterinary drug residues in Egyptian poultry meat by high performance liquid chromatography. *Journal of Veterinary Medicine and Allied Sciences*, 1(1), 51–57.
3. Bacanlı, M. (2019). Importance of antibiotic residues in animal food. *Food and Chemical Toxicology*. <https://doi.org/10.1016/j.fct.2019.01.033>
4. Central Bureau of Statistics. (2015). *Nepal Commercial Poultry Survey*. <https://microdata.cbs.gov.np/index.php/catalog/72>
5. Cheong, C. K., Hajeb, P., Jinap, S., & Ismail-Fitry, M. R. (2010). Sulfonamides determination in chicken meat products from Malaysia. *International Food Research Journal*, 17, 885–892.
6. Chopra, I., & Roberts, M. (2001). Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews*, 65(2), 232–260.
7. Eliopoulos, G. M., & Roberts, M. C. (2003). Tetracycline therapy: Update. *Clinical Infectious Diseases*, 36(4), 462–467. <https://doi.org/10.1086/367622>
8. Fanning, S., Wang, J., & Leonard, N. (2014). Veterinary drugs residues: Antibacterials. In Y. Motarjemi (Ed.), *Encyclopedia of Food Safety* (pp. 142–149). Academic Press. <https://www.sciencedirect.com/science/article/pii/B9780123786128002420>
9. Food and Agriculture Organization. (2014). *Poultry sector Nepal (FAO Animal Production and Health Livestock Country Reviews)*.
10. GARP-Nepal. (2015). *Situation analysis and recommendations: Antibiotic use and resistance in Nepal*. https://cddep.org/wp-content/uploads/2017/08/garp-nepal_sa.pdf
11. Gombo, T. R., Sapkota, R., Subedi, M., Koirala, P., & Bhatta, D. D. (2020). Monitoring of antibiotic residues in chicken meat, cow and buffalo milk samples in Nepal. *International Journal of Applied Sciences and Biotechnology*, 8(3), 355–362. <https://doi.org/10.3126/ijasbt.v8i3.31314>
12. Goren, E., De Jong, W. A., & Doornenbal, P. (1984). Some pharmacokinetic aspects of four sulphonamides and trimethoprim, and their therapeutic efficacy in experimental Escherichia coli infection in poultry. *Veterinary Quarterly*, 6(3), 134–140.
13. Khatun, R., Howlader, A. J., Ahmed, S., Islam, N., Alam, K., Haider, S., ... & Hasan, M. A. (2018). Validation of the declared withdrawal periods of antibiotics. *Universal Journal of Public Health*, 6(1), 14–22.
14. Koirala, A., Bhandari, P., Shewade, H. D., Tao, W., Thapa, B., Terry, R., ... & Karki, S. (2021). Antibiotic use in broiler poultry farms in Kathmandu Valley of Nepal: Which antibiotics and why? *Tropical Medicine and Infectious Disease*, 6(2), 47.
15. Kumar Yadav, R., & Yadav, R. (2021). A review on poultry population, production (egg and meat) and distribution in Nepal. *Food & Agribusiness Management (FABM)*, 2(1), 10–12.
16. Lambrou, A. S., Innes, G. K., O'Sullivan, L., Luitel, H., Bhattarai, R. K., Basnet, H. B., & Heaney, C. D. (2021). Policy implications for awareness gaps in antimicrobial resistance (AMR) and antimicrobial use among commercial Nepalese poultry producers. *Global Health Research and Policy*, 6(1), 1–9.
17. Maharjan, B., Neupane, R., & Bhatta, D. D. (2020). Antibiotic residue in marketed broiler meat of Kathmandu Metropolitan City. *Advances in Veterinary Science and Microbiology*, 3(1), 1–10. <https://doi.org/10.26502/avsm.010>
18. Mashak, Z., Mojaddar Langroodi, A., Mehdizadeh, T., EbadiFathabad, A., & HoomanAsadi, A. (2022). Detection of quinolone residues in beef and chicken meat in hypermarkets of Urmia, Iran using ELISA. *Iran Agricultural Research*, 36(1), 73–77.
19. Ministry of Agriculture and Livestock Development. (2021). *Livestock statistics of Nepal*. Department of Livestock Services. [PDF file].
20. MSD Veterinary Manual. (2022). Sulfonamides and sulfonamide combinations. <https://www.msddvetmanual.com/pharmacology/antibacterial-agents/sulfonamides-and-sulfonamide-combinations>
21. Mund, M. D., Khan, U. H., Tahir, U., Mustafa, B. E., & Fayyaz, A. (2017). Antimicrobial drug residues in poultry products and implications on public health: A review. *International Journal of*

Food Properties, 20(7), 1433–1446.

22. Mungroo, N. A., & Neethirajan, S. (2014). Biosensors for the detection of antibiotics in poultry industry: A review. *Biosensors*, 4(4), 472–493.
23. Nelson, A., Manandhar, S., Ruzante, J., Gywali, A., Dhakal, B., Dulal, S., ... & Dixit, S. M. (2020). Antimicrobial drug resistant non-typhoidal *Salmonella enterica* in commercial poultry value chain in Chitwan, Nepal. *One Health Outlook*, 2(1), 1–8.
24. O'Brien, J., Campbell, N., & Conaghan, T. (1981). Effect of cooking and cold storage on biologically active antibiotic residues in meat. *Journal of Hygiene*, 87(3), 511–523.
25. Osti, R., Bhattarai, D., Chaudhary, H., & Singh, V. (2017). Poultry production in Nepal: Characteristics, productivity and constraints. *International Journal of Applied Sciences and Biotechnology*, 5(2), 222–226.
26. Pantha, C., Thapaliya, S., Aryal, B., Bhattarai, N., & Sharma, S. (2019). Evaluation of antibiotic residues in marketed broiler meat of Kathmandu Valley of Nepal. *EC Veterinary Science*, 4(9), 26–35.
27. Patra, G., Biswas, S., & Das, A. K. (2019). Antibiotic residues in meat products and public health importance in the perspective of drug resistance. *Indian Journal of Animal Health*, 58(2SPL), 87–104. <https://doi.org/10.36062/ijah.58.2SPL.2019.87-104>
28. Patrabansh, S., Parajuli, N., & Jha, V. K. (2020). Rapid detection of tetracycline residues in chicken. *International Journal of Applied Sciences and Biotechnology*, 8(1), 14–20. <https://doi.org/10.3126/ijasbt.v8i1.27201>
29. Prajapati, M., Ranjit, E., Shrestha, R., Shrestha, S. P., Adhikari, S. K., & Khanal, D. R. (2018). Status of antibiotic residues in poultry meat of Nepal. *Nepalese Veterinary Journal*, 35, 55–62.
30. Puchades, R., & Cha, C. (2010). Fast screening methods to detect antibiotic residues in food samples. *Trends in Analytical Chemistry*, 29(9). <https://doi.org/10.1016/j.trac.2010.06.004>
31. Ramatla, T., Ngoma, L., Adetunji, M., & Mwanza, M. (2017). Evaluation of antibiotic residues in raw meat using different analytical methods. *Antibiotics*, 6(4), 34.
32. Ranganathan, V. (2017). Ethno veterinary practices for combating antimicrobial resistance. *International Journal of Science, Environment and Technology*, 1(6), 840–844.
33. Raut, R., Mandal, R. K., Kaphle, K., Pant, D., Nepali, S., & Shrestha, A. (2017). Assessment of antibiotic residues in the marketed meat of Kailali and Kavre of Nepal. *International Journal of Applied Sciences and Biotechnology*, 5(3), 386–389.
34. Sapkota, R., Raut, R., Khanal, S., & Gyawali, M. (2019). Screening of antibiotic residue in poultry in Kathmandu Valley of Nepal: A cross-sectional study. *Archives of Pharmacy and Pharmaceutical Sciences*, 3(1), 79–81.
35. Saxena, P., Prof. A., & Prof. M. (2014). Antibiotics in chicken meat: Pollution monitoring laboratory of CSE. *Antimicrobial Resistance and Regulations in Chicken Meat*.
36. Şahin, S. (2018). Determination of some antibiotic residues by HPLC method in chicken meats prepared for consumption. *Erciyes Üniversitesi Veteriner Fakültesi Dergisi*. <https://doi.org/10.32707/ercivet.477315>
37. Shahbazi, Y., Ahmadi, F., & Karami, N. (2015). Screening, determination and confirmation of tetracycline residues in chicken tissues using four-plate test, ELISA and HPLC-UV methods: Comparison between correlation results. *Food and Agricultural Immunology*, 26(6), 821–834.
38. Thaker, M., Spanogiannopoulos, P., & Wright, G. D. (2010). The tetracycline resistome. *Cellular and Molecular Life Sciences*, 67(3), 419–431.
39. Thapa, S. (2021). *Antibiotic residues in broiler meat sold in Dharan* [Doctoral dissertation, Tribhuvan University].
40. Veterinary Standard and Drug Regulatory Laboratory. (2020). *Technical Bulletin*. http://www.vsdril.gov.np/downloadfile/VSDRL%20Technical%20Bulletin_2020_1595343270.pdf

The Impact of Varying Ivermectin, Albendazole and Piperazine Dosages on the Elimination of Strongyle Eggs in Horse Feces

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ABSTRACT

Strongyles are one of the most common internal parasites affecting horses. However, there is a lack of comprehensive information on the prevalence, biological traits, and effective treatments against anthelmintic-resistant strongyle infestations in horses. This research aims to evaluate the effectiveness of various anthelmintics and identify factors influencing these infestations. The study was conducted on 96 horses of different ages, body weights, and sex. The egg per gram (EPG) of all horses was calculated initially, and the horses with EPG with very high level of infestation were divided into 7 groups, each group with 6 horses. Each group were treated with two different doses of ivermectin (@0.1 and @0.2 mg/kg body weight), albendazole (@20 and @30 mg/kg body weight) and piperazine (@200 and @300 mg/kg body weight) except the control. EPG of each horse at day 0 (before treatment) and on day 14 post-treatment was calculated. The fecal egg count reduction (FECR) was calculated based on the EPG. The result showed that age, sex, weight and pregnancy status did not have a significant influence on EPG counts. Moreover, ivermectin at both doses, albendazole @30 and piperazine @300 were effective (FECR $\geq$ 95%), whereas other groups had reduced efficacy (FECR ranging from 85 to 95%) against strongyles. In conclusion, ivermectin was highly effective even at low doses, while albendazole showed variable efficacy with emerging resistance concerns, and piperazine was effective only at recommended dosages.

Keywords: Anthelmintic resistance; EPG; FECR; Strongyles

INTRODUCTION

Horses are highly social animals that thrives on heard interactions, mental and physical activities (Sigurjónsdóttir & Haraldsson, 2019). Horse health and welfare are important issues for maintaining healthy populations irrespective of their purpose or use. To maintain the welfare, it is crucial to provide adequate nutrition, shelter, social interaction, and healthcare (Mueller *et al.*, 2018). Parasitic infections are a common disease and is an important problem for equine health (Singh *et al.*, 2019). Equines are exposed to various types of gastro-intestinal parasites which includes *strongylus spp*, *parascaris equorum*, *strongyloides westeri*, *oxyuris equi*, *anoplocephala perfoliata*, *gasterophilus spp*, *dictyocaulus arnfieldi*, *paranoplocephala mamillana* (Belay & Teshome, 2016;

Gebremariam *et al.*, 2015; Nigusu & Mohammed, 2017). Strongyles are one of the most common and important internal parasites that affect equine health (Scare *et al.*, 2018; Singh *et al.*, 2019). Strongyles are internal parasites that infect equines and are of two types, small and large. Small strongyles also called as cyathostomins, cause a range of health problems including weight loss, diarrhea, colic and can be deadly, while large strongyles mainly *strongylus vulgaris*, damages vital organs and can lead to anemia or even death (Pfister & van Doorn, 2018; Scare *et al.*, 2018; Singh *et al.*, 2019).

It is essential to prevent and treat strongyle infections in horses in order to maintain welfare and good health of equines. Ivermectin, albendazole and piperazine are commonly available anthelmintic drugs available to treat parasite infections in horses. Ivermectin is extensively used and well-known for its efficacy and safety and is the most popular drug used for treating internal and external parasites in equines (Asquith & Kulwich, 1981; Rosanowski *et al.*, 2017). Nevertheless, the emergence of anthelmintic resistance has major implications for equine health.

Many factors, including misuse or incorrect use of anthelmintic agents, inappropriate dose, and a lack of diversity in the management of parasite infections, all could contribute to the development of anthelmintic resistance (Fissiha & Kinde, 2021; Shalaby, 2013). Resistant to ivermectin has been reported in breeding horses in the United Kingdom, as well as in cyathostomins in New Zealand (Bolwell *et al.*, 2015; Relf *et al.*, 2014). Similarly, the recommended dose of albendazole and piperazine has shown reduced efficacy and is likely resistant against strongyles (Baranova *et al.*, 2022; Elmeligy *et al.*, 2021; Saeed *et al.*, 2008). There is a lack of comprehensive information on the prevalence, risk factors, and effective treatments against anthelmintic-resistant strongyle infestations in horses. The research aims to determine the prevalence of strongyle infestations in horses, evaluate the effectiveness of various anthelmintic treatments, and identify factors influencing these infestations, such as age, sex and pregnancy status. By evaluating the efficacy of different dosages, horse owners and veterinarians can make intelligent decisions regarding the most appropriate treatment options for their horses, ensuring optimal health and welfare.

MATERIALS AND METHODS

Study area and animal selection

The study was conducted on 96 horses of different ages and sex. The horses were selected from a single stud farm located in Bharatpur 15, Nepal (27.68°N, 84.42°E). The area has a tropical monsoon climate with a peak summer temperature of 42°C and a winter minimum temperature of 3°C. The horses were kept in different stables under a loose housing system according to their sex and pregnancy status. The feed, feeding system and management practices were the same for all horses in this study. Egg per gram (EPG) of all horses was calculated initially (regarded as day 0 of the study), and was divided into 4 groups (Table 1) (McNeill, 2023). The horses with levels of infestation very high were selected for further study.

Study design

Forty-two horses (female = 27, male = 15) were randomly assigned to 7 treatment groups each with 6 horses as shown in Table 2. The weight of the horses was determined using the girth tape method. The ivermectin (Hitek Ivermectin oral solution BP 0.08% w/v), Albendazole (Albomar-Albendazole 25mg/ml), and Piperazine (Pipraz 45% w/v) were administered orally to each treatment group as shown in Table 2. Finally, after the 14th day of treatment, EPG was again calculated.

Fecal sample collection

Fresh fecal samples were collected rectally in clean zip-lock plastic bags from each horse at day 0 (before treatment) and on day 14 post-treatment. The samples were collected in the early morning period to ensure that the fecal samples were fresh.

Determination of egg per gram (EPG)

The fecal samples were analyzed for strongyle egg count using a McMaster egg counting technique. In summary, 26 ml of floatation solution (40% salt solution) was mixed vigorously with 4 grams of fecal sample for 1-2 minutes. Immediately after mixing, with the help of a pipette, both chambers of McMaster slides were filled. After 20-30 minutes, the slide was examined under a microscope. The EPG count was calculated by multiplying the number of eggs present in both chambers of the McMaster slide by 25 (Coles *et al.*, 1992). The processing of all samples was completed within 12 hours following their collection.

Table 1. Level of infestation of strongyles according to EPG

Groups	EPG less than 200	EPG between 200 - 500	EPG between 500 - 1000	EPG above 1000
Level of infestation	Low shedder (n = 25)	Moderate shedder (n = 8)	High shedder (n = 12)	Very high shedder (n = 47)

Table 2. Experimental design of the study (McNeill, 2023)

Treatment Groups	Anthelmintics	Dose (mg/kg BW)
Ivermectin @ 0.1 (n = 6)	Ivermectin	0.1
Ivermectin @ 0.2* (n = 6)	Ivermectin	0.2
Albendazole @ 20 (n = 6)	Albendazole	20
Albendazole @ 30 (n = 6)	Albendazole	30
Piperazine @ 200 (n = 6)	Piperazine	200
Piperazine @ 300* (n = 6)	Piperazine	300
Control (n = 6)	Clean water	-

Note: Recommended dose by manufacturer is indicated by *; recommended dose by manufacturer for Albendazole is 10 mg/kg body weight.

Calculation of fecal egg count reduction (FECR)

The FECR was calculated by the following formula to determine the efficacy of anthelmintic drugs (Coles *et al.*, 2006). After calculation, the effectiveness was assessed based on the following criterion (Kaplan, 2020).

Table 3. Criterion for the interpretation of FECR

FECR	Interpretation
≥95	Effective, No resistance
≥90 to <95	Reduced Efficacy, Suspected resistance
≥80 to <90	Reduced Efficacy, Likely resistance
<80	Ineffective, highly resistance

Statistical analysis

Statistical analysis was performed using Excel (Microsoft 365) and R version 4.3.2. Mann Whitney U test was used to compare EPG before treatment between male and female, and pregnant and non-pregnant. Pearson correlation coefficient was determined to establish the relation between age and body weight with EPG. Kruskal Wallis test along with the Dunn-Bonferroni post-hoc test was performed to compare the FECR between different treatment groups.

RESULTS

Out of 96 horses, 87 were found to shed strongyles eggs in the feces, among which, 43 had high infestations (EPG>1200) as shown in Table 1. In this study, the correlation between the age and weight of horses with EPG was established as shown in Fig. 1. The result showed higher EPG in the young horses but no significant association among these parameters.

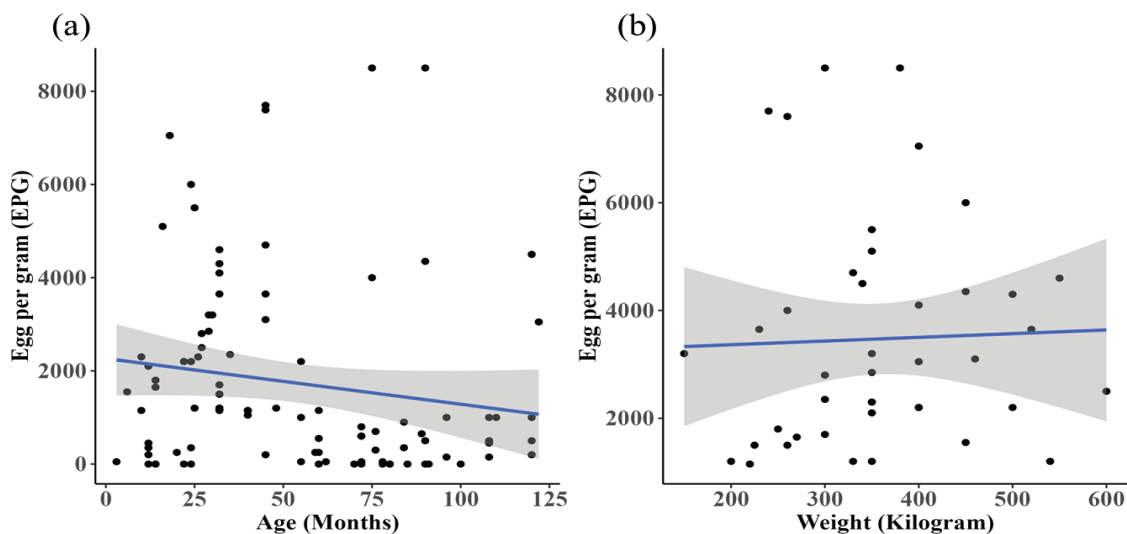


Fig. 1. A scatter plot illustrating the relationship between (a) age (in months) and (b) body

weight (in kilogram) with EPG (eggs per gram) of horses. Each data point represents an individual, with age plotted on the x-axis and EPG on the y-axis. The plot shows no significant correlation with EPG.

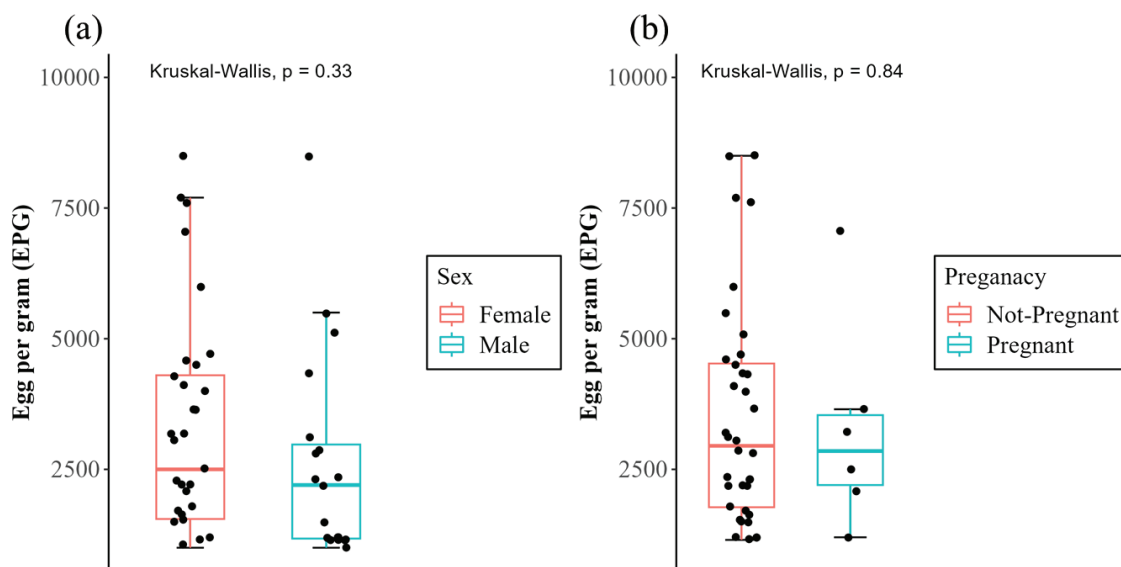


Fig. 2. Boxplot illustrating the comparison of EPG between sex (a) and pregnancy status (b) using Mann-Whitney U test. The plot shows no significant difference in EPG.

The EPG before treatment among sex and pregnancy status was compared using the Mann-Whitney U test as shown in Fig. 2. The result showed no significant difference in EPG (mean $\pm$ SD) between males and females (1363.41 ± 1795.06 and 2018.182 ± 2240.151 ; $p = 0.33$). Similarly, EPG (mean $\pm$ SD) among pregnant horses was 3283.33 ± 2033.88 and 3502.78 ± 2113.15 for non-pregnant horses. The comparison showed no significant difference ($p = 0.84$) between the groups.

Table 4. Comparative FECR test using arithmetic means and confidence intervals

Groups	Mean EPG (mean +SEM)		95% confidence level		
	Day 0	Day 14	FECR%	LCL	UCL
I1	3091.67 $\pm$ 977.19	6.67 $\pm$ 4.41	99.76	99.36	100
I2	2441.67 $\pm$ 430.97	25.00 $\pm$ 25.00	98.95	96.61	100
A1	3066.67 $\pm$ 706.83	174.17 $\pm$ 48.59	94.05	89.35	97.25
A2	5616.67 $\pm$ 784.93	144.83 $\pm$ 8.41	97.37	96.53	97.98
P1	4291.67 $\pm$ 1798.44	1050.00 $\pm$ 617.25	70.63	7.92	98.75
P2	3466.67 $\pm$ 965.29	69.17 $\pm$ 43.41	97.88	94.63	99.93
Control	3525.00 $\pm$ 370.53	2295.00 $\pm$ 214.97	34.52	16.22	49.59

FECR, fecal egg count reduction; SEM, standard error of mean; LCL, lower confidence limit; UCL, upper confidence limit

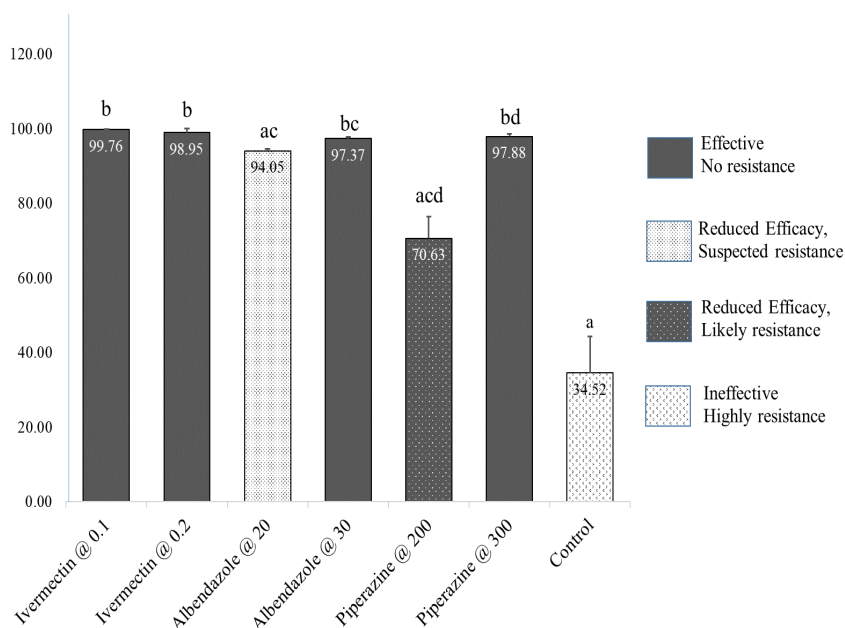


Fig. 3. Bar plot illustrating the comparison of FECR (y-axis) between each treatment group (x-axis). The plot shows a significant difference in FECR between groups. Superscripts indicate test results of the Kruskal-Wallis test followed by the Dunn-Bonferroni post-hoc test (any group with shared superscript letters are not significantly different from each other at $P < 0.05$).

The FECR between each treatment group was compared using the Kruskal-Wallis test as shown in Fig. 3. Every treatment group had significantly lower FECR than the control group. Treatment groups ivermectin @ 0.1, ivermectin @ 0.2, albendazole @ 30 and piperazine @ 300 reduced the fecal egg count most effectively ($\text{FECR} \geq 95\%$). Likewise, reduced efficacy with suspected resistance was observed in the albendazole @ 20 group (FECR ranging from 90 to 95%). The piperazine @ 200 group with FECR 85.81% showed reduced efficacy against strongyles and is likely resistant. However, in this study, none of the treatment groups was completely ineffective (FECR less than 80) against the strongyles.

DISCUSSION

The results of this study provide a valuable understanding of the prevalence of strongyle infestations and the efficacy of different anthelmintic treatments in horses. Despite this high prevalence in young horses, no significant correlation was observed between the ages of horses with EPG. This aligns with the finding that shows no significant correlation but is higher in young horses (Mostafa *et al.*, 2023). However, several other research has shown a significant correlation between EPG and age showing lower in older horses

(Fesseha *et al.*, 2020; Scala *et al.*, 2020). Young horses have poorly developed immune systems, which can increase their susceptibility to parasite infestations (Perkins & Wagner, 2015). The lack of a significant correlation between age and EPG counts might be attributed to the limited geographical areas studied. Likewise, the horse's weight did not appear to be significantly related to EPG counts. This might be due to the estimation of horse weights using measuring tapes instead of a more precise weighing scale.

The comparison of EPG between different sexes and pregnancy status using the Mann-Whitney U test showed no significant differences. Female horses had slightly higher EPG levels but were not significantly different from males. This finding aligns with the previous research showing no significant difference in sex but higher in females (Mostafa *et al.*, 2023). Similarly, some research has shown significant differences in EPG between sexes with higher in females as well (Scala *et al.*, 2020). Since male and female horses were housed separately, and females had access to a large pasture during the day while males were confined to smaller separate areas, females were more likely to be exposed to parasites in the environment. Because strongyle infestations begin after ingesting larvae from the environment (Marchiondo *et al.*, 2019), and females have greater access to open spaces, they may have higher EPG counts than males. Moreover, the similar EPG levels between pregnant and non-pregnant horses suggest that parasite infestation is not significantly influenced by pregnancy status.

The efficacy of various anthelmintic treatments was evaluated using the FECR test, with comparisons made between treatment and control groups using the Kruskal-Wallis test. The results showed that ivermectin at both doses showed highly effective treatments, achieving excellent efficacy against strongyles. Ivermectin is widely recognized for its safety and effectiveness against strongyles, with FECR often exceeding 98% (Apolinário *et al.*, 2022; Dauparaité *et al.*, 2021; Fesseha *et al.*, 2020). While there have been occasional reports of lower FECR (below 95%) (de Favare *et al.*, 2023; Rosanowski *et al.*, 2017), the strongyles in the current study demonstrated high susceptibility to ivermectin. The study also found that even when given at half the recommended dose, ivermectin was still highly effective against strongyles. This could be because ivermectin was rarely used on this farm, reducing the likelihood of developing resistance against it. Exposure of parasites to drugs and a lack of drug diversity can contribute to the development of resistance (Fissiha & Kinde, 2021; Shalaby, 2013). This suggests that ivermectin's effectiveness against these parasites is unlikely to decrease in the near future. Furthermore, long-term studies should be conducted to investigate the efficacy of ivermectin against parasites on this farm in order to better understand anthelmintic resistance patterns.

Similarly, albendazole was effective against strongyles when administered at a dosage of 30 mg/kg, but it was ineffective at a dosage of 20 mg/kg. Several reports indicate that albendazole can be completely ineffective against certain parasites (Baranova *et al.*, 2022; Molento & Canever, 2017; Saeed *et al.*, 2008). The albendazole dosage used in this study was significantly higher than the standard dose. This was necessary because previous data from the farms indicated that the recommended albendazole dose was ineffective against the local strongyle population, leading to the use of double the recommended dose. This

might be due to the lack of diverse anthelmintics in the management of parasite infections (Fissiha & Kinde, 2021; Shalaby, 2013). Additionally, it can be inferred that the strongyles in this study were resistant to albendazole. The reproduction of these resistant parasites in the environment further contaminates the pasture, contributing to the development of additional anthelmintic resistant strongyles (Fissiha & Kinde, 2021). The findings suggest that repeated use of albendazole, even at three times the recommended dose, may lead to future resistance. Therefore, it is recommended to use various combinations of anthelmintics to reduce resistance, as combinations can reduce selective pressure (Bartram *et al.*, 2012).

The study revealed that piperazine, when administered at the recommended dosage, was effective against strongyles. Other studies have also indicated that piperazine is generally effective against these parasites (Elmeligy *et al.*, 2021). However, when the dose was reduced by just 20% (@ 200 mg/kg body weight), it became completely ineffective against these parasites. This finding is significantly different from previous research that demonstrated the effectiveness of piperazine against parasites even when the dose was reduced by 50% (Saes *et al.*, 2016). As various factors affect the pharmacodynamics of anthelmintics, this difference is because of the geographic region and the history of frequent exposure to piperazine that influences its effectiveness (Fissiha & Kinde, 2021; Shalaby, 2013). To minimize resistance, it is advisable to follow the recommended dosage for piperazine.

CONCLUSION

While strongyle infestations were common among younger horses, most variables concerning age, sex and pregnancy status did not have a deep influence on EPG counts. Ivermectin was particularly effective against strongyles even at low dosages, further indicating the possibility of long-term efficacy. However, albendazole proved variably effective and resistance to this anthelmintic has been a cause of concern. Piperazine was effective only at the recommended dosage rate and proved to be very sensitive to dosage rate reductions. A strategic deworming program should be used to reduce the risk of anthelmintic resistance, including the use of combination therapies and adherence to recommended dosages. The monitoring of EPG levels regularly and the introduction of necessary prevention measures are, therefore, important for health maintenance in horse populations.

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Animal Welfare/Ethical approval

This study was granted ethical approval by the University Ethical Committee of Agriculture and Forestry University (AFU), Nepal. Also, the owner gave consent for their animals' inclusion in the study.

Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Apolinário, E. C. F., Oliveira, L. L. dos S., Rocha, R. H. F., Silva, F. V. e., Santos, I. S., Alves, C. A., Dias Júnior, J. A., Mendes, L. de J., & Lima, W. dos S. (2022). Anthelmintic efficacy in strongyles of horses in Northern Minas Gerais, Brazil. *Veterinary Parasitology: Regional Studies and Reports*, 36, 100810. <https://doi.org/10.1016/j.vprsr.2022.100810>
2. Asquith, R. L., & Kulwich, R. (1981). Safety and therapeutic activity of ivermectin as an equine anthelmintic. *Journal of Equine Veterinary Science*, 1(1), 18–20. [https://doi.org/10.1016/S0737-0806\(81\)80013-5](https://doi.org/10.1016/S0737-0806(81)80013-5)
3. Baranova, M. V., Panova, O. A., Polukhina, D. N., & Panova, D. S. (2022). Reduction of the nematode egg reappearance period in horses after anthelmintic therapy. *Veterinary World*, 15(6), 1530–1534. <https://doi.org/10.14202/vetworld.2022.1530-1534>
4. Bartram, D. J., Leathwick, D. M., Taylor, M. A., Geurden, T., & Maeder, S. J. (2012). The role of combination anthelmintic formulations in the sustainable control of sheep nematodes. *Veterinary Parasitology*, 186(3–4), 151–158. <https://doi.org/10.1016/j.vetpar.2011.11.030>
5. Belay, W., & Teshome, D. (2016). Study on the prevalence of gastrointestinal helminthes infection in equines in and around Kombolcha. *Journal of Veterinary Science & Technology*, 7(5). <https://doi.org/10.4172/2157-7579.1000372>
6. Bolwell, C. F., Rosanowski, S. M., Scott, I., Sells, P. D., & Rogers, C. W. (2015). Questionnaire study on parasite control practices on Thoroughbred and Standardbred breeding farms in New Zealand. *Veterinary Parasitology*, 209(1–2), 62–69. <https://doi.org/10.1016/j.vetpar.2015.02.011>
7. Coles, G. C., Bauer, C., Borgsteede, F. H. M., Geerts, S., Klei, T. R., Taylor, M. A., & Waller, P. J. (1992). World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology*, 44(1–2), 35–44. [https://doi.org/10.1016/0304-4017\(92\)90141-U](https://doi.org/10.1016/0304-4017(92)90141-U)
8. Coles, G. C., Jackson, F., Pomroy, W. E., Prichard, R. K., Von Samson-Himmelstjerna, G., Silvestre, A., Taylor, M. A., & Vercruysse, J. (2006). The detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology*, 136(3–4), 167–185. <https://doi.org/10.1016/j.vetpar.2005.11.019>
9. Dauparaitė, E., Kupčinskis, T., von Samson-Himmelstjerna, G., & Petkevičius, S. (2021). Anthelmintic resistance of horse strongyle nematodes to ivermectin and pyrantel in Lithuania. *Acta Veterinaria Scandinavica*, 63(1), 1–7. <https://doi.org/10.1186/s13028-021-00569-z>
10. de Favare, G. M., de Almeida Cipriano, I., do Carmo, T. A., Mena, M. O., Guelpa, G. J., do Amarante, A. F. T., & de Soutello, R. V. G. (2023). Anthelmintic resistance of horse strongyle nematodes to ivermectin in São Paulo state, Brazil. *Veterinary Parasitology: Regional Studies and Reports*, 41, 100864. <https://doi.org/10.1016/j.vprsr.2023.100864>
11. Elmeligy, E., Abdelbaset, A., Elsayed, H. K., Bayomi, S. A., Hafez, A., Abu-Seida, A. M., El-Khabaz,

- K. A. S., Hassan, D., Ghandour, R. A., & Khalphallah, A. (2021). Oxidative stress in *Strongylus* spp. infected donkeys treated with piperazine citrate versus doramectin. *Open Veterinary Journal*, 11(2), 238–250. <https://doi.org/10.5455/ovj.2021.v11.i2.8>
12. Fesseha, H., Mathewos, M., & Kidanemariam, F. (2020). Anthelmintic efficacy of strongyle nematodes to ivermectin and fenbendazole on working donkeys (*Equus asinus*) in and around Hosaena Town, Southern Ethiopia. *Veterinary Medicine International*, 2020, 4868797. <https://doi.org/10.1155/2020/4868797>
13. Fissiha, W., & Kinde, M. Z. (2021). Anthelmintic resistance and its mechanism: A review. *Infection and Drug Resistance*, 14, 5403–5410. <https://doi.org/10.2147/IDR.S332378>
14. Gebremariam, A. A., Assefa, A., Enigidaw, S., Mekonnen, N., & Belete, S. (2015). Prevalence of gastrointestinal nematode parasitic infections of horses and donkeys in and around Kombolcha Town. *Journal of Scientific Research*, 10(4), 228–234. <https://doi.org/10.5829/idosi.ajejsr.2015.10.4.95266>
15. Kaplan, R. M. (2020). Biology, epidemiology, diagnosis, and management of anthelmintic resistance in gastrointestinal nematodes of livestock. *The Veterinary Clinics of North America: Food Animal Practice*, 36(1), 17–30. <https://doi.org/10.1016/j.cvfa.2019.12.001>
16. Marchiondo, A. A., Cruthers, L. R., Reinemeyer, C. R., Snyder, D. E., Yazwinski, T. A., Dzimianski, M. T., & Zarlenga, D. S. (2019). Nematoda. In *Parasiticide screening: Volume 2: In vitro and in vivo tests with relevant parasite rearing and host infection/infestation methods* (pp. 135–335). <https://doi.org/10.1016/B978-0-12-816577-5.00007-7>
17. McNeill, C. (2023, July 26). Strongyles in horses: Symptoms, treatment & prevention. *Mad Barn*. <https://madbarn.com/strongyles-in-horses/>
18. Molento, M. B., & Canevar, R. J. (2017). In vitro evaluation of ivermectin, moxidectin, albendazole and pyrantel against cyathostomins of horses. *Revista Brasileira de Parasitologia Veterinária*, 27(1), 90–93. <https://doi.org/10.1590/s1984-29612017055>
19. Mostafa, W., Felefel, W., Abd El-Rady, A., Ahmed Khalifa, F., Gahlan Youseef, A., El-Dakroury, M. F., Selim, S., Morsi Elkamshishi, M., & Sayed Mohammed, E. (2023). Prevalence of intestinal nematodes in equines with an assessment of the therapy using albendazole and doramectin. *Research Square*. <https://doi.org/10.21203/rs.3.rs-2889716/v1>
20. Mueller, M. K., Sween, C., Frank, N., & Paradis, M. R. (2018). Survey of human-horse relationships and veterinary care for geriatric horses. *Journal of the American Veterinary Medical Association*, 253(3), 337–345. <https://doi.org/10.2460/javma.253.3.337>
21. Nigusu, K., & Mohammed, T. (2017). Assessment of gastrointestinal tract parasites of equines in Asella Town, Ethiopia. *East African Journal of Veterinary and Animal Sciences*, 1(2), 93–100. <https://doi.org/10.20372/eajvas.v1i2.430>
22. Perkins, G. A., & Wagner, B. (2015). The development of equine immunity: Current knowledge on immunology in the young horse. *Equine Veterinary Journal*, 47(3), 267–274. <https://doi.org/10.1111/evj.12387>
23. Pfister, K., & van Doorn, D. (2018). New perspectives in equine intestinal parasitic disease: Insights in monitoring helminth infections. *The Veterinary Clinics of North America: Equine Practice*, 34(1), 141–153. <https://doi.org/10.1016/j.cveq.2017.11.009>
24. Relf, V. E., Lester, H. E., Morgan, E. R., Hodgkinson, J. E., & Matthews, J. B. (2014). Anthelmintic efficacy on UK Thoroughbred stud farms. *International Journal for Parasitology*, 44(8), 507–514. <https://doi.org/10.1016/j.ijpara.2014.03.006>
25. Rosanowski, S. M., Bolwell, C. F., Scott, I., Sells, P. D., & Rogers, C. W. (2017). The efficacy of ivermectin against strongyles in yearlings on Thoroughbred breeding farms in New Zealand. *Veterinary Parasitology: Regional Studies and Reports*, 8, 70–74. <https://doi.org/10.1016/j.vprsr.2017.02.001>
26. Saeed, K., Qadir, Z., Khan, S. A., Ashraf, K., & Nazir, S. (2008). Evaluation of some broad spectrum antiparasitic drugs against natural strongyle infection in horse. *Journal of Animal and Plant Sciences*,

18(3), 64–66.

27. Saes, I. de L., Vera, J. H. S., Fachioli, D. F., Yamada, P. H., Dellaqua, J. V. T., Saes, R. de L., Amarante, A. F. T., & Soutello, R. V. G. (2016). Time required by different anthelmintics to reach expected efficacy levels in horses infected by strongyles. *Veterinary Parasitology*, 229, 90–92. <https://doi.org/10.1016/j.vetpar.2016.10.002>
28. Scala, A., Tamponi, C., Sanna, G., Predieri, G., Dessì, G., Sedda, G., Buono, F., Cappai, M. G., Veneziano, V., & Varcasia, A. (2020). Gastrointestinal strongyles egg excretion in relation to age, gender, and management of horses in Italy. *Animals*, 10(12), 2283. <https://doi.org/10.3390/ani10122283>
29. Scare, J. A., Steuer, A. E., Gravatte, H. S., Kálmán, C., Ramires, L., Dias de Castro, L. L., Norris, J. K., Miller, F., Camargo, F., Lawyer, A., De Pedro, P., Jolly, B., & Nielsen, M. K. (2018). Management practices associated with strongylid parasite prevalence on horse farms in rural counties of Kentucky. *Veterinary Parasitology: Regional Studies and Reports*, 14, 25–31. <https://doi.org/10.1016/j.vprsr.2018.08.002>
30. Shalaby, H. A. (2013). Anthelmintics resistance; How to overcome it? *Iranian Journal of Parasitology*, 8(1), 18. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3655236/>
31. Sigurjónsdóttir, H., & Haraldsson, H. (2019). Significance of group composition for the welfare of pastured horses. *Animals*, 9(1), 14. <https://doi.org/10.3390/ani9010014>
32. Singh, E., Singh, C., Kaur, S., Singh, E., & Singla, L. (2019). Strongylosis in equine: An overview. *Journal of Entomology and Zoology Studies*, 7(5), 43–46.

Pattern of Canine Cases Brought at Veterinary Teaching Hospital of Agriculture and Forestry University from 2018/19 to 2022/23

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ABSTRACT

A study was conducted to understand the canine cases flow pattern brought at the Veterinary Teaching Hospital (VTH) of Agriculture and Forestry University (AFU), Rampur, Chitwan during last five years period. Canine cases from the case-record books of the VTH AFU from 2018/19 to 2022/23 were analyzed retrospectively. Total of 803 canine cases were presented during a five years period. The result showed an increasing trend with the highest number of cases in 2022/23 (34.3%) and the lowest being in 2018/19 (9.3%). The winter season (31.6%) and March/April month (18.9%) had the highest, while autumn (18.6%) had the lowest number of cases. Non-specific breed accounted for the highest number of cases (44.5%), followed by Japanese spitz (31.3%). The number of cases decreased as the age of the dogs increased, with dogs under one year accounting for 46.6% of total cases. A greater number of male cases were presented than the females (60% and 40%, respectively). The hospital's surrounding areas accounted for 90% of the overall case flow. The majority of the dogs (23.2%) showed non-specific general signs, followed by parasitic diseases (16.9%). External parasitic diseases were the most prevalent parasitic diseases, with demodicosis alone constituting 26.5% of total parasitic diseases. Parvovirus infection was the most common viral disease of canine (59.6 %) while canine distemper mainly occurred during the winter season (61.0%). Despite the modest number of surgical cases, the incidences of fracture and accident (n= 26) were higher than castration and spaying (n= 13). The case flow of the veterinary teaching hospital, Rampur can be increased by improving diagnostic facilities, increasing extension activities, and establishing an in-patient department facility.

Keywords: Dog cases; Veterinary Teaching Hospital; Chitwan; Parvovirus infection

INTRODUCTION

Humans and dogs share an everlasting and profound affinity that is also deeply established in history and culture. Dogs are the most frequent household pets found abundantly in almost every human settlement globally (Chomel, 1992). Dogs serve as companions, working animals, and guardians, thus understanding and addressing their health needs is paramount. Good pet ownership comes with responsibilities like proper housing facilities, disease management, and balanced nutrition for the dog. Various clinical conditions and diseases severely affect the health of dogs because of which dog owners abandon them

rendering them stray. The close association between humans and dogs also poses a major threat to public health as dogs harbor a bewildering number of infectious zoonotic diseases (Kornblatt & Schantz, 1980)

A wide variety of clinical conditions and diseases affect the dogs including bacterial, viral, and fungal diseases, ecto and endo parasites, blood parasites, allergies, and nutritional deficiencies. Common viral diseases include parvovirus infection, canine distemper, infectious canine hepatitis, and rabies whereas frequently encountered bacterial diseases are brucellosis, salmonellosis, and leptospirosis. Parasitic infection includes mange, scabies, roundworm, hookworm, and blood protozoans while nutritional deficiency disorders include alopecia, dermatitis, rickets, and ophthalmic diseases (Joshi et al., 2007).

The Veterinary Teaching Hospital (VTH) at Agriculture and Forestry University (AFU) in Chitwan, Nepal serves as a critical center for canine healthcare of dogs in the surrounding and also outside the district. It seems that the number of households keeping dogs has been increasing day by day and thus the number of canine cases brought to the VTH might have increased over the last few years. However, there is a lack of comprehensive study on clinical cases presented at veterinary institutions in Nepal. This knowledge gap restricts our ability to properly appreciate the health difficulties that these devoted companions experience and impedes the development of specialized healthcare treatments to improve their quality of life. To bridge this void, we conducted a retrospective study of canine clinical cases reported at the Veterinary Teaching Hospital, Rampur from 2018/19 to 2022/23. The objectives of the study was to assess the trend of canine cases being presented at the VTH, Rampur for five years.

MATERIALS AND METHODS

The study was based on the analysis of clinical cases of dogs recorded at the Veterinary Teaching Hospital (VTH), Agriculture and Forestry University, Rampur, Chitwan from April/May 2018 (Baishakh 2075 B.S) to March/April 2023 (Chaitra 2079 B.S.). A total of five years of data were acquired from the case record book of the hospital. The hospital is located at an elevation of 415 m above sea level at 27°39'23.6" N latitude and 84°21'26.8" E longitude. It serves clients with animal disease diagnosis and treatment services such as medical, surgical, gynecological, and laboratory services by specialized veterinary experts.

The canine cases of the last five years, 2018/19 to 2022/23 were gathered using a retrospective epidemiological study method. All the cases brought to the VTH were registered in a log book on a daily basis. The diseases were mainly diagnosed based on the history and clinical signs along with limited laboratory tests and some use of certain rapid diagnostic kits. The distribution of canine cases under the following categories was analyzed: month, season, breed, age, and location. Total canine cases presented at VTH were categorized into surgical cases, parasitic diseases, viral diseases, bacterial infections, eye-related problems, poisoning, canine transmissible venereal tumor (CTVT), other skin-

related problems, anti-rabies vaccinations, nerve-related problems, non-specific general signs, and other less frequent cases. The winter season includes December, January, and February; the spring season includes March, April, and May; the summer season includes June, July, and August; and the autumn season includes September, October, and November. The data were analyzed using Microsoft Excel 2016 and results were shown via different descriptive statistics like tables, bar diagrams, and pie charts.

RESULTS

A total of 803 canine cases were presented at VTH during a period from 2018/19 to 2022/23 of which the highest case flow was in 2022/23 (34.3%), followed by 2021/22 (31.3%), 2019/20 (14.6%), 2020/21 (10.6%) and lowest in 2018/19 (9.3%) as shown in Table 1.

Table 1. Monthly case flow pattern at VTH, Rampur during 5 years period from 2018/19 to 2022/23

Year / Months	2018/19 (2075)	2019/20 (2076)	2020/21 (2077)	2021/22 (2078)	2022/23 (2079)	Total Cases	Percentage (%)
April/May (Baishakh)	0	12	0	3	16	31	3.9
May/June (Jeth)	0	10	0	2	18	30	3.7
June/July (Asar)	8	10	7	12	21	58	7.2
July/Aug (Shrawan)	10	9	6	16	26	67	8.3
Aug/Sept (Bhadra)	7	7	0	8	19	41	5.1
Sept/Oct (Asoj)	2	9	6	19	14	50	6.2
Oct/Nov (Kartik)	3	7	2	19	16	47	5.9
Nov/Dec (Mangsir)	9	4	8	32	8	61	7.6
Dec/Jan (Paush)	10	11	9	37	15	82	10.2
Jan/Feb (Magh)	3	17	9	21	26	76	9.5
Feb/March (Falgun)	8	17	19	33	31	108	13.5
March/April (Chaitra)	15	4	19	49	65	152	18.9
	75	117	85	251	275	803	100.00

Monthly case flow

During the five years, the highest number of cases flow was in March/April, 152 (18.9%) and the lowest number of cases flow was in May/June, 30 (3.7%).

Seasonal case flow

Fig. 1 shows the seasonal case flow pattern during five years' time, in which, the highest number of cases were recorded in the spring season (31.6%), followed by winter (30.0%), summer 19.8%, and autumn (18.6%). However, winter had the highest number of cases in 2018/19, 2019/20, 2020/21, and 2021/22 whereas spring had the highest number of cases inflow in 2021/22. The autumn season had the lowest case flow except in 2021/22.

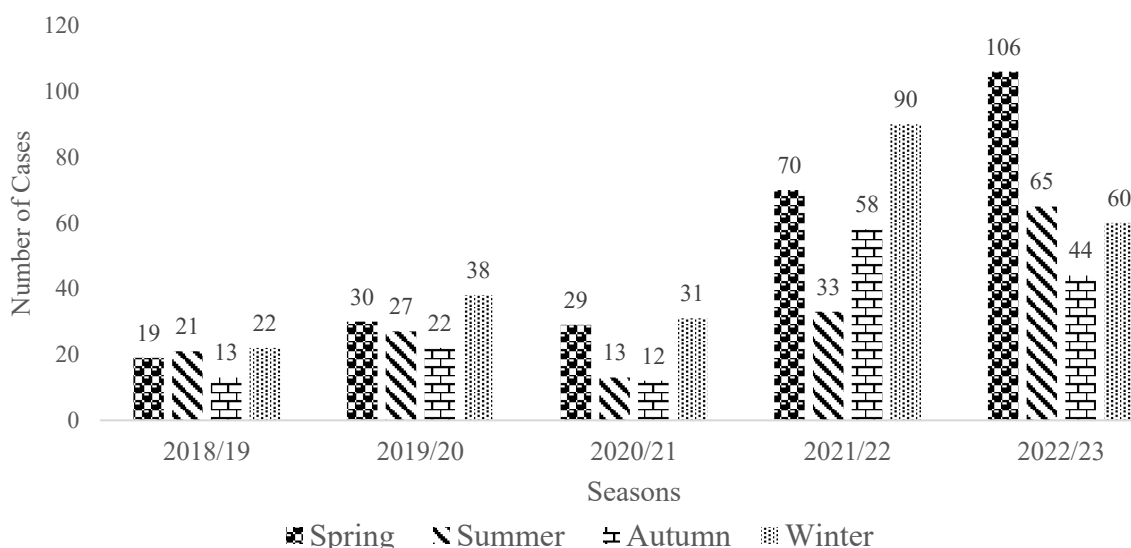


Fig. 1. Season-wise case flow pattern of canine disease at VTH, Rampur from 2018/19 to 2022/23

Breed-wise case flow pattern of canine

A total of sixteen different dog breeds were brought to the VTH, Rampur for five years, from 2018/19 to 2022/23. Among them, the non-specific breed (NSB) had the highest number (44.8%), followed by Japanese spitz (31.3%), German shepherd (7.7%), Pug (4.6%), Tibetan mastiff (3.5%), Labrador retriever (3.0%), Cocker spaniel (1.6%), Boxer (1.0%), Golden retriever (1.0%), Doberman (0.5%) and Husky (0.4%) as shown in Fig. 2. The other breeds infrequently admitted to the hospital were Bulldog, Chihuahua, Dachshund, Dalmatian, and Rottweiler.

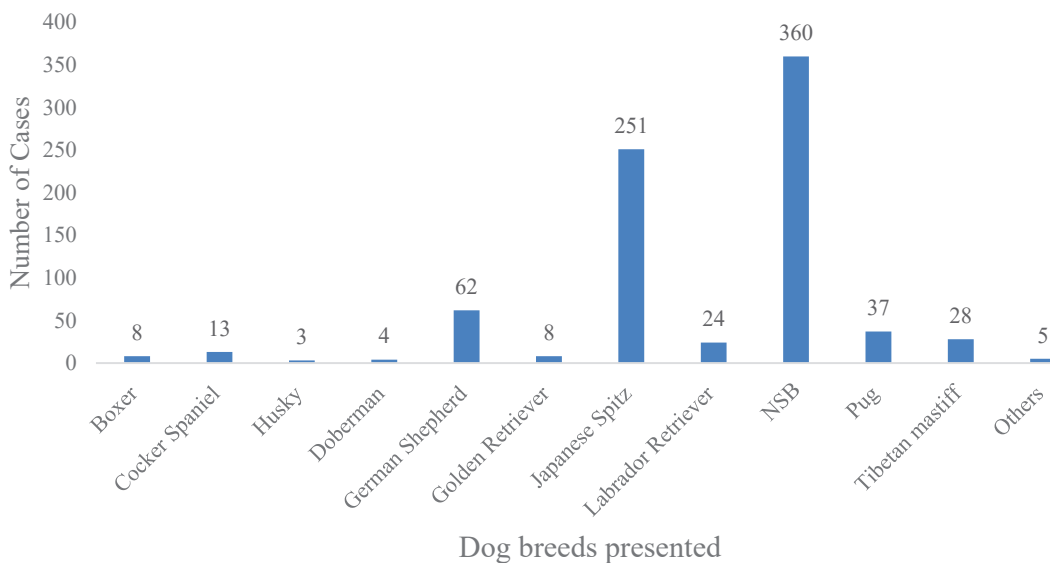


Fig. 2. Breed-wise case flow pattern of canine diseases at VTH, Rampur from 2018/19 to 2022/23

Age-wise case flow pattern

Fig. 3 depicts the decreasing pattern of cases flow as age increases. The highest number of cases (n=374) were below the age of one year old, accounting for 46.6% of total cases. Similarly, proportions of cases belonging to age group 1-2 yr, 2-3 yr, 3-4 yr, 4-5 yr, 5-6 yr, 6-7 yr, 7-8 yr, 8-9 yr and above 9 yr were 16.8%, 11.8%, 9.3%, 5.4%, 3.5%, 1.4%, 2.6%, 1.6%, and 1.0% respectively.

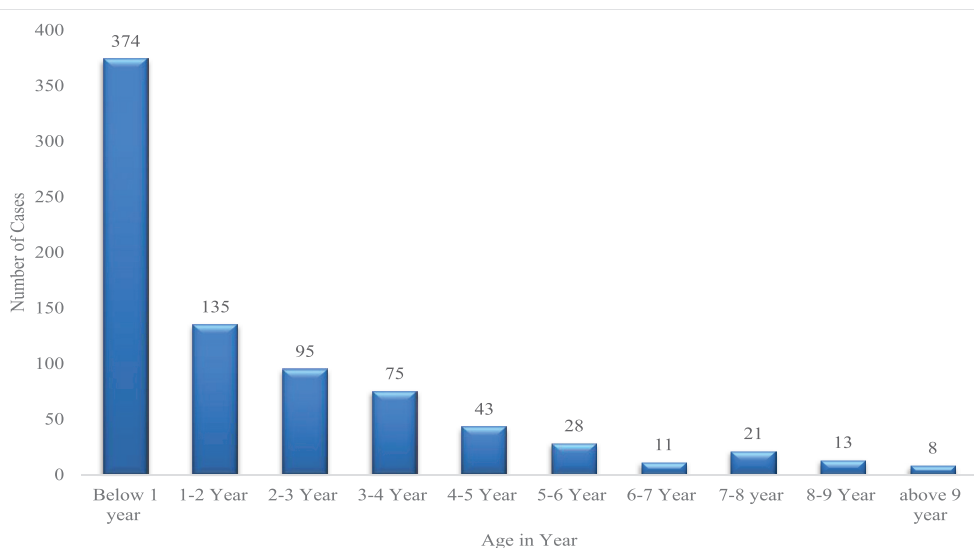


Fig. 3. Age-wise case flow pattern of canine diseases at VTH, Rampur from 2018/19 to 2022/23

Sex-wise and location -wise case flow

Higher number of male cases (60%) were brought as compared to female cases (40%). The highest number of cases were from the adjoining areas of the VTH (90.0%), followed by the core urban area (8.0%) and the least were from outside Bharatpur metropolitan city (2.0%). Adjoining areas of VTH included wards numbers 15, 16, 17, 18, 19, 20, 21, and 26 of Bharatpur metropolitan city whereas the core urban area included wards numbers 3, 4, 5, 7, 9, 10, 11, and 12.

Disease-wise case flow

Table 2 shows the year-wise number of different diseases documented at VTH, Rampur during five years' time. The highest number of cases were of non-specific general signs 186 (23.2%), followed by parasitic diseases 136 (16.9%), surgical cases 117 (14.6%), viral diseases 109 (13.6%), anti-rabies vaccination 100 (12.4%), bacterial diseases 40 (5.0%), other skin related problems 33 (4.1%), nerve-related problems 18 (2.2%), eye related problems 8 (1.0%), canine transmissible venereal tumor 8 (1.0%), and lowest being poisoning cases 7 (0.9%). There was an increasing trend in the number of cases flow from 2018/19 to 2022/23, except 2020/21.

Bacterial infections included bacterial-originated respiratory tract infections, urinary tract infections, mastitis, and listeriosis. Conjunctivitis, hydrophthamlos, ophthalmoplegia, and cloudy eyes were categorized as eye-related problems. Aside from parasitic diseases, skin problems such as alopecia, allergic dermatitis, atopic dermatitis, zinc deficiency, and ringworm infection were ground as other skin-related problems. Non-specific general signs included anorexia, pyrexia of unknown origin, gastritis, indigestion, diarrhea, vomiting, and constipation. Other less frequently seen cases included anemia, cold stress, leukorrhea, ascites, acute hepatitis, and euthanasia. Euthanasia was performed by veterinary experts under the consent of owners to avoid further suffering of the animals.

Table 2. Disease-wise case flow pattern at VTH, Rampur from 2018/19 to 2022/23

S.N.	Diagnosis	Number of Cases					Total no.	Percentage (%)
		2018/19	2019/20	2020/21	2021/22	2022/23		
1.	Surgical Cases	11	23	13	30	40	117	14.6
2.	Parasitic Diseases	12	29	13	39	43	136	16.9
3.	Viral Diseases	10	6	4	33	56	109	13.6
4.	Bacterial Infections	7	3	6	17	7	40	5.0
5.	Other Skin-Related Problems	7	5	3	8	10	33	4.1
6.	Eye Related Problems	0	2	1	1	4	8	1.0
7.	Nerve Related Problems	4	3	1	3	7	18	2.2
8.	Poisoning	3	3	0	0	1	7	0.9

9.	CTVT	0	2	0	2	4	8	1.0
10.	Anti-Rabies Vaccination	2	11	10	26	51	100	12.4
11.	Non-Specific General Signs	12	22	26	82	44	186	23.2
12.	Others	7	8	8	10	8	41	5.1
		75	117	85	251	275	803	100

Surgical case flow pattern

Among the surgical cases, the highest number of cases were of open wounds and traumatic injuries (n= 59), followed by fracture and accidents (n= 26), castration and spaying (n= 13), aural hematoma (n = 8) and abscess (n = 5) as shown in Fig. 6. The other six miscellaneous surgical cases included leg amputation, tail docking, paraphimosis, hernia, and intestinal obstructions.

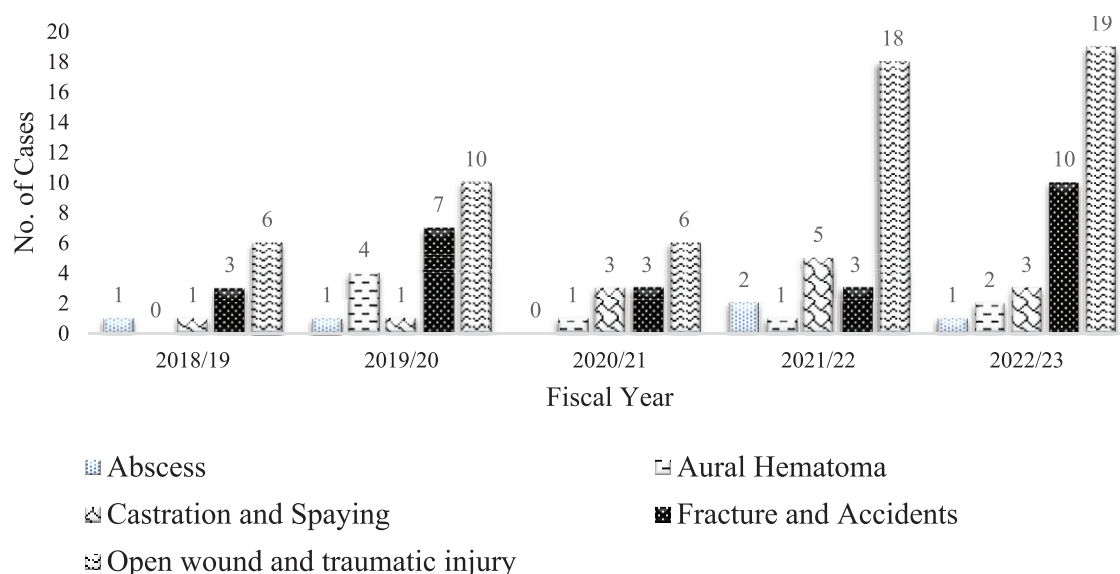


Fig. 6. Pattern of surgical cases inflow at VTH, Rampur from 2018/19 to 2022/23

Parasitic disease cases flow

Blood parasitic infections, gastrointestinal parasites, and external parasitic infections were broadly categorized under parasitic diseases. Anaplasmosis and Ehrlichiosis were reported blood parasitic infections, whereas demodicosis, scabies, myiasis, tick infestation, and ear mites were reported external parasitic problems. Table 3 shows that gastrointestinal was the most prevalent parasitic disease i.e. (30.9%) of total parasitic cases encountered over a five-year period, and maggot infestation (4.4%) was the least. Demodicosis constituted 26.5% of total parasitic diseases, followed by tick infestation (16.9%), ear mites (10.3%), blood parasites (5.9%), scabies (5.1%), and myiasis (4.4%) respectively.

Table 3. Parasitic disease case-flow pattern at VTH, Rampur during 5 years period (from 2018/19 to 2022/23)

S.N.	Parasitic Diseases	Number of Cases					Total no.	Percentage (%)
		2018/19	2019/20	2020/21	2021/22	2022/23		
1.	Blood parasites	2	0	1	1	4	8	5.9
2.	GI parasites	2	6	6	15	13	42	30.9
3.	Demodicosis	1	5	4	17	9	36	26.5
4.	Scabies	0	2	1	0	4	7	5.1
5.	Myiasis	2	2	0	0	2	6	4.4
6.	Tick infestation	4	10	0	3	6	23	16.9
7.	Ear mites	1	4	1	3	5	14	10.3
		12	29	13	39	43	136	100.0

Major viral disease cases

There were a total of 109 viral cases of canine presented at VTH during five years. Among them, the highest number of cases were recorded in 2022/23 with 37 parvovirus infections, 17 canine distempers, 1 rabies suspected case, and 1 papillomavirus infection, and the lowest was in 2020/21 with 4 parvovirus infection cases only. Apart from this one more case of rabies was admitted to VTH, in 2021/22.

Season-wise Canine Distemper Cases

Winter had the highest number of canine distemper cases 25 (61.0%), followed by spring 10 (24.4%), summer 3 (7.3%), and autumn 3 (7.3%) respectively.

DISCUSSION

This study tried to analyze the trend of the case flow pattern of canine cases brought at VTH Rampur during a period of five years. A total of 803 cases were presented to the hospital from fiscal year 2018/19 to 2022/23 which showed an increasing trend, except in 2020/21. The drop in the number of canine cases in the year 2020/21 may be due to student and employee strikes which impede the operations of the hospital. The zero case inflow in April/May and May/June of 2018/19 and 2020/21 was due to student and employee strikes that kept the hospital closed. The low-case inflow between September/October and October/November was attributed to Nepal's Dashain and Tihar festivities. During that time, the hospital remained closed for a few days. Due to transition seasonal impacts, the highest number of case flows were in March/April. This conclusion was consistent with the findings of Gautam et al. (2017).

Except in 2022/23, winter had the highest number of cases, which could be attributed to a variety of factors, including an increased prevalence of canine distemper during the cold season (71.43%) (Mousafarkhani et al., 2023). Furthermore, extreme environmental conditions such as non-humid and arid airflow lead to tough, dull, and delicate skin tone, which was quickly affected by even tiny tears, and injury (Hasan et al., 2022). The poor visibility in the morning and evening of winter also increased the cases of accidents.

The fracture and accidents also increased the cases of nerve-related problems in dogs. Fighting and mating behavior also rose during this period. Dogs were prone to lice and tick infestations due to filthy and uneven hairs caused by infrequent washing and showering during cold weather (Foreyt, 2013).

The work of Panth & Poudel (2020) also supports the finding of this study that the highest number of dog cases presented at VTH were under one year of age. Canine distemper and parvovirus infection were common in puppies under the age of a year. Younger puppies (≤ 3 months) had a higher risk of parvovirus infection since the virus had a greater affinity for replicating at the intestinal crypt with a higher mitotic index at weaning age (Hasan et al., 2017). Also, the recovery rate was higher in older dogs (Pradhan, 2005). After two months of age, the maternally derived antibodies declined, and the puppy's immune system was not completely developed; as a result, the odds of pathogen attack increased (Pereira et al., 2019). Further, because of the unvaccinated mothers, the puppies were unable to get maternal antibodies against common diseases like parvovirus and canine distemper, increasing the chances of infection.

The higher number of male cases admitted supports the work done by previous studies (Hasan et al., 2022; Panth & Poudel, 2020). In contrast, the number of female cases (51.3%) was higher than the males (48.7%) (Islam et al., 2019). Male dogs are more likely to fight, bite, and self-inflicted injuries than female dogs (Hasan et al., 2022), resulting in secondary bacterial, fungal, or viral infections. The Veterinary Teaching Hospital, Rampur is located at a distance from the core urban area which makes owner difficult to bring their pets to the hospital. Pet owners found it inconvenient to bring their pets to the hospital on a daily basis due to the lack of an in-patient department facility. The lack of different diagnostic facilities made it difficult to diagnose the clinical condition. Furthermore, the establishment of new modern veterinary clinics in the urban area of Bharatpur also decreased the flow of patients in the hospital.

Diarrhea and vomiting were the most prevalent conditions in dogs and were caused by gastrointestinal disorders (Hall, 2009). Dietary indiscretion, food intolerance, abrupt changes in diet, and various infectious pathogens were the most likely causes of diarrhea. Similarly, vomiting mainly occurred due to acute hepatic and renal failure, pancreatitis, motion sickness, dietary irregularities, poisoning, certain gastrointestinal ailments, and congenital abnormalities (Simpson, 2005). The lower castration and spaying rates were attributed to frequent animal birth control camps (ABC camps) organized by HART, Nepal, and lower awareness among the local people regarding the benefits of castration and spaying. Castration prevents male aggressive behavior, urine sprinkling, and mounting whereas spaying controls unwanted breeding and prevents potential female reproductive diseases like pyometra, metritis, dystocia, vaginal hemorrhage, and neoplasms (Kustritz, 2007). The majority of fractures were caused by being hit by a car, falling from a height, fighting, nutritional imbalances, and tumors of bone.

The higher prevalence of demodicosis might be due to a higher number of community and stray dogs that come in contact with each other more frequently, transferring mites from one to another. Bindari et al. (2012) showed that 56% and 67% of suspected cases were positive for mites in owned and stray dogs, respectively. In owned dogs, 68% had demodicosis and 32% had scabies, while 70% had demodicosis and 30% had scabies in stray dogs. Nutritional and allergic alopecia were also common problem of dogs especially during the summer season (Siam et al., 2022). Nutritional alopecia might be due to copper or zinc deficiency. The highest prevalence of gastrointestinal parasites might be attributed to pet owners unaware of the regular deworming of their pets. A study conducted in Kathmandu Valley showed that the prevalence of gastrointestinal helminths was 46.7%, with *Ancylostoma* spp being the most common, followed by *Toxocara canis*, *Taenia/Echinococcus* spp, *Dipylidium canium* and *Trichuris vulpis* (Satyal et al., 2013).

CONCLUSION

The number of canine clinical cases brought to the Veterinary Teaching Hospital in Rampur was increasing in comparison to previous years, although the total number of cases was not sufficient. There were less than 10% canine cases from areas away from VTH. The teaching hospital should concentrate on improving disease diagnostic techniques and expanding the types of services offered, such as in-patient department facilities. To increase the number of cases flow in the hospital, pet owners should be made aware of the benefits of frequent health check-ups, and advertisements of available amenities in the hospital should be made.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Bindari, Y. R., Shrestha, S., & Shrestha, M. N. (2012). *Prevalence of mange infestation in canines of Kathmandu Valley*.
2. Chomel, B. B. (1992). Zoonoses of house pets other than dogs, cats and birds. *The Pediatric Infectious Disease Journal*, 11(6), 479–487.
3. Foreyt, W. J. (2013). *Veterinary parasitology reference manual*. John Wiley & Sons.
4. Gautam, G., Devkota, B., & Thapaliya, S. (2017). Recent case flow pattern in Veterinary Teaching Hospital. *Journal of Agriculture and Forestry University*, 1, 119–128.
5. Hall, E. (2009). Canine diarrhoea: A rational approach to diagnostic and therapeutic dilemmas. *In Practice*, 31(1), 8–16. <https://doi.org/10.1136/inpract.31.1.8>

6. Hasan, M. M., Jalal, M. S., Bayzid, M., Sharif, M. A. M., & Masuduzzaman, M. (2017). A comparative study on canine parvovirus infection of dog in Bangladesh and India. *Bangladesh Journal of Veterinary Medicine*, 14(2), 237–241. <https://doi.org/10.3329/bjvm.v14i2.31403>
7. Hasan, N., Munif, M. R., Rahman, M. M., & Alam, M. M. (2022). Diseases of dogs and cats recorded in the Bangladesh Agricultural University Veterinary Teaching Hospital during 2009–2020. *Bangladesh Journal of Veterinary Medicine*, 20(2), 65–79. <https://doi.org/10.33109/bjvmjd2022sam1>
8. Islam, O., Khatun, S., & Famous, M. (2019). Comparative hospital prevalence study of different diseases of dog at Dhaka, Chittagong and Sylhet District of Bangladesh. *Veterinary Sciences: Research and Reviews*, 5(2), 53–57. <https://doi.org/10.17582/journal.vsr/2019/5.2.53.57>
9. Joshi, M., Mehta, M. K., & Sharma, S. K. (2007). Feeding practices and common nutritional deficiency disorders in dogs. *Veterinary Practitioner*, 8(1), 83–84.
10. Kornblatt, A. N., & Schantz, P. M. (1980). Veterinary and public health considerations in canine roundworm control: A survey of practicing veterinarians. *Journal of the American Veterinary Medical Association*, 177(12), 1212–1215.
11. Kustritz, M. V. R. (2007). Determining the optimal age for gonadectomy of dogs and cats. *Journal of the American Veterinary Medical Association*, 231(11), 1665–1675.
12. Mousafarkhani, F., Sarchahi, A. A., Mohebalian, H., Khoshnegah, J., & Arbabi, M. (2023). Prevalence of canine distemper in dogs referred to Veterinary Hospital of Ferdowsi University of Mashhad, Mashhad, Iran. *Veterinary Research Forum*. Advance online publication. <https://doi.org/10.30466/vrf.2022.541661.3269>
13. Panth, Y., & Poudel, B. (2020). Retrospective study of clinical cases of dogs presented at Veterinary Teaching Hospital, Agriculture and Forestry University, Chitwan, Nepal. *Miscellaneous View Project*. March.
14. Pereira, M., Valério-Bolas, A., Saraiva-Marques, C., Alexandre-Pires, G., Pereira Da Fonseca, I., & Santos-Gomes, G. (2019). Development of dog immune system: From in uterus to elderly. *Veterinary Sciences*, 6(4), 83. <https://doi.org/10.3390/vetsci6040083>
15. Pradhan, P. (2005). Study on suspected Parvo virus infection in dogs. *Journal of Himalayan College of Agricultural Science and Technology*, 3, 57–60.
16. Satyal, R., Manandhar, S., Dhakal, S., Mahato, B., Chaulagain, S., Ghimire, L., & Pandeya, Y. (2013). Prevalence of gastrointestinal zoonotic helminths in dogs of Kathmandu, Nepal. *International Journal of Infection and Microbiology*, 2(3), 91–94. <https://doi.org/10.3126/ijim.v2i3.8211>
17. Siam, M. O., El-Sheikh, A. E.-K., & Attia, N. E. (2022). Alopecia in dogs: Causes, incidence and clinical signs with a special reference to nutritional alopecia. *Zagazig Veterinary Journal*, 50(2), 151–160. <https://doi.org/10.21608/zvjz.2022.125735.1175>
18. Simpson, K. W. (2005). Acute and chronic vomiting. In *BSAVA manual of canine and feline gastroenterology* (pp. 73–77). BSAVA Library. <https://www.bsavalibrary.com/content/chapter/10.22233/9781910443361.chap7?crawler=true>

Nutritional Evaluation of Water Hyacinth (*Eichhornia crassipes*) and Water Lettuce (*Pistia stratiotes*) in Chitwan, Nepal

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ABSTRACT

Water hyacinth (*Eichhornia crassipes*) and Water lettuce (*Pistia stratiotes*) are invasive aquatic plant in Nepal. This study assessed their potential as alternative livestock feed by analyzing their proximate composition across five different water bodies (Batuli lake, Bishazari lake, Dhungre river, Kingfisher lake, and Rampur wetland) in Chitwan, Nepal and environmental parameters (temperature, dissolved oxygen, pH, total dissolved solids, and electrical conductivity). Water quality analysis revealed significant spatial variations in total dissolved solids (TDS) and electrical conductivity (EC), with Rampur wetland exhibiting the highest values (TDS: 286.50 ppm, EC: 573.50 μ S/cm) and Bishazari lake the lowest (TDS: 53.00 ppm, EC: 106.25 μ S/cm), while temperature, pH, and dissolved oxygen (DO) remained stable. Proximate analysis showed that Water lettuce had significantly higher crude protein (27.56% in Rampur) and gross energy (3474.71 kcal/kg) compared to Water hyacinth (max protein: 18.97%). In contrast, Water hyacinth exhibited higher crude fiber (25.47% in Kingfisher lake), indicating its fibrous nature. Environmental factors, particularly TDS and EC, strongly influenced nutritional composition, with mineral-rich waters enhancing protein and fat content in Water lettuce while reducing fiber in Water hyacinth. These findings highlight Water lettuce's potential as protein-rich feed and Water hyacinth's suitability for fiber uses, with nutritional composition varying by water conditions. It further suggests that site-specific conditions must be considered when utilizing these invasive species for livestock feed and further research is needed to assess anti-nutritional factors and optimize their practical use.

Keywords: Water hyacinth; Water lettuce; Proximate analysis; Livestock feed; Environmental factors

INTRODUCTION

Water hyacinth (*Eichhornia crassipes*) and water lettuce (*Pistia stratiotes*) are highly invasive aquatic plants known for their adaptability to diverse environmental conditions, including temperature, pH, and salinity, water hyacinth, in particular, spreads rapidly through vegetative reproduction, often dominating water bodies and causing ecological

imbalances (Suleiman et al., 2019). Water lettuce, a floating macrophyte common in tropical regions, plays a significant role in nutrient absorption and water quality improvement (Purnamawati et al., 2020). Both plants have garnered attention for their potential as alternative feed sources due to their high protein content and other nutritional attributes (Lu et al., 2010).

Ecologically, these plants contribute to nutrient cycling by absorbing excess nitrogen and phosphorus, thereby enhancing water quality (Aguilar-Trigueros et al., 2019). They also provide habitat for aquatic organisms, supporting biodiversity (Sutton et al., 2018). Economically, they offer opportunities in waste water treatment, bioenergy production, and cultural applications, such as handicrafts and traditional medicine (Thapa et al., 2020). Recent studies highlight their potential in environmental remediation, renewable energy, and biomaterials (Chen et al., 2020; Pandey et al., 2021).

Despite their ecological benefits, water hyacinth and water lettuce are invasive species that cause significant environmental challenges in Nepal. Their rapid growth leads to dense mat formations, blocking waterways, reducing water quality, and obstructing irrigation and navigation channels (Dissanayaka et al., 2023). These plants also diminish the aesthetic and recreational value of water bodies and disrupt aquatic ecosystems. However, they hold potential as a nutritional resource for livestock and humans. The lack of comprehensive data on their nutritional composition and potential anti-nutritional factors limits their utilization as feed. Accurate information on their nutritional value is essential to assess their feasibility as a sustainable feed resource. Removing these plants from water bodies and repurposing them for livestock feed could mitigate their environmental impact while enhancing animal production.

Water hyacinth and water lettuce are abundant in Nepal but pose severe environmental challenges, such as waterway blockages and disruption of aquatic ecosystems. During dry seasons, feed shortages significantly constrain livestock production (Duguma and Janssens, 2021). The quality of livestock performance is directly linked to feed quality (Coleman and Moore, 2003), yet the high cost and limited availability of conventional feeds hinder sustainable livestock development. Aquatic plants like Water hyacinth and Water lettuce, rich in protein, minerals, and vitamins, offer a promising alternative (Ekunseitan et al., 2013; KM et al., 2016). Proximate analysis of these plants, including crude protein, crude fiber, ether extract, ash, and nitrogen-free extract, is crucial to evaluate their nutritional potential. This study aims to provide valuable insights into the nutritional composition of water hyacinth and water lettuce, enabling their utilization as livestock feed. Specifically, the study seeks to: assess the proximate composition of these plants in Chitwan district, Nepal; analyze their mineral content, focusing on calcium and phosphorus; and evaluate the influence of environmental factors on their nutritional composition. By repurposing these invasive plants, the study addresses feed shortages, enhances animal production, and mitigates environmental impacts, contributing to sustainable livestock practices in Nepal.

MATERIALS AND METHODS

Study area

The study was conducted in water bodies of Chitwan district, Nepal, known for its rich biodiversity and proximity to Chitwan National Park. Sampling sites were selected based on land use patterns and the extent of invasion by Water hyacinth and Water lettuce. Samples were collected from Bishazari lake (27.61605°N, 84.4373°E), Kingfisher lake (27.61712°N, 84.41953°E), Batuli lake (27.58715°N, 84.39768°E), and Dhungre river (27.5738°N, 84.4939°E) for water hyacinth, and from Rampur wetland (27.65728°N, 84.34964°E) and Dhungre river (27.5738°N, 84.4939°E) for water lettuce. These sites represent diverse ecological conditions influenced by human settlements, agricultural activities, and surrounding landscapes. Dhungre river, near the tourist hub of Sauraha, and Rampur wetland in Bharatpur Metropolitan City were chosen to capture variations due to anthropogenic impacts.



Fig. 1. Map of the study area showing sampling sites in Chitwan district, Nepal

Sampling

Sampling was conducted in February 2024 using a 1m x 1m quadrant. Plants were harvested randomly from each site using hand-held equipment and stored in labeled polythene bags. Dissolved oxygen (DO) of the water was recorded using Lutron® PDO-519 DO meter. Other environmental parameters including water temperature, pH, total dissolved solids (TDS), and electrical conductivity (EC), were recorded onsite using Hanna® HI-98129 meter. Samples were washed with water to remove debris, sealed with plastic, and transported to the laboratory for analysis.

Sample preparation

Roots were removed, and plant samples were cleaned with tap water to eliminate debris. The samples were chopped into small pieces, dried at 60°C for 72 hours, and ground into a fine powder using a laboratory grinder. The powdered samples were stored in airtight ziplock bag and transported to the lab for further analysis.

Laboratory analysis

Proximate analysis was conducted at the National Animal Feed and Livestock Quality Management Laboratory, Hariharbhawan, Lalitpur, following standard methods. Parameters such as dry matter (AOAC, 2005, Method 930.15), ash (AOAC, 2005, Method 942.05), crude protein (AOAC, 2005, Method 2001.11), crude fiber (AOAC, 2005, Method 978.10), ether extract (AOAC, 2005, Method 920.39) were determined.

RESULTS

Different environmental parameters across sampling sites

The study investigated the different environmental parameters across five sampling sites: Batuli lake, Bishazari lake, Dhungre River, Kingfisher lake, and Rampur wetland. The parameters examined were temperature, total dissolved solid (TDS), dissolved oxygen (DO), pH, and electrical conductivity (EC).

The water quality analysis across five locations revealed significant variations in TDS and EC, with Rampur showing the highest values (TDS: 286.50 ppm, EC: 573.50 μ S/cm) and Bishazari the lowest (TDS: 53.00 ppm, EC: 106.25 μ S/cm). Temperature and pH remained relatively stable with no significant differences (NS), DO averaged 6.99 ppm. TDS and EC exhibited highly significant differences ($P < 0.01$), indicating these parameters are strongly influenced by location-specific factors, such as mineral content or pollution.

Table 1. Physical parameters of water during the sampling period across different water bodies.

Location	Temperature (°C)	pH	TDS (ppm)	EC (μ S/cm)	DO (ppm.)
Bishazari	20.03 $\pm$ 0.74	7.15 $\pm$ 0.03	53.00 ^d $\pm$ 1.23	106.25 ^d $\pm$ 2.40	6.53 $\pm$ 0.60
Batuli	19.70 $\pm$ 0.31	7.48 $\pm$ 0.17	142.25 ^c $\pm$ 4.33	285.00 ^c $\pm$ 8.86	7.00 $\pm$ 1.23
Kingfisher	18.65 $\pm$ 0.73	7.35 $\pm$ 0.26	62.00 ^d $\pm$ 7.01	125.75 ^d $\pm$ 14.18	7.48 $\pm$ 0.57
Dhungre	19.75 $\pm$ 0.86	7.50 $\pm$ 0.17	233.50 ^b $\pm$ 10.54	467.50 ^b $\pm$ 21.13	7.35 $\pm$ 0.34
Rampur	20.30 $\pm$ 0.57	7.03 $\pm$ 0.09	286.50 ^a $\pm$ 13.41	573.50 ^a $\pm$ 26.71	6.60 $\pm$ 0.44
Grand Mean	19.69	7.3	155.45	311.61	6.99
LSD (0.05)	2.01	0.48	25.58	57.87	2.12
SEM ($\pm$)	0.67	0.16	8.49	18.78	0.7
P-value	NS	NS	**	**	NS

Figures followed by the same letter in a column are not significantly different by DMRT at a 5% confidence level. LSD= Least Significant Difference, NS= non-significant, * significant at 5% level and ** significant at 1%.

Proximate analysis of the water hyacinth

Water hyacinth nutritional content varied by location, with crude fiber showing significant differences ($P < 0.05$), peaking in Kingfisher (25.47%) and lowest in Bishazari (17.37%). Crude protein ranged from 14.42% (Batuli) to 18.97% (Bishazari), though differences were non-significant (NS). Fat, ash, and gross energy (GE) also showed no significant variations, suggesting Water hyacinth's nutritional profile is moderately consistent across sites, with fiber being the most variable component.

Table 2. Proximate analysis of Water hyacinth collected from the water bodies of Chitwan, Nepal

Location	Dry Matter (%)	Crude Protein (%)	Crude Fiber (%)	Fat(%)	Ash(%)	NFE(%)	GE (kcal/kg)
Bishazari	8.94±0.62	18.97±0.61	17.37 ^c ±1.27	1.92±0.08	10.12±0.79	42.68±1.97	3284.19±18.82
Batuli	11.33±0.73	14.42±1.59	24.60 ^{ab} ±2.01	2.96±0.68	8.62±0.28	38.08±1.78	3193.41±43.18
Kingfisher	9.83±1.10	15.54±1.62	25.47 ^a ±1.94	2.40±0.32	9.54±0.43	37.23±3.38	3180.31±29.90
Dhungre	12.41±0.96	17.06±2.35	19.97 ^{bc} ±1.24	2.52±0.31	10.12±0.61	37.92±1.83	3213.41±23.00
Grand Mean	10.63	16.50	21.85	2.45	9.60	38.98	3217.83
LSD (0.05)	2.68	5.11	5.09	1.25	1.72	7.19	92.96
SEM (±)	0.87	1.66	1.65	0.41	0.56	2.33	30.17
P-value	NS	NS	*	NS	NS	NS	NS

Figures followed by the same letter in a column are not significantly different by DMRT at a 5% confidence level. LSD= Least Significant Difference, NS= non-significant, * significant at 5% level and ** significant at 1% level.

Proximate analysis of the water lettuce

Water lettuce exhibited stark contrasts between Dhungre and Rampur. Rampur's WL had significantly higher crude protein (27.56%) and fat (4.08%) but lower fiber (14.61%) compared to Dhungre (protein: 19.43%, fiber: 21.97%). Dry matter and GE were also significantly higher in Rampur ($P < 0.01$), likely linked to its high-TDS environment (Table 1). These findings highlight Water lettuce's adaptability, with Rampur's conditions favoring protein and energy accumulation over fiber.

Table 3. Proximate composition of water lettuce

Location	Dry Matter (%)	Crude Protein (%)	Crude Fiber (%)	Fat(%)	Ash(%)	NFE(%)	GE (kcal/kg)
Dhungre	10.10 ^a ±0.38	19.43 ^b ±1.49	21.97 ^a ±2.11	2.27 ^a ±0.31	11.36±1.10	34.87±2.65	3364.17 ^b ±10.12
Rampur	6.73 ^b ±0.30	27.56 ^a ±2.00	14.61 ^b ±0.94	4.08 ^b ±0.23	8.70±0.32	38.33±1.78	3474.71 ^a ±12.16
Grand mean	8.42	23.49	18.29	3.18	10.03	36.60	3419.44
LSD (0.05)	1.17	6.09	5.64	0.94	2.80	7.81	38.73
SEM (±)	0.34	1.76	1.63	0.27	0.80	2.26	11.19
P-value	**	*	*	**	NS	NS	**

Figures followed by the same letter in a column are not significantly different by DMRT at a 5% confidence level. LSD= Least Significant Difference, NS= non-significant, * significant at 5% level and ** significant at 1% level.

Comparison between water hyacinth and water lettuce

The data reveals notable differences in the proximate composition of water hyacinth and Water lettuce collected from water bodies in Chitwan influenced by their growth environments. Water lettuce exhibited a significantly higher crude protein content (27.56% in Rampur and average 23.49%) compared to water hyacinth (maximum of 19.43% in Dhungre and average 16.50%), indicating its potential as a superior protein source. Conversely, water hyacinth had higher crude fiber content (25.47% in Kingfisher and average 21.85%) than Water lettuce (21.97% in Dhungre and average 18.29%), suggesting Water hyacinth may be more fibrous. Fat content was also higher in Water lettuce (Average 3.18%) than in water hyacinth. Both plants showed non-significant differences in ash and NFE, but water lettuce had a significantly higher GE value (3474.71 kcal/kg in Rampur and average 3419.44 kcal/kg) compared to water hyacinth (maximum of 3284.19 kcal/kg in Bishazari and average 3217.83 kcal/kg).

Environmental influence on nutritional values

The nutritional composition of water hyacinth and water lettuce is strongly influenced by the physicochemical properties of their aquatic habitats. TDS and EC exhibit a notable positive correlation with crude protein and fat content, particularly in Water lettuce from Rampur (TDS: 286.50 ppm, crude protein: 27.56%), suggesting that mineral-rich waters enhance protein synthesis. Conversely, higher TDS and EC levels appear to reduce crude fiber in Water hyacinth, as seen in Batuli (TDS: 142.25 ppm, crude fiber: 24.60%) compared to Bishazari (TDS: 53.00 ppm, crude fiber: 17.37%). Temperature and pH show weaker correlations with nutritional values in both plants. DO has mixed effects, higher DO sometimes aligns with elevated protein, but has inconsistencies. GE is highest in water lettuce from Rampur (3474.71 kcal/kg), likely due to its high fat and protein content.

DISCUSSION

The findings of this study demonstrate a pronounced influence of environmental conditions on both water quality and the nutritional composition of aquatic plants. Significant spatial variations in TDS and EC highlight the role of localized factors such as geological substrate, anthropogenic pollution, and agricultural runoff in shaping water chemistry (Nyenje et al., 2010). The elevated TDS/EC in Rampur likely reflects inputs from surrounding land use, consistent with studies linking nutrient-rich runoff to increased mineral loads in wetlands (Awasthi et al., 2023).

Water hyacinth exhibited relative stability in most nutritional parameters across sites, but crude fiber varied significantly, suggesting fiber synthesis is more sensitive to environmental fluctuations. Notably, fiber content was inversely correlated with TDS/EC. This aligns with evidence that mineral stress influence metabolic processes and can alter carbon allocation in aquatic plants, prioritizing structural components like fiber under resource limited conditions (Silber & Bar-Tal, 2019; Trejo-Téllez & Gómez-

Merino, 2012). In contrast, water lettuce displayed marked nutritional plasticity, with Rampur samples accumulating significantly higher crude protein and fat but lower fiber compared to Dhungre. This suggests that mineral-rich environments (like Rampur) may enhance protein and energy storage while suppressing fiber production, possibly due to shifts in metabolic pathways under high nutrient availability (Huang et al., 2017; Bloem et al., 2017). The species' adaptability underscores its potential for cultivation in nutrient-dense waters for protein-rich biomass. Also, the divergent nutritional profiles of the two species offer distinct applications. Water lettuce's superior protein and energy content make it a promising candidate than water hyacinth for animal feed, particularly in areas with mineral-enriched waters. Conversely, water hyacinth's superior fiber content makes it a potential candidate for fiber-based uses and making their nutritional profiles different.

While this study establishes clear links between water quality and nutritional composition, unmeasured factors such as plant age and genetic variability may contribute to observed variations. For instance, younger plants typically prioritize protein synthesis over fiber (Trejo-Téllez & Gómez-Merino, 2012). Future research should employ controlled experiments to isolate the effects of specific parameters while accounting for those unmeasured factors.

CONCLUSION

Key findings revealed significant site-specific variations in water quality (e.g., TDS, EC) and plant nutritional composition. Water lettuce exhibited higher crude protein content than water hyacinth. Both species showed variations to local conditions, with their nutritional profiles strongly influenced by environmental factors and proximity to human activities. From the study we have a conclusion that, despite their invasive nature, water hyacinth and Water lettuce offer sustainable, low-cost feed potential for livestock, particularly in resource-scarce regions. However, challenges including anti-nutritional compounds, contamination risks, and palatability must be addressed before large-scale adoption.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Aguilar-Trigueros, C. A., Santos, G. M., Campos, J. H., & Assis, L. C. (2019). Water hyacinth (*Eichhornia crassipes*): A review of its biology, ecology, distribution, benefits, and risks. In M. A. Pérez-Carrera (Ed.), *New perspectives in forage crops* (pp. 231–256).
2. Association of Official Analytical Chemists. (2005). *Official methods of analysis* (18th ed.). AOAC International.
3. Awasthi, P., Chataut, G., & Khatri, R. (2023). Solid waste composition and its management: A case study of Kirtipur Municipality-10. *Heliyon*, 9(11), e21831. <https://doi.org/10.1016/j.heliyon.2023.e21360>
4. Bloem, E., Albiñ, A., Elving, J., Hermann, L., Lehmann, L., Sarvi, M., Schaaf, T., Schick, J., Turtola, E., & Ylivainio, K. (2017). Contamination of organic nutrient sources with potentially toxic elements, antibiotics and pathogen microorganisms in relation to P fertilizer potential and treatment options for the production of sustainable fertilizers: A review. *Science of the Total Environment*, 607–608, 225–242. <https://doi.org/10.1016/j.scitotenv.2017.06.274>
5. Chen, Y., Sun, J., Shen, L., Wu, Y., & Li, J. (2020). Current utilization and future prospects of water hyacinth (*Eichhornia crassipes*) and water lettuce (*Pistia stratiotes*) for environmental remediation. *Frontiers in Environmental Science*, 8, 150.
6. Coleman, S. W., & Moore, J. E. (2003). Feed quality and animal performance. *Field Crops Research*, 84(1–2), 17–29. [https://doi.org/10.1016/S0378-4290\(03\)00138-2](https://doi.org/10.1016/S0378-4290(03)00138-2)
7. Dissanayaka, D. M. N. S., Udumann, S. S., Dissanayake, D. K. R. P. L., Nuwarapaksha, T. D., & Atapattu, A. J. (2023). Review on aquatic weeds as potential source for compost production to meet sustainable plant nutrient management needs. *Waste*, 1(1), 264–280. <https://doi.org/10.3390/waste1010017>
8. Duguma, B., & Janssens, G. P. J. (2021). Assessment of livestock feed resources and coping strategies with dry season feed scarcity in mixed crop-livestock farming systems around the Gilgel Gibe catchment, Southwest Ethiopia. *Sustainability*, 13(19), 10745. <https://doi.org/10.3390/su131910713>
9. Ekunseitan, D. A., Yusuf, A. O., & Odesanmi, A. F. (2013). Assessment of nutritive values of some waterweeds. *International Institute for Science, Technology and Education*. <https://www.iiste.org>
10. Huang, L., Liu, X., Wang, Z., Liang, Z., Wang, M., Liu, M., & Suarez, D. L. (2017). Interactive effects of pH, EC and nitrogen on yields and nutrient absorption of rice (*Oryza sativa* L.). *Agricultural Water Management*, 194, 48–57. <https://doi.org/10.1016/j.agwat.2017.08.012>
11. KM, A., AS, K., & RP, A. (2016). Nutritional and phytochemical quality of some tropical aquatic plants. *Poultry, Fisheries & Wildlife Sciences*, 4(2), 1–6. <https://doi.org/10.4172/2375-446X.1000164>
12. Lu, Q., He, Z. L., Graetz, D. A., Stoffella, P. J., & Yang, X. (2010). Phytoremediation to remove nutrients and improve eutrophic stormwaters using water lettuce (*Pistia stratiotes* L.). *Environmental Science and Pollution Research*, 17(1), 84–96. <https://doi.org/10.1007/s11356-008-0094-0>
13. Nyenje, P. M., Foppen, J. W., Uhlenbrook, S., Kulabako, R., & Muwanga, A. (2010). Eutrophication and nutrient release in urban areas of sub-Saharan Africa—A review. *Science of the Total Environment*, 408(3), 447–455. <https://doi.org/10.1016/j.scitotenv.2009.10.020>
14. Pandey, K. K., Maiti, S., & Ghosh, S. (2021). Emerging trends in utilization of water hyacinth: A sustainable approach for circular economy. *Resources, Conservation and Recycling*, 167, 105389. <https://doi.org/10.1016/j.resconrec.2020.105389>
15. Purnamawati, R., Taufikurrahman, T., Rahmawati, A., Putra, C. R., Rahmatilah, D. D. F., & Ashgi, F. (2020). The physiological responses of water hyacinth (*Eichhornia crassipes* (Mart.) Solms) and water lettuce (*Pistia stratiotes* L.) as trivalent chromium bioaccumulator. *Journal of Biological Science, Technology and Management*, 2(1), 7–15. <https://doi.org/10.5614/3bio.2020.2.1.2>
16. Silber, A., & Bar-Tal, A. (2019). Nutrition of substrate-grown plants. In M. Raviv, J. H. Lieth, & A. Bar-Tal (Eds.), *Soilless culture: Theory and practice* (2nd ed., pp. 291–339). Elsevier. <https://doi.org/10.1016/j.sci.2019.05.001>

org/10.1016/B978-044452975-6.50010-1

17. Suleiman, M., Khadija, A. Y., Nasiru, Y., Garba, A. A., Alhassan, M., & Bello, H. J. (2019). Proximate, minerals and anti-nutritional composition of water hyacinth (*Eichhornia crassipes*) grass. *Earthline Journal of Chemical Sciences*, 2(1), 51–59. <https://doi.org/10.34198/ejcs.3120.5159>
18. Sutton, D. L., Center, T. D., & Li, L. (2018). Water lettuce (*Pistia stratiotes* L.) and water hyacinth (*Eichhornia crassipes* (Mart.) Solms): Emerging threats to aquatic ecosystems in Florida. *Invasive Plant Science and Management*, 11(1), 1–17.
19. Thapa, A., Pradhan, S., Timilsina, Y. P., & Neupane, K. R. (2020). Water hyacinth: A resource or threat for management of the environment. *Journal of Maize Research and Development*, 6(1), 41–50.
20. Trejo-Téllez, L. I., & Gómez-Merino, F. C. (2012). Nutrient solutions for hydroponic systems. In T. Asao (Ed.), *Hydroponics: A standard methodology for plant biological researches* (pp. 1–22). IntechOpen. <https://doi.org/10.5772/37578>

Knowledge, Attitude, and Practice on Lumpy Skin Disease among Livestock Farmers in Bharatpur Metropolitan City, Chitwan, Nepal

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ABSTRACT

Lumpy Skin Disease (LSD) represents a significant transboundary viral infection affecting livestock, caused by a double-stranded DNA virus belonging to the genus Capripox within the Poxviridae family. This study aimed to evaluate the Knowledge, Attitudes, and Practices (KAP) regarding LSD among livestock farmers in Bharatpur, Metropolitan City, Chitwan, Nepal. A structured questionnaire was implemented, gathering data from 387 farmers selected through purposive sampling for face-to-face interviews. The findings starkly indicated that while many farmers were aware of LSD, their understanding of its transmission and prevention was severely lacking. Specifically, in Bharatpur, the data revealed a concerning gap in knowledge about the disease. Statistical analysis using the Chi-square test highlighted significant relationships between several factors and the farmers' KAP: gender of the respondent ($X^2 = 10.510$, $p = 0.001$), primary occupation ($X^2 = 24.057$, $p < 0.001$), education level ($X^2 = 14.881$, $p = 0.002$), type of buffalo breeds ($X^2 = 6.109$, $p = 0.013$), herd size ($X^2 = 55.878$, $p < 0.001$), and previous encounters with LSD ($X^2 = 98.377$, $p < 0.001$). These results underscore the urgent need for targeted awareness programs for livestock farmers. It is imperative that the government organizes mass vaccination campaigns and takes swift, decisive action to control and prevent outbreaks of LSD, ultimately safeguarding the livelihoods of farmers and the health of livestock in the region.

Keywords: Awareness; KAP Survey; LSD; Transboundary

INTRODUCTION

LSD possess an urgent and formidable challenge on livestock health along with the economic stability of farming communities, particularly within the cattle and buffalo

populations (Brenner et al., 2006). The disease is caused by a double-stranded DNA virus within the genus Capripoxvirus which manifests a variety of clinical symptoms, including fever, nodular skin lesions, and potentially fatal outcomes (Molla et al., 2018) respectively and varied significantly between districts. The true animal level and herd level prevalences were estimated as 26.5% (95% CI: 24.7–28.3; (OIE, 2017)genus Capripoxvirus Sheep pox virus (SPV). In India, a molecular assay study revealed that seroprevalence rate of 55.85% among screened cattle, indicating widespread prior exposure to the disease (Gayal, et al., 2022). Other report highlighted a dramatic reduction in milk production of 50% to 20% (Manjunatha Reddy, et al., 2023). A comprehensive analysis estimated that the economic toll from the July-October 2022 outbreak reached approximately INR 18,337.76 crores (around 2.26 billion USD) (Reddy, et al., 2025). A study conducted between October 2021 and March 2022, the overall seroprevalence of LSD in cattle across Bangladesh was found to be 24.59% (Begum, et al., 2024). These findings underscore the significant economic and agricultural impact of the outbreaks.

Despite the established recognition of LSD as a critical veterinary concern, a substantial research gap exists regarding the knowledge, attitudes, and practices (KAP) of livestock farmers, particularly emphasis on Nepal. Although the disease's impact is undeniable, current studies addressing farmers' awareness and responses to LSD are nonexistent in Nepal. This gap poses a significant hindrance to the development of tailored interventions that could educate farmers on preventive measures and effective management strategies. The consequences of LSD on livestock health are undeniably severe, leading to reduced productivity, increased veterinary costs, and potential loss of livelihoods for farmers. Therefore, conducting thorough research to assess the KAP of livestock farmers concerning this disease is not merely beneficial but essential. Such research would not only bridge a critical knowledge gap but also crucial for mitigating the spread and impact of LSD.

This study aims to assess farmers' KAP regarding LSD in Bharatpur Metropolitan City, Chitwan. It will analyze the demographic and farm characteristics of respondents, evaluate their understanding of the disease's causes, transmission, and symptoms, and assess their attitudes towards its impact on livestock health and economic implications. Additionally, the research will investigate management and prevention practices adopted by farmers and identify factors associated with the prevalence of the disease.

MATERIALS AND METHODS

Study areas

This study was conducted between 1 July 2023-1September in Bharatpur Metropolitan City, situated in the Chitwan district of Bagmati Province, Nepal (Figure 1). The region is part of the Terai area, renowned for its substantial livestock population, which consists of approximately 102,997 cattle and 78,833 buffaloes (MoALD, 2021).

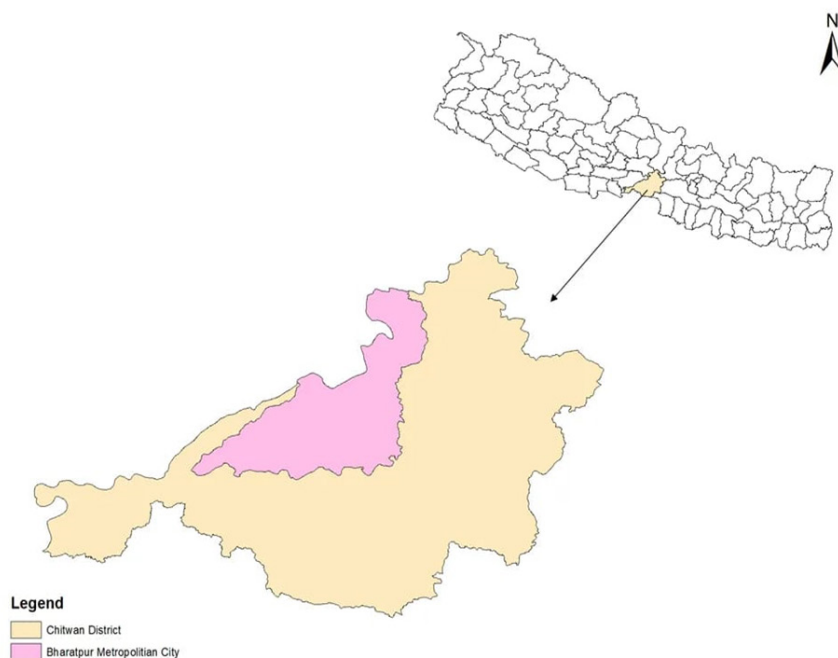


Fig. 1. Map of Nepal showing Bharatpur Metropolitan City, Chitwan district. The Map prepared using Arc GIS 10.8.

Study design and sample size

A cross-sectional study design was employed for this research. The sample size was calculated based on the assumption that 50% of livestock farmers were aware of LSD, utilizing a 95% confidence level and a 5% margin of error. According to these parameters, a total sample size of 385 was determined following the formula outlined by Daniel (1999). Ultimately, surveys were conducted with a total of 387 livestock farmers using a purposive sampling method.

The sample size calculation using Daniel formula is given below.

$$\begin{aligned} \text{i.e. } n &= \\ &= (1.96^2 \times 0.5 \times (1-0.5)) / 0.05^2 \\ &= 384.16, \text{ i.e., } 385 \end{aligned}$$

Where,

n = Sample Size

Z = At 95% level of confidence interval, Z value is 1.960

p = Anticipated level of awareness

d = Precision level (i.e., 5%)

Questionnaire design and data collection

The questionnaire used for data collection contains a set of structured close-ended questions on demographics of farmers, knowledge, attitude and practice regarding disease. The oral consent was obtained from all respondents prior to participation in the interviews. A preliminary interviews questionnaire was conducted among 10 farmers to improve the quality of questionnaire, but their data were not included in the analysis.

Data analysis

After data collection, it was imported into IBM SPSS version 25 and Microsoft Excel 2016 for analysis. The data were meticulously checked for completeness, and the subsequent analysis included descriptive statistics and chi-square tests. In knowledge and practice sections, a response of yes was score as 1 while no was scored as 0. In the attitude section, agree was score as 1 and neutral and disagree score as 0. Multiple-choice questions were scored based on the number of correct selections. For example, a question with four options, 2 points for giving two correct answers, and 4 points all four. A binary outcome for KAP was calculated based on the total scores obtained respondents KAP level was evaluated. The total scores of each respondent in all sections were converted into percentages. Based on the modified Bloom's cut-off point, respondents scoring 80% or above were classified as good knowledge, positive attitude, or good practice whereas below 80% were considered to have poor knowledge, negative attitude, or poor practice.

RESULTS

Demographic detail of respondents and farm characteristics

Majority of the respondents were male 56.8%, (n=220) of which individual aged ≥ 41 years formed the largest proportion of the participants 22%, (n=302). Large number of respondents 65.9% (n=255) had attained school level education (SLC). Majority 80.1% (n=309) of the respondents were subsistence farmer. Exotic breed of cattle and buffalo are dominant in the study area 97.5% (n=310) and 84.1% (n=116) respectively, where over half of the respondents have been raising livestock with herd size ≥ 4 . Details of socio demographic information are included in Table 1.

Table 1. Socio-demographics of respondents and farm characteristics (n=387)

		Frequency	Percentage
Gender	Male	220	56.8%
	Female	167	43.2%
Age category	≤ 40 years	85	22%
	≥ 41 years	302	78%
Occupation	Subsistence Farmer	310	80.1%
	Commercial Farmer	65	16.8%
	Others	12	3.1%
Education level	School (upto SEE/SLC)	225	65.9%
	College (upto +2 Level)	36	9.3%
	University (Bachleor or Above)	17	4.4%
	No formal education	79	20.4%
Breed of cattle	Exotic	310	97.5%
	Local	8	2.5%
Breed of buffalo	Exotic	116	84.1%
	Local	22	15.9%
Herd size	≤ 3	158	40.8%
	≥ 4	229	59.2%

Knowledge, Attitude and Practices toward LSD

Knowledge of respondents towards LSD

Our study looked at the KAP of an individual who had learned about LSD from any relevant sources. It was found that, 80.36% (n=311) of respondents were known about LSD. Furthermore, major source of information was from radio, television and mobile 63.3% (n=245) (Fig. 2).

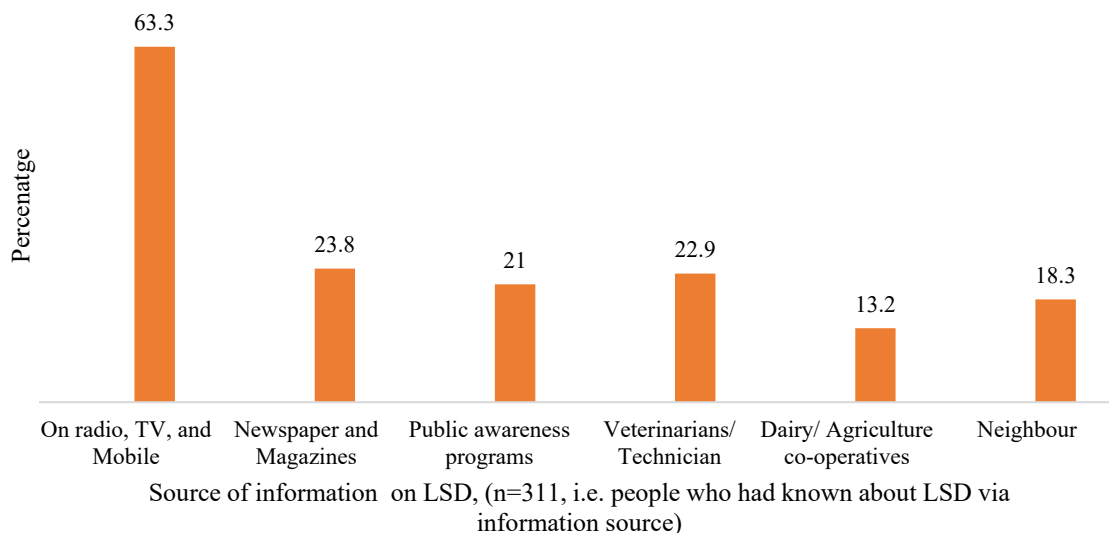


Fig.2. Source of information of LSD

Our study finding reveal that, 55.3% (n=172) of respondents did not know the cause of LSD whereas nearly one third of respondent were informed about the causative agent of LSD to be virus 31.2% (n= 97). Individuals' knowledge in regards to cause of LSD is included in Table 2.

Table 2. Knowledge of individual on etiology of LSD (n=311).

Cause of LSD	Frequency	Percentage
Bacteria	23	7.4
Virus	97	31.2
Fungus	15	4.8
Parasites	4	1.3
Don't know	172	55.3

Majority of the respondents claimed direct contact as route of transmission of LSD 82.6%, n=257), whereas over half 55.30% (n=172) of the respondent were aware of the role of biting vectors in LSD transmission (Fig. 3).

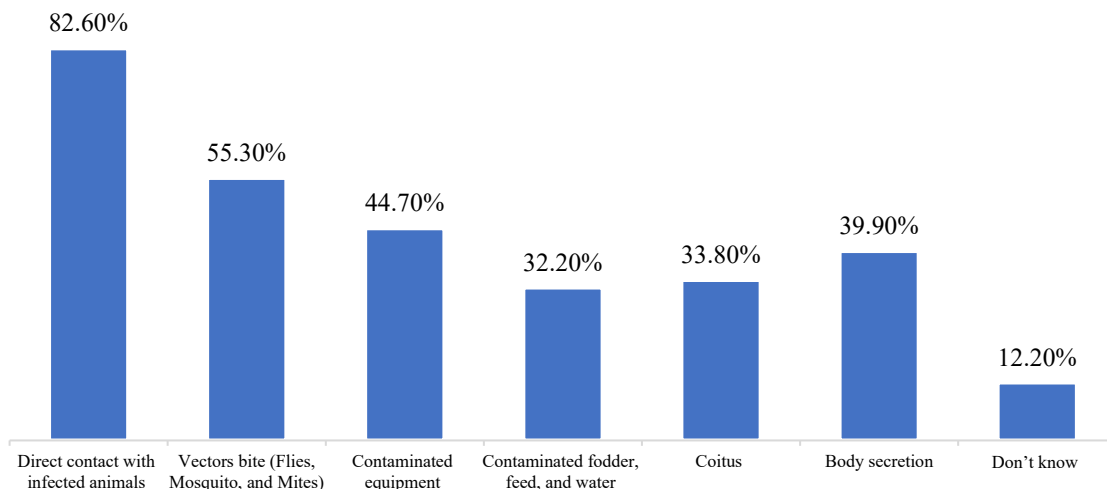


Fig. 3. Knowledge on transmission route of LSD (n=311).

Our study further revealed that majority of respondent (97.7%, n=304) claimed blister / nodules occurs in animal body during LSD period (Fig. 4).

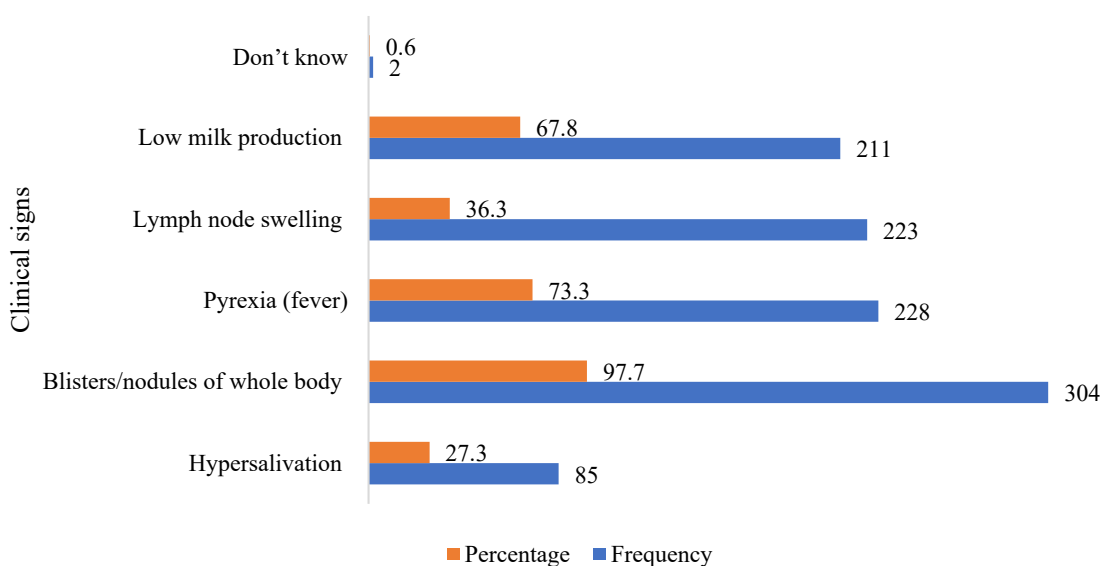


Fig. 4. Clinical signs and symptoms of LSD (n=311).

Attitude towards LSD

Finding on attitude of individual towards LSD revealed, most of the individual 99.40% (n=309) thought LSD poses threat to livestock health and productivity, whereas only 71.70 % (n=223) believed vaccination as effective preventive measure against the disease.

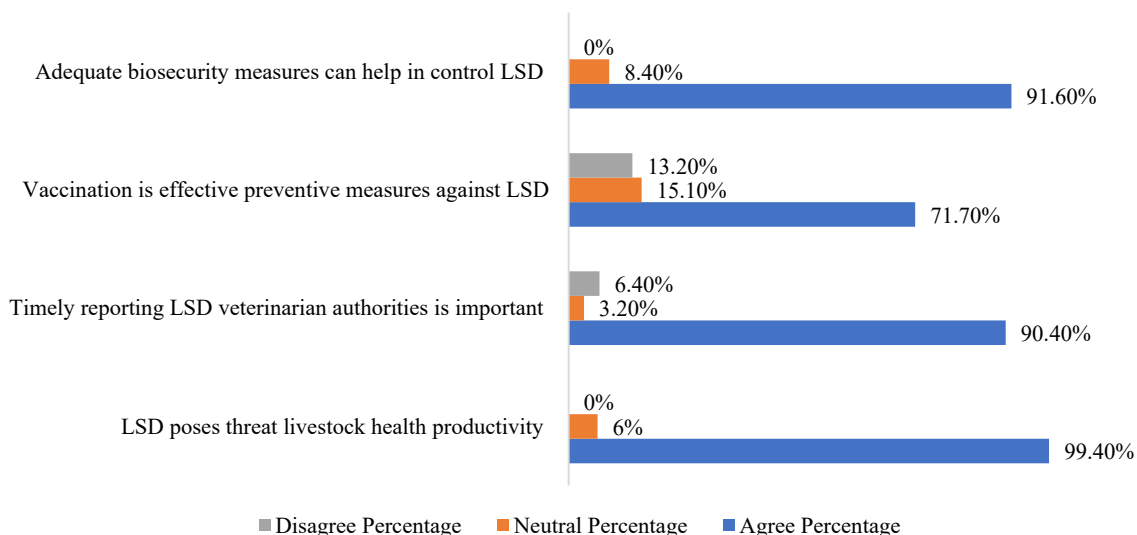


Fig. 5. Attitude of respondents towards LSD (n=311)

Practice towards LSD

Among the individual, majority 73.3% (n=73.3%) followed supportive treatment, whereas only 14.5% (n=45).

Table 3. Practices existed among individual against LSD (n=311).

Practices	Yes	No
Vaccination	45 (14.5%)	266 (85.5%)
Biosecurity Implementation	109 (35%)	202 (65%)
Isolation	110 (35.4%)	201 (64.6%)
Supportive treatment	228 (73.3%)	83 (26.7%)
Restriction of animal movement	194 (62.4%)	117 (37.6%)

Reason for no vaccination, among the respondent not vaccinating animals (n=226), most of the individual, 96.20%, claimed there was no availability of the vaccine against the disease, where as 63.20% individual did not know about LSD vaccination.

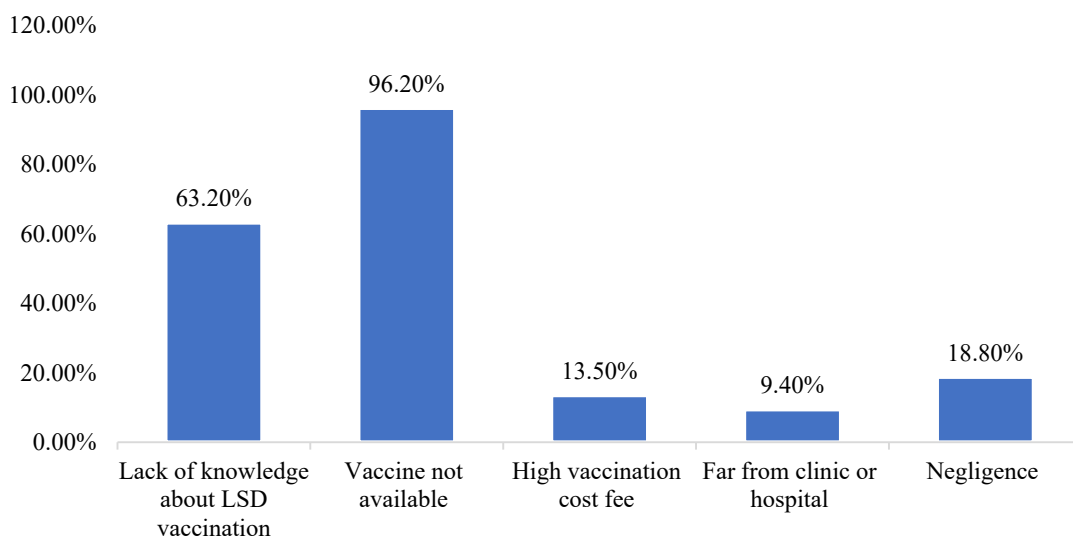


Fig. 6. Reasons for not vaccinating their animal against LSD (n=226 i.e., number of respondents not vaccinating animals)

DISCUSSION

The findings of our study indicate a significant acceptance of mass media channels, such as radio, television, and mobile, as reliable sources of information regarding Lumpy Skin Disease (LSD) among respondents. This aligns with previous reports suggesting that mass media plays a crucial role in disseminating information about animal diseases (Nesar et al., 2022). The increasing accessibility of the internet, with 49.2% of the total population in Nepal reported as internet users (Kemp, 2024), alongside proactive information dissemination by government agencies, likely enhances public awareness about LSD.

Many individuals demonstrate a lack of understanding regarding the causative agents of LSD. Our data reveals that most respondents were middle-aged and possessed educational qualifications up to the primary level. It is noteworthy that LSD was first detected in Nepal in 2020 (WOAH - Asia, 2023), yet courses relating to this disease have not incorporated relevant information. This, consequently contributes to a generally low knowledge level surrounding the disease's etiology.

Interestingly, while individuals accepted that LSD is transmitted through direct contact, this finding contrasts with Sudhakar et al. (2020), where a majority (39%) identified biting flies as the primary source of infection. This discrepancy likely reflects variations in knowledge levels among different groups. LSD is known for its characteristic nodular skin lesions and discharge, which may lead to the belief that direct contact with infected animals is a primary route of transmission. However, LSD predominantly spreads through mechanical transmission via blood-feeding insects that flourish in warm, moist environments (Chihota et al., 2001), highlighting a gap in public understanding that needs to be addressed.

Moreover, it was observed that individuals showed poor uptake of vaccination as a control measure against LSD. Among respondents who did not vaccinate their animals, the predominant reasons cited were the unavailability of the vaccine and insufficient knowledge regarding vaccination practices. However, according to WOAH, a significant quantity of LSD vaccine, specifically 1519000 doses, has been imported into Nepal, with 1.2 million doses reportedly used. Given that the total population of cattle and buffalo in Nepal amounts to approximately 7.48 million (Central Statistics Office, 2023), the available vaccine supply is insufficient to meet the coverage needs of the livestock population. This inadequacy likely explains the low vaccination rates observed among respondents.

Majority of the respondents were male, suggesting that livestock farming in Chitwan is male dominated and is in agreement with previous studies by (Parvin *et al.*, 2011). This can be explained by the tradition and culture of most of Nepalese societies where male have a responsibility of taking care of animals and making decision of various family matters. Male and female respondents demonstrate varying levels of knowledge and practices regarding disease management. Similar findings have been reported in studies on antimicrobial resistance in livestock, which highlights gender disparities in both awareness and practices (Parvin *et al.*, 2022). Such disparities may stem from traditional gender roles within animal husbandry, differential access to information, and disparities in decision-making power.

Individuals directly involved in livestock farming exhibit higher awareness and more proactive measures in disease prevention and control. These findings resonate with those observed in Thailand, where farmers primarily engaged in cattle raising displayed significantly improved KAP regarding Lumpy Skin Disease (LSD) (Sricharern, Thongratsakul, & Kongkaew, 2024). This is because literate respondents affirm their ability to adapt to new technologies in livestock farming and disease control. Higher levels of formal education appear to enhance the understanding of disease prevention and management. However, advanced educational attainment does not always correlate with improved practices, underscoring the necessity for specialised training in veterinary health (Sricharern *et al.*, 2024). Farmers with larger herds exhibit heightened vigilance towards disease risks, likely due to increased financial stakes. The effect of herd size as a factor influencing preventive behaviour has been well established (Sricharern *et al.*, 2024). Disease risk can be high due to high chance of spreading in a flock over a short period of time. Vectors population and distribution is also high in terai region of Nepal. Age category or cattle breed may not exert a direct influence on LSD-related knowledge or behavior, contrasting with the more substantial impacts of education, experience, and occupation.

CONCLUSION

We found poor KAP towards LSD in Bharatpur Metropolitan City, Chitwan Nepal and different farm characteristics were associated with knowledge level of LSD. There is great need of conducting awareness program related to LSD targeting livestock farmers. We

found majority of farmers did not vaccinate their animal against LSD because vaccine is not easily accessible to farmers. So, government should provide necessary arrangements for mass LSD vaccination camps. Ultimately, enhancing farmers' awareness & engagement can significantly contribute to the control and prevention of LSD outbreaks.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Acharya, K. P., & Subedi, D. (2020). First outbreak of lumpy skin disease in Nepal. *Transboundary and Emerging Diseases*, 67(6), 2280–2281. <https://doi.org/10.1111/tbed.13815>
2. Addise Ambilo, A. M. (2013). Major skin diseases of cattle: Prevalence and risk factors in and around. *Journal Name*, 3, 147–153. (*Journal name missing; please update.*)
3. Al-Salihi, K. A. (2014). Lumpy skin disease: Review of literature. *Mirror of Research in Veterinary Sciences and Animals*, 3(3), 6–23.
4. Annandale, C. H., Holm, D. E., Ebersohn, K., & Venter, E. H. (2014). Seminal transmission of lumpy skin disease virus in heifers. *Transboundary and Emerging Diseases*, 61(5), 443–448. <https://doi.org/10.1111/tbed.12045>
5. Ayelet, G., Abate, Y., Sisay, T., Nigussie, H., Gelaye, E., Jemberie, S., & Asmare, K. (2013). Lumpy skin disease: Preliminary vaccine efficacy assessment and overview on outbreak impact in dairy cattle at Debre Zeit, central Ethiopia. *Antiviral Research*, 98(2), 261–265. <https://doi.org/10.1016/j.antiviral.2013.02.008>
6. Babiuk, S., Bowden, T. R., Boyle, D. B., Wallace, D. B., & Kitching, R. P. (2008). Capripoxviruses: An emerging worldwide threat to sheep, goats and cattle. *Transboundary and Emerging Diseases*, 55(7), 263–272. <https://doi.org/10.1111/j.1865-1682.2008.01043.x>
7. Bean, W. J. (1991). Virology. *Archives of Virology*, 120, 309–323.
8. Begum, R., Uddin, M. B., Rahman, M. M., Roy, M., Rahman, A., Rafe-Ush-Shan, S. M., & Hossain, M. M. (2024). Lumpy skin disease in Bangladesh: Seroprevalence and risk factor of LSD in cattle. *International Journal of Veterinary Science*. <https://doi.org/10.47278/journal.ijvs/2024.207>
9. Brenner, J., et al. (2006). Lumpy skin disease (LSD) in a large dairy herd in Israel, June 2006. *Israel Journal of Veterinary Medicine*, 61(1), 73–77.
10. Chihota, C. M., Rennie, L. F., Kitching, R. P., & Mellor, P. S. (2001). Mechanical transmission of lumpy skin disease virus by Aedes. *Epidemiology and Infection*, 126, 317–321.
11. Das, M., Chowdhury, M. S. R., Akter, S., Mondal, A. K., Uddin, M. J., Rahman, M. M., & Rahman, M. M. (2021). An updated review on lumpy skin disease: Perspective of Southeast Asian countries. *Journal of Advanced Biotechnology and Experimental Therapeutics*, 4(3), 322–333. <https://doi.org/10.5455/JABET.2021.D133>
12. Davies, F. G. (1991). Lumpy skin disease, an African capripox virus disease of cattle. *British Veterinary Journal*, 147, 489–503.

13. Dietze, K., Moritz, T., Alexandrov, T., Krstevski, K., Schlottau, K., Milovanovic, M., Hoffmann, D., & Hoffmann, B. (2018). Suitability of group-level oral fluid sampling in ruminant populations for lumpy skin disease virus detection. *Veterinary Microbiology*, 221, 44–48. <https://doi.org/10.1016/j.vetmic.2018.05.022>
14. Duguma, B., Kechero, Y., & Janssens, G. P. J. (2012). Survey of major diseases affecting dairy cattle in Jimma town, Oromia, Ethiopia. *Global Veterinaria*, 8(1), 62–66.
15. Farah Gumbe, A. A. (2018). Review on lumpy skin disease and its economic impacts in Ethiopia. *Journal of Dairy, Veterinary & Animal Research*, 7(2), 39–46. <https://doi.org/10.15406/jdvar.2018.07.00187>
16. Gari, G., Waret-Szkuta, A., Grosbois, V., Jacquiet, P., & Roger, F. (2010). Risk factors associated with observed clinical lumpy skin disease in Ethiopia. *Epidemiology and Infection*, 138(11), 1657–1666. <https://doi.org/10.1017/S0950268810000506>
17. Gautam, M., Kattel, P., & Kaphle, K. (2022). Review on lumpy skin disease and its emerging threat to livestock in Nepal. *Veterinary Sciences: Research and Reviews*, 8(1), 43–51. <https://doi.org/10.17582/journal.vsr/2022.8.1.43.51>
18. Gayal, S. D., Sakhare, M. P., Suman, P., Shafi, T. A., Siddiqui, M. F., & Yeotikar, P. V. (2022). Molecular detection and seroprevalence of lumpy skin disease in cattle. *Indian Journal of Animal Research*. <https://doi.org/10.18805/IJAR.B-4933>
19. Hirpa, E., Gnare, F., Balcha, E., Hagos, Y., Duguma, M., Amante, M., & Abdeta, D. (2015). Assessment of community awareness and retrospective study on lumpy skin disease in Guto Gida, Wayu Tuka and Gidaayana Districts, Eastern Wollega, Ethiopia. *African Journal of Basic & Applied Sciences*, 7(4), 194–199. <https://doi.org/10.5829/idosi.ajbas.2015.7.4.9685>
20. Hunter, P., & Wallace, D. (2001). Lumpy skin disease in Southern Africa: A review of the disease and aspects of control. *Journal of the South African Veterinary Association*, 72(2), 68–71. <https://doi.org/10.4102/jsava.v72i2.619>
21. Kasem, S., Saleh, M., Qasim, I., Hashim, O., Alkarar, A., Abu-Obeida, A., Gaafer, A., Hussien, R., AL-Sahaf, A., Al-Doweriej, A., Bayoumi, F., Hodhood, A., & Abdelatif, M. (2018). Outbreak investigation and molecular diagnosis of lumpy skin disease among livestock in Saudi Arabia 2016. *Transboundary and Emerging Diseases*, 65(2), e494–e500. <https://doi.org/10.1111/tbed.12769>
22. Kemp, S. (2024, February 23). *Digital 2024: Nepal — DataReportal – Global Digital Insights*. <https://datareportal.com/reports/digital-2024-nepal>
23. Manjunatha Reddy, G. B., Nagaraj, S., Bijalwan, S., Tadakod, S., Bhutia, Z., Palmu, D., ... Gulati, B. R. (2023). Lumpy skin disease (LSD) in yak (*Bos grunniens*): An evidence of species spillover from cattle in India. *Microorganisms*, 11(12), 2823. <https://doi.org/10.3390/microorganisms11122823>
24. MoALD. (2021). *Statistical information on Nepalese agriculture (2077/78)*. Nepal in Data Portal. <https://nepalindata.com/resource/statistical-information-nepalese-agriculture-207374-201617/>
25. Molla, W., Frankena, K., Gari, G., Kidane, M., Shegu, D., & de Jong, M. C. M. (2018). Seroprevalence and risk factors of lumpy skin disease in Ethiopia. *Preventive Veterinary Medicine*, 160, 99–104. <https://doi.org/10.1016/j.prevetmed.2018.09.029>
26. Mulatu, E., & Feyisa, A. (2018). Review: Lumpy skin disease. *Journal of Veterinary Science & Technology*, 9(3). <https://doi.org/10.4172/2157-7579.1000535>
27. Nesar, S., Rafiq, K., Ansari, A., Ali, S., Usmani, M. S., & Bhutto, M. (n.d.). The awareness of lumpy skin disease (LSD) among health care practitioners of Karachi, Pakistan. *Journal Name, Volume(Issue)*, 61–67. (Details incomplete; update with journal name and volume.)
28. OIE. (2017). *Lumpy skin disease card*. <https://www.oie.int/wahis/public.php?page=home>
29. OIE. (2021). *Technical meeting on lumpy skin disease (LSD): LSD situation in Viet Nam 2021*.
30. Poudel, U. (2020). Lumpy skin disease (LSD): Recent outbreak and threatening smallholder cattle

- farmer in Nepal. *Turkish Veterinary Journal*, 2(2), 84–86.
31. Reddy, M., Baijalwan, G. B., Jacob, S. S., Tadakod, S., Maharana, S. M., Nagaraj, S., ... Gulati, B. R. (2025). Investigation of comorbidity and risk factors analysis during lumpy skin disease outbreaks in India. *Microorganisms*, 13(3), 472. <https://doi.org/10.3390/microorganisms13030472>
32. Singh, S. B., Singh, N., Sector, L., & Specialist, D. (2019). Nepal livestock feed balance and strategies to address the feed deficit. *Journal of Agriculture and Forestry University*, 3, 159–171.
33. Sprygin, A., Artyuchova, E., Babin, Y., Prutnikov, P., Kostrova, E., Byadovskaya, O., & Kononov, A. (2018). Epidemiological characterization of lumpy skin disease outbreaks in Russia in 2016. *Transboundary and Emerging Diseases*, 65(6), 1514–1521. <https://doi.org/10.1111/tbed.12889>
34. Sudhakar, S. B., Mishra, N., Kalaiyarasu, S., Jhade, S. K., Hemadri, D., Sood, R., Bal, G. C., Nayak, M. K., Pradhan, S. K., & Singh, V. P. (2020). Lumpy skin disease (LSD) outbreaks in cattle in Odisha state, India in August 2019: Epidemiological features and molecular studies. *Transboundary and Emerging Diseases*, 67(6), 2408–2422. <https://doi.org/10.1111/tbed.13579>
35. Thota, R., & Manda, A. (2023). A short review on transmission of lumpy skin disease. *Journal Name*, 12(7), 1154–1159. (*Journal name missing; please update.*)
36. Tuppurainen, E., Alexandrov, T., & Beltrán-Alcrudo, D. (2017). *Lumpy skin disease field manual – A manual for veterinarians* (FAO Animal Production and Health Manual No. 20). Food and Agriculture Organization of the United Nations. <https://www.fao.org/publications>
37. Tuppurainen, E. S. M., & Oura, C. A. L. (2012). Lumpy skin disease: An emerging threat to Europe, the Middle East and Asia. *Transboundary and Emerging Diseases*, 59(1), 40–48. <https://doi.org/10.1111/j.1865-1682.2011.01242.x>
38. WOAHA - Asia. (2023). *Livestock and animal health situation in Nepal: Opportunities, challenges and way forward*. <https://rr-asia.woah.org/app/uploads/2023/11/5-country-report-nepal.pdf>

Seroprevalence of Brucellosis in Cattle and Yaks of Nepal

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ABSTRACT

Cross-sectional studies were conducted from April 2021 to October 2024 to determine the prevalence of Brucella abortus antibody in raw milk and serum of cows and Yak/Nak in Tanahu, Mustang, Chitwan, Rupandehi, Nawalparasi Bardaghat Susta East and Kanchanpur district. A total of 130 milk samples and 997 serum samples were collected using a purposive sampling technique from different household and farms. Thus, collected samples were transported to National Cattle Research Program Laboratory, Chitwan for indirect ELISA test by maintaining cold chain. Data entry was done in Microsoft excel and statistical analysis was done by using SPSS version 19 and Fisher exact test was used. These samples showed an overall prevalence of brucellosis to be 0.62% (7/1127) in the study area. Considering district wise prevalence of brucellosis, 3% (4/130) in Tanahu, 0% (0/92) in Mustang, 0.2% (1/498) in Chitwan, 0.72% (1/139) in Nawalparasi Bardaghat Susta East, 0% (0/141) in Rupandehi and 0.78% (1/127) in Kanchanpur was found. The result showed no statistical significance ($P > 0.05$) to any of the factors including age, breed, breeding method, location and history of abortion/repeat breeding/ROP. With no history of vaccination, positive reactors were asymptomatic animals that harbored natural infection. Thus, further studies need to be done in other remaining areas to detect animals in the carrier state with molecular testing facility like PCR. Start of vaccination programs to the animals along with awareness among people may aid in the prevention of disease transmission and public health problems.

Keywords: *B. abortus; Indirect ELISA; Public health; Zoonosis*

INTRODUCTION

Brucellosis is an infectious disease caused by gram negative bacteria characterized by abortion and infertility in several mammalian species, and is one of the most important zoonosis worldwide (Cutler et al., 2005). Various species of *Brucella* affects different livestock species as *Brucella abortus* (bovines), *Brucella melitensis* (sheep and goats) and *Brucella suis* (pigs) (Mufflihanah et al., 2013). Brucellosis creates significant economic losses to the livestock industry worldwide because it usually results in abortion, infertility, decreased milk and meat production. The clinical signs of disease in male animals includes orchitis, epididymitis and seminal vesiculitis (Cutler et al 2005). Ruminants are highly susceptible to brucellosis compared to other domestic animals (Moreno, 2014). Transmission occurs to human mainly through contact with placenta, fetus, fetal fluids and vaginal discharges from an infected animal. Entry into the body occurs by ingestion of unpasteurized dairy products, undercooked meat and through the mucous membranes, broken skin and possibly through intact skin (Xavier et al., 2010). Brucellosis has been an occupational risk for farmers, veterinarians and employees in the meat packing business. Non occupational sources of infection include consumption of fresh, unpasteurized raw fresh milk and milk products (Hartigan, 1997). In human, brucellosis can cause multisystemic disease with varying spectrum of symptoms. The clinical signs may in human include intermittent or irregular fever, headache, weakness, profuse sweating, chills, weight loss and general aching. Organ infections including liver and spleen may also occur (Corbel, 2006).

In Nepal, endemic bovine and caprine brucellosis pose a serious public health hazard. Recent cases of human brucellosis in veterinary and para-veterinary practitioners have been shocking. However, a systematic and large-scale brucellosis control program targeting the eradication of the disease has not been adopted in Nepal. Financial limitations, inadequate laboratory facilities, lack of obedience by farmers and religious restrictions may impede the success of *Brucella* eradication in Nepal (Acharya et al., 2016). The disease has been successfully managed or eradicated from several developed countries due to routine screening of disease in domestic animals and animal vaccination program, but it is still endemic in livestock and human populations in resource-poor countries (Lopes et al., 2010 and McDermott et al., 2013). Currently, awareness creating program on zoonotic disease and sero- surveillance monitoring of zoonotic disease including brucellosis is in progress.

Although there are some published studies describing the seroprevalence of animal and human brucellosis in Nepal, there are very few studies that describe the risk factors related to animal brucellosis in Nepal. Identifying risk factors and implementing prevention and control programs could lead to a decrease in the disease burden in ruminants especially cattle, with consequent improvements in human health. Effective control and preventive measures can be applied once the risk factors are identified.

MATERIALS AND METHODS

Different cross-sectional studies were conducted from July 2021 to September 2021 (Tanahu), April 2021 to May 2021 (Mustang), November 2022 to January 2023 (Chitwan), May to June 2023 (Rupandehi), June to August 2023 (Kanchanpur) and August to October 2024 (Nawalparasi Bardaghat Susta East and Chitwan) to determine the prevalence of *Brucella abortus* antibody in raw milk of cows in Tanahu district, serum of yak/nak in Mustang district and serum of cattle in Chitwan, Rupandehi, Nawalparasi Bardaghat Susta East and Kanchanpur district. A total of 130 milk samples and 997 serum samples were collected using a purposive sampling technique from different household and farms.

Thus, collected samples were transported to National Cattle Research Program laboratory, Chitwan for indirect ELISA test by maintaining cold chain. Questionnaire survey regarding personnel information and different risk factors were also conducted during the sampling time. Data entry was done in Microsoft excel and statistical analysis was done by using SPSS version 19 and Fisher exact test was used.

RESULTS

These samples showed an overall prevalence of brucellosis to be 0.62% (7/1127) in the study area. Considering district wise prevalence of brucellosis, 3% (4/130) in Tanahu, 0% (0/92) in Mustang, 0.2% (1/498) in Chitwan, 0.72% (1/139) in Nawalparasi Bardaghat Susta East, 0% (0/141) in Rupandehi and 0.78% (1/127) in Kanchanpur was found as shown in Table 1. The result showed no statistical significance ($P>0.05$) to any of the factors including age, breed, breeding method, location, history of abortion/repeat breeding/ROP etc. on seroprevalence as shown in Table 2.

Table 1. District wise prevalence of brucellosis in cattle and yak

Location	Species	Number	Positive	P value
Tanahu	Cattle	130	4 (3.00%)	0.24
Chitwan	Cattle	498	1 (0.20%)	
Rupandehi	Cattle	141	0 (0.00%)	
Kanchanpur	Cattle	127	1 (0.78%)	
Nawalparasi Bardaghat Susta East	Cattle	139	1 (0.72%)	
Mustang	Yak	92	0 (0.00%)	

In breed wise prevalence of brucellosis, HF cross had 0.67% (3/449) prevalence, Jersey cross had 0.74% (3/404), local breed had 0.55% (1/182) and Yak had 0% (0/92). The result showed no statistical significance ($P>0.05$) in different breeds of cattle. Considering age wise prevalence of brucellosis in cattle, 0-4 years age group had prevalence of 0.50% (2/396), 4-8 years age group had prevalence of 0.91% (5/545) and 8-12 years age group had prevalence of 0% (0/186). The result showed no statistical significance ($P>0.05$) in different age groups of cattle.

In breeding method wise comparison of brucellosis prevalence in cattle, artificial insemination practicing cattle had 0.85% (6/703) and natural insemination practicing cattle had 0.24% (1/424). The result showed no statistical significance ($P>0.05$) in different breeding method. Regarding history of abortion in cow, history with presence of abortion had prevalence of 1.72% (5/290) and without abortion had prevalence of 0.24% (2/837). The result showed no statistical significance ($P>0.05$) with history of abortion. Regarding history of repeat breeding in cow, history with presence of repeat breeding had prevalence of 1.5% (6/399) and without repeat breeding had prevalence of 0.13% (1/728). The result showed no statistical significance ($P>0.05$) with history of repeat breeding. Regarding history of retention of placenta in cow, history with presence of retention of placenta had prevalence of 0.62% (2/322) and without retention of placenta had prevalence of 0.62% (5/805). The result showed no statistical significance ($P>0.05$) with history of abortion.

Table 2. Effect of various factors on seroprevalence of brucellosis

Factors		Number	Positive	P value
Breed	HF cross	449	3 (0.67%)	0.27
	Jersey cross	404	3 (0.74%)	
	Local	182	1 (0.55%)	
	Yak/Nak	92	0 (0.00%)	
Age (Years)	0-4	396	2 (0.50%)	0.29
	4-8	545	5 (0.91%)	
	8-12	186	0 (0.00%)	
Breeding method	AI	703	6 (0.85%)	0.16
	NI	424	1 (0.24%)	
History of abortion	Yes	290	5 (1.72%)	0.16
	No	837	2 (0.24%)	
History of repeat breeding	Yes	399	6 (1.5%)	0.16
	No	728	1 (0.13%)	
History of ROP	Yes	322	2 (0.62%)	0.25
	No	805	5 (0.62%)	

DISCUSSION

Overall prevalence of *B. abortus* in our study is 0.62% ($n=7/1127$). Previous studies in Nepal showed prevalence of brucellosis in cattle as 3% (Pradhan, 1996), 1.38% (Pyakurel and Mishra, 1977), 32% (Pandeya et al., 2013), 10.86% (Subedi et al., 2016) and 2.33% (Acharya et al., 2020). Less prevalence in present study than previous studies may be

due to increased awareness in farmers and more trend of using artificial insemination in cattle than natural insemination in recent days. Result showed no statistical significance ($P>0.05$) to any of the factors including age, breed, location, breeding method, history of abortion, history of retention of placenta and history of repeat breeding in cows.

Result of our study showed prevalence higher in Jersey cross (0.74%) as compared to Holstein Friesian cross (0.67%) which is in contrary with (Chaulagain and Bowen 2016) that showed higher prevalence (31.57%) in Holstein Friesian (HF) crossbred cattle than Jersey cross (12.12%). This could be due to less sample size of Jersey cross than HF cross in our study.

A study conducted in Chitwan, age group of (3-5) year had 1.09% (1/92) positive. On age group of (5-7) year, 3% showed the positive. The age group (7-9) year, 6% cattle showed positive. In the same way the age group (9 year above), 0% was positive (Subedi et al., 2016). There was no statistically significant difference in age wise prevalence of brucellosis in cattle ($p>0.05$). This study had also similar finding as our study.

CONCLUSION

Among six districts studied, four districts had prevalence of brucellosis. Tanahu, Bardaghat Susta East and Kanchanpur were three new districts for study and all of these districts showed prevalence of brucellosis. It signifies brucellosis is endemic in Nepal. As in other under-developed and developing countries of world, the situation involving brucellosis is alarming in Nepal too. Thus, further studies need to be done in different areas considering more risk factors to detect animals in the carrier state with molecular testing facility like PCR. Extensive research on the epidemiology and public health impacts of brucellosis is urgently necessary. Efforts should be made to control brucellosis in animals and to reduce its transmission to human. Vaccination to animals and awareness program to public should be strengthened by government sector as soon as possible with due importance.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Acharya, K. P., Kaphle, K., Shrestha, K., Garin-Bastuji, B., & Smits, H. L. (2016). Review of brucellosis in Nepal. *International Journal of Veterinary Science and Medicine*, 4(2), 54–62. <https://doi.org/10.1016/j.ijvsm.2016.08.001>
2. Acharya, M. P., Pathak, C. R., Pandey, G., Pandeya, Y. R., & Pathak, L. R. (2020). Screening of major infectious causes of infertility in dairy cattle of Nepal. In *11th National Workshop on Livestock and*

Fisheries Research in Nepal (p. 62).

3. Cárdenas, L., Awada, L., Tizzani, P., Cáceres, P., & Casal, J. (2019). Characterization and evolution of countries affected by bovine brucellosis (1996–2014). *Transboundary and Emerging Diseases*, 66(3), 1280–1290. <https://doi.org/10.1111/tbed.13139>
4. Chaulagain, R., & Bowen, R. A. (2016). Seroprevalence and epidemiological study of bovine brucellosis in dairy cattle of Kavrepalanchowk district of Nepal. [*Unpublished manuscript*].
5. Corbel, M. J. (2006). *Brucellosis in humans and animals*. World Health Organization, Food and Agriculture Organization of the United Nations, & World Organization for Animal Health (OIE).
6. Cutler, S. J., Whatmore, A. M., & Commander, N. J. (2005). Brucellosis: New aspects of an old disease. *Journal of Applied Microbiology*, 98(6), 1270–1281. <https://doi.org/10.1111/j.1365-2672.2005.02622.x>
7. Hartigan, P. (1997). Human brucellosis: Epidemiology and clinical manifestations. *Irish Veterinary Journal*, 50, 179–180.
8. Lopes, L. B., Nicolino, R., & Haddad, J. P. A. (2010). Brucellosis-risk factors and prevalence: A review. *The Open Veterinary Science Journal*, 4(1), 24–28. <https://doi.org/10.2174/1874318801004010024>
9. McDermott, J., Grace, D., & Zinsstag, J. (2013). Economics of brucellosis impact and control in low-income countries. *Revue Scientifique et Technique (International Office of Epizootics)*, 32(1), 249–261. <https://doi.org/10.20506/rst.32.1.2197>
10. Moreno, E. (2014). Retrospective and prospective perspectives on zoonotic brucellosis. *Frontiers in Microbiology*, 5, Article 213. <https://doi.org/10.3389/fmicb.2014.00213>
11. Muflihanah, H., Hatta, M., Rood, E., Scheelbeek, P., Abdoel, T. H., & Smits, H. L. (2013). Brucellosis seroprevalence in Bali cattle with reproductive failure in South Sulawesi and *Brucella abortus* biovar 1 genotypes in the Eastern Indonesian archipelago. *BMC Veterinary Research*, 9, Article 233. <https://doi.org/10.1186/1746-6148-9-233>
12. Pandeya, Y. R., Joshi, D. D., Dhakal, S., Ghimire, L., Mahato, B. R., & Chaulagain, S. (2013). Seroprevalence of brucellosis in different animal species of Kailali district, Nepal. *International Journal of Infection and Microbiology*, 2(1), 22–25. <https://doi.org/10.3126/ijim.v2i1.8320>
13. Pradhan, A. (1996). Sero-surveillance of brucellosis in cattle and buffaloes in Chitwan. In *Proceedings of the First National Workshop on Livestock and Fisheries Research in Nepal* (pp. 227–231).
14. Pyakurel, S., & Mishra, U. (1977). Sero-epidemiological evidence of animal brucellosis in Nepal. *Bulletin of Veterinary Science and Animal Husbandry, Nepal*, 6, 1–5.
15. Subedi, S., Prajapati, M., & Dhakal, B. (2016). Sero-surveillance of brucellosis in cattle of Chitwan District, Nepal. *International Journal of Applied Sciences and Biotechnology*, 4(3), 365–371. <https://doi.org/10.3126/ijasbt.v4i3.15538>
16. Xavier, M. N., Paixão, T. A., den Hartigh, A. B., Tsolis, R. M., & Santos, R. L. (2010). Pathogenesis of *Brucella* spp. *The Open Veterinary Science Journal*, 4, 109–118. <https://doi.org/10.2174/1874318801004010109>

Serological and Genomic Evidence of *Brucella* in Nepalese Dairy Cattle

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ABSTRACT

Starting in 2015, research was undertaken to estimate the prevalence of brucellosis in a non-vaccinated herd of apparently healthy dairy cattle with a history of infertility and repeat breeding using blood/serum and milk samples. A Rose Bengal Plate Test (RBPT) was done to screen the blood samples, followed by a DOT ELISA using Immunocomb Assay in serum samples. Representative samples positive on both tests were then subjected to PCR for the confirmation of Brucella species. Out of the total 1456 samples collected in the last 10 years, 242 non-repetitive samples were found to be positive for Brucella antigen, with a prevalence of 16.62%. End-point conventional PCR was done on the selected samples to identify the organism, and the results showed the presence of B. abortus in these animals. Though Nepal does not allow vaccination of animals against brucellosis, the serological evidence showed a high prevalence of antibodies among the dairy cattle. Confirmed by PCR, it can be inferred that B. abortus is actively circulating in the Nepalese dairy herd, which could be the major cause of decreased fertility and allied problems in such animals. Suitable control strategies, including the possibility of access to vaccines, should be considered for mitigating the adverse effects. Future research should differentiate field and vaccine strains and explore molecular epidemiology to support targeted vaccination policies.

Keywords: *Brucella; Cattle; Serology; Prevalence; Molecular*

INTRODUCTION

The disease brucellosis is an infectious and economically important zoonotic disease caused by bacteria from the genus *Brucella*. *Brucella abortus* is the primary causal agent in cattle, while *B. melitensis* and *B. suis* can seldom infect them (Corbel et al., 2006). The condition is generally defined by reproductive issues such as abortion, retention of placenta, infertility, orchitis, and impaired milk production. These manifestations result in significant economic losses for the livestock industry due to decreased productivity,

reduced fertility, and increased culling rates (McDermott et al., 2013). Brucellosis in cattle is transmitted mostly through direct contact with aborted fetuses, placenta, vaginal secretions, and contaminated surroundings. The organism may survive in the environment for extended periods, particularly in cool and moist circumstances, allowing it to spread inside and between herds (OIE, 2016). Infected animals may be asymptomatic carriers, spilling bacteria intermittently, complicating early detection and control attempts.

The likelihood of transmission is particularly high in places such as Nepal, where conventional methods of farming, communal grazing, and intimate animal contacts are frequent. Cattle are an important part of Nepalese agriculture, contributing to milk production, draft power, dung, and money generation. However, diseases such as brucellosis frequently go undiscovered or underreported due to a lack of awareness, insufficient diagnostic facilities, and the lack of a national control or eradication effort (Pandeya et al., 2013). Furthermore, animal transportation without proper screening and the lack of frequent surveillance measures contribute to the silent spread of the disease. Serological tests are still the primary method of diagnosing brucellosis in the context of Nepal. The Rose Bengal Plate Test (RBPT) and ELISA are commonly used for detecting anti-Brucella antibodies and estimating seroprevalence (Acharya et al., 2016). These tests are simple to perform, inexpensive, and suitable for large-scale epidemiological research. However, their drawbacks include the inability to distinguish between vaccinated and infected animals, as well as potential interactions with other gram-negative bacteria (Godfroid et al., 2011). In addition to serological investigation, molecular methods such as polymerase chain reaction (PCR) have been developed as useful tools for the quick and specific identification of Brucella DNA in clinical samples (Acharya et al., 2016). PCR can identify the pathogen even in latent infections or in animals with low or no antibody titers, resulting in a more conclusive diagnosis than conventional approaches (Baddour & Alkhalifa, 2008). A combination of these techniques improves diagnosis accuracy and allows for better comprehension of the infection status of animals.

Although a few studies have been conducted to detect bovine brucellosis in Nepal (Chalise & Pandeya, 2024; Subedi et al., 2016), comprehensive information on both serological and molecular detection is limited. Therefore, this study was conducted to determine the sero-prevalence of brucellosis in cattle from different geographic locations of Nepal using serological assays, and to confirm using PCR-based molecular techniques. The findings will help to improve our understanding of the epidemiology of bovine brucellosis in Nepal and serve as a foundation for developing effective disease surveillance and control measures in the national livestock industry.

MATERIALS AND METHODS

Study area and sample collection

A total of 1456 blood and milk samples were collected from non-vaccinated apparently healthy cattle with a history of abortion, infertility, and repeat breeding, over 10 years, from 2015 to 2025. The samples were collected together from Jhapa, Sunsari, Saptari, Makawanpur, Tanahun, Kaski, and Kavre districts of Nepal with a range of tropical,

subtropical, and temperate climatic conditions. Blood was collected aseptically from the jugular vein, and 3 ml was transferred into an EDTA tube and a serum separation tube for DNA extraction and serum extraction, respectively. Milk of cattle from four quarters was collected in a tube containing 0.1% sodium azide as a preservative. The samples were transported to the laboratory of the National Animal Health Research Center (NAHRC), keeping them in an ice box. At the laboratory, serum was separated by centrifuging the blood samples at 1,000 x g for 15 minutes. The separated serum samples were then transferred to 2 ml sample tubes and stored at -80°C until testing.

Serological testing

Rose Bengal Plate Test (RBPT)

The Rose Bengal antigen test was used as a rapid test method (*Brucella abortus* biovar 1) (ID Vet) to screen brucellosis. Briefly, all the samples and the antigen were defrosted and brought to room temperature. Then, 0.03 ml of serum samples was dispensed into a clean white tile. An equal volume of antigen was added beside each serum. The negative and positive controls were also prepared for each tile as provided with the kit. The serum and antigen were then mixed immediately using a glass rod for 3-4 minutes and observed for agglutination. Samples were considered positive when any agglutination was observed.

Dot Enzyme-Linked Immunosorbent Assay (Dot-ELISA)

Following the initial Rose Bengal Plate Test, an additional serological analysis was performed using the ImmunoComb Bovine Brucella Antibody Test Kit (Biogal Galed Laboratories, Israel). The diagnostic assay is a self-contained portable kit. It detects antibody levels in serum/plasma or milk, is highly sensitive, and provides results within 38 minutes. The samples were tested in strict accordance with the manufacturer's guidelines provided with the kit. The controls and samples were kept in different wells of a developing plate and mixed thoroughly with the help of a comb. After several steps of mixing, the ImmunoCombs were developed, which were ready for observation. The developed combs were observed, and the sample dots were compared with the negative and positive control serum dots to determine whether the given samples were positive or not. Thus, the results of all samples were noted.

Molecular diagnosis

Representative samples among positive results upon RBPT and dot-ELISA were subjected to molecular detection. DNA was extracted using Qiagen DNeasy Blood and Tissue Extraction kit following the protocols provided. The purity of the extracted DNA was determined and stored at -45°C until performing PCR. A conventional PCR was performed subsequently on the extracted DNA samples using a Techne 3Prime Thermal Cycler for species identification. A final volume of 25 µl of Master mix was used for the amplification, as shown in Table 1. The forward and reverse primer sequences used were 5'-GAGGGCTGACTCCGAATTTG -3' and 5'-ACGCTTCTCTGCCTTATGGC -3', respectively. The PCR conditions are shown in Table 2. The PCR product was electrophoresed in 1.5% agarose prepared in 1X TAE and 3µL Red safe (10mg/ml) using 10 µL volume. It was run at 90V for 45 minutes. The bands were then observed in a UV transilluminator, and the size was compared with the molecular ladder (100- 1000bp).

Table 1. Preparation of PCR reaction mixture for amplification of *Brucella* spp.

SN	Components	Volume (μ L)	Concentration
1.	Bioneer AccuPower PCR Master Mix	13	2x
2.	Forward primer	1	10 μ M
3.	Reverse primer	1	10 μ M
4.	DNA template	5	Varied
5.	Nuclease-free Water	5	
Total		25 μ L	

Table 2. PCR cycling conditions used for the detection of *Brucella* spp.

SN	Steps	Temperature	Time	Cycles
1.	Initial Denaturation	94°C	5 min	1
2.	Denaturation	94°C	1 min	35
3.	Annealing	50°C	40 sec	35
4.	Extension	72°C	1 min	35
5.	Final extension	72°C	10 min	1

RESULTS

Rapid diagnostic test

The total number of samples collected and the percentage of positive samples for brucellosis over ten years have been shown in Fig 1. The highest number of samples tested was in the fiscal year 2078/79, i.e., 339, followed by 329 samples in 2079/80. Whereas, in the year 2073/74, only 34 samples were tested. Out of the total samples tested, 242 (16.62%) showed a positive result for *Brucella* from both the Rose Bengal Plate Test and ImmunoComb Bovine *Brucella* Antibody Test. The highest number of positive cases was recorded in the year 2079/80, i.e., 27.90%, whereas only 2.50% positive cases were recorded in the year 2074/75.

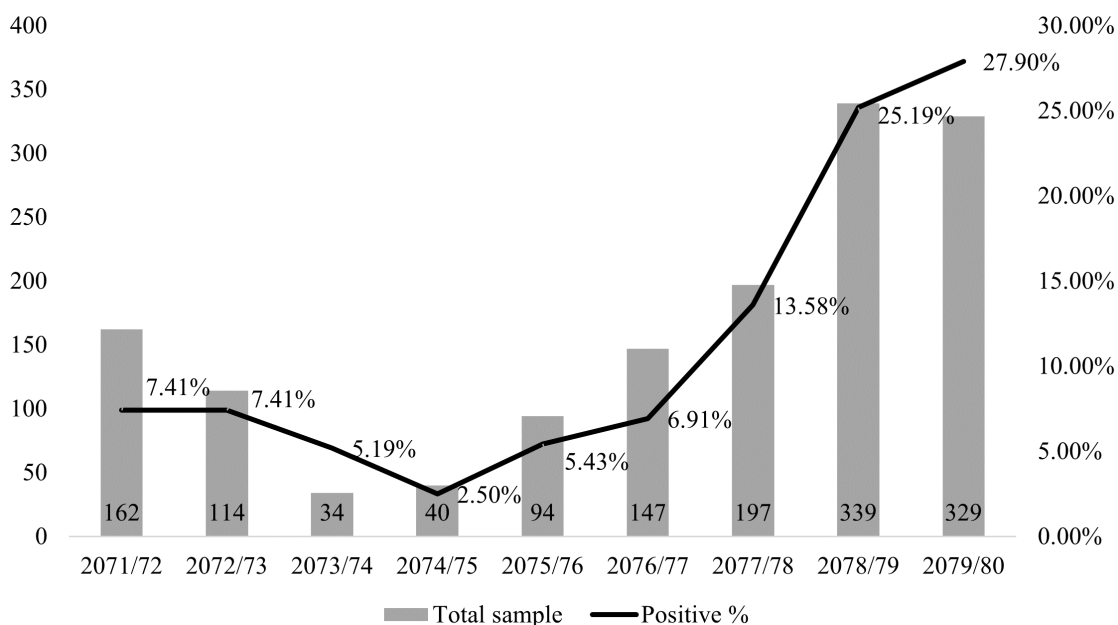


Fig. 1. Seroprevalence of brucellosis observed in different fiscal years

Molecular diagnosis

Agarose gel electrophoresis of the PCR products confirmed the presence of *Brucella abortus*, showing a distinct band at approximately 556 bp shown in Fig. 2.

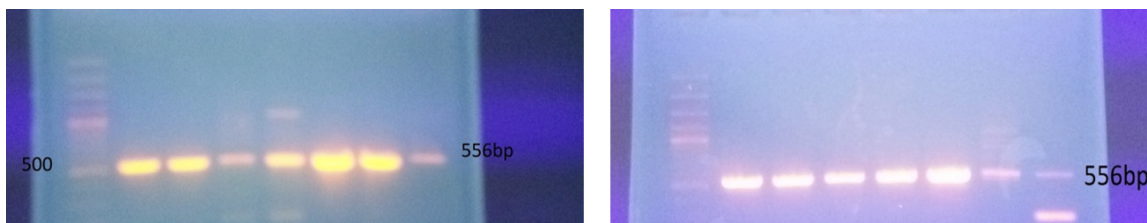


Fig. 2. Bands observed in samples at the amplicon size of around 556bp

DISCUSSION

This study found an overall seroprevalence of 16.62% for brucellosis, with yearly prevalence rates ranging from 2.50% to 27.90% among cattle in the selected regions of Nepal, with molecular confirmation of *Brucella abortus* via PCR. The observed serological prevalence in this study is consistent with past data from Nepal. A prevalence of 16.89% in yaks and 26% in cattle was observed in the very first study regarding serological evidence of brucellosis in Nepal (Pyakural & Mishra, 1977).

Similar prevalence rates in Chitwan, with 10.86% positive by RBPT and 14.13% by iELISA, were observed after decades, confirming the disease's continued existence in

Nepalese cattle herds (Subedi et al., 2016). Acharya et al. (2016) found a significantly reduced sero-prevalence of 3.23% in cattle from Chitwan, Nawalpur, Rupandehi, and Makawanpur districts. On the other hand, Pandeya et al. (2013) discovered a remarkably high rate of 32% in Kailali district, most likely due to localized outbreaks or risk factors such as communal grazing.

The molecular detection of *B. abortus* in this study is consistent with findings from other regions, where the *bcbp31* gene is regarded as a suitable target for genus-level recognition (Baddour & Alkhalifa, 2008; Refai, 2002). PCR confirmation improves diagnostic specificity and offers a solid foundation for detecting strains that are prevalent in endemic areas (García-Yoldi et al., 2006).

The wide range in reported prevalence can be attributable to changes in geographical zones, livestock farming practices, herd numbers, diagnostic methods, and biosecurity measures (Deka et al., 2018). Studies using more sensitive methods, such as iELISA and PCR, tend to report a greater prevalence compared to those using RBPT alone, which could alter the infection rates (Nielsen, 2002).

Importantly, while Nepal does not currently provide routine immunization against bovine brucellosis, the existence of circulating *Brucella* antibodies in cattle, as well as PCR-based detection of *B. abortus*, suggests that natural infections continue in the field. This raises issues regarding silent transmission and underdiagnosis in the absence of clinical symptoms or immunization history. As a result, future research should differentiate between field strains and vaccine strains to rule out any potential misleading seropositivity because of unrecorded vaccination and to better understand infection epidemiology.

CONCLUSION

The present study confirms the presence of circulating *Brucella* antibodies (16.62% seroprevalence) and the molecular detection of *B. abortus* in cattle, despite not having a vaccination program in Nepal. The findings point to current field infections and emphasize the need for better surveillance and control techniques. Future research should focus on distinguishing field strains from vaccine strains to inform prospective vaccination programs.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Acharya, K. P., Niroula, N., & Kaphle, K. (2016). Review of Brucellosis in Nepal. *Epidemiology and Health*, 38, e2016042. <https://doi.org/10.4178/epih.e2016042>
2. Baddour, M. M., & Alkhalifa, D. H. (2008). Evaluation of three polymerase chain reaction techniques for detection of Brucella DNA in peripheral human blood. *Canadian Journal of Microbiology*, 54(5), 352–357. <https://doi.org/10.1139/w08-017>
3. Chalise, S., & Pandeya, Y. (2024). Seroprevalence of Brucellosis in Cattle of Rupandehi District, Nepal. *NPI Journal of Science and Technology*, 1, 174–184. <https://doi.org/10.3126/npijst.v1i1.68953>
4. Corbel, M. J., Food, of the United Nations, A. O., Organization, W. H., & for Animal Health, W. O. (2006). *Brucellosis in humans and animals* (p. Produced by the Food and Agriculture Organization of the United Nations, World Organisation for Animal Health and the World Health Organization). World Health Organization.
5. Deka, R. P., Ulf, M., Delia, G., & and Lindahl, J. (2018). Bovine brucellosis: prevalence, risk factors, economic cost and control options with particular reference to India- a review. *Infection Ecology & Epidemiology*, 8(1), 1556548. <https://doi.org/10.1080/20008686.2018.1556548>
6. García-Yoldi, D., Marín, C. M., de Miguel, M. J., Muñoz, P. M., Vizmanos, J. L., & López-Goñi, I. (2006). Multiplex PCR assay for the identification and differentiation of all Brucella species and the vaccine strains Brucella abortus S19 and RB51, and Brucella melitensis Rev1. In *Clinical chemistry* (Vol. 52, Issue 4, pp. 779–781). <https://doi.org/10.1373/clinchem.2005.062596>
7. Godfroid, J., Scholz, H. C., Barbier, T., Nicolas, C., Wattiau, P., Fretin, D., Whatmore, A. M., Cloeckaert, A., Blasco, J. M., Moriyon, I., Saegerman, C., Muma, J. B., Al Dahouk, S., Neubauer, H., & Letesson, J.-J. (2011). Brucellosis at the animal/ecosystem/human interface at the beginning of the 21st century. *Preventive Veterinary Medicine*, 102(2), 118–131. <https://doi.org/10.1016/j.prevetmed.2011.04.007>
8. McDermott, J., Grace, D., & Zinsstag, J. (2013). Economics of brucellosis impact and control in low-income countries. *Revue Scientifique et Technique (International Office of Epizootics)*, 32(1), 249–261. <https://doi.org/10.20506/rst.32.1.2197>
9. Nielsen, K. (2002). Diagnosis of brucellosis by serology. *Veterinary Microbiology*, 90(1–4), 447–459. [https://doi.org/10.1016/s0378-1135\(02\)00229-8](https://doi.org/10.1016/s0378-1135(02)00229-8)
10. OIE. (2016). Brucellosis (Brucella abortus, B. melitensis, and B. suis)(infection with B. abortus, B. melitensis, and B. suis). In *Manual of diagnostic tests and vaccines for terrestrial animals* (pp. 1–44). World Organisation for Animal Health (OIE), Paris.
11. Pandeya, Y., Joshi, D., Dhakal, S., Ghimire, L., Mahato, B., Chaulagain, S., Satyal, R., & Sah, S. (2013). Seroprevalence of brucellosis in different animal species of Kailali district, Nepal. *International Journal of Infection and Microbiology*, 2(1), 22–25. <https://doi.org/10.3126/ijim.v2i1.8005>
12. Pyakural, S., & Mishra, U. (1977). Sero-epidemiological evidence of animal brucellosis in Nepal. *Bulletin of Veterinary Science and Animal Husbandry Nepal*, 6.
13. Refai, M. (2002). Incidence and control of brucellosis in the Near East region. *Veterinary Microbiology*, 90(1–4), 81–110. [https://doi.org/10.1016/s0378-1135\(02\)00248-1](https://doi.org/10.1016/s0378-1135(02)00248-1)
14. Subedi, S., Prajapati, M., & Dhakal, B. (2016). Sero-Surveillance of Brucellosis in Cattle of Chitwan District, Nepal. *International Journal of Applied Sciences and Biotechnology*, 4(3), 365–371. <https://doi.org/10.3126/ijasbt.v4i3.15774>

Effect of Phenotypic Influences on the Reproductive Traits of Crossbred Murrah Buffaloes in Nepal

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ABSTRACT

This study focuses on the factors influencing reproductive traits, such as birth weight, age at conception, age at first calving, gestation length, and calving interval and to analyze the impact of various factors-location, year, season, and parity on the reproductive traits of crossbred Murrah buffaloes. The research was conducted Terai and mid-hill district over three years during 2017 to 2020 and different seasons (summer, autumn, winter, spring). The study included 16,912 pregnant and milking buffaloes, with 10,987 from Terai- Dhanusha district and 5,925 from Mid-hill- Kaski district. Data on desired traits were collected from the Veterinary Hospital Livestock Specialties Center's Artificial Insemination record book and the livestock service section from the Rural Municipality using a set of questionnaires. The buffaloes were categorized by parity (first, second, third). The data were analyzed by using the mixed technique of the Harvey model. The findings revealed that the overall least squares mean of birth weight, age at conception, age at first calving, gestation length and calving interval were 35.10 ± 0.56 kg, 975.14 ± 10.54 days, 1287.88 ± 10.50 days, 312.72 ± 0.41 days, and 420.00 ± 3.60 days respectively with significant ($p < 0.01$) effects of year, season, and parity. The research findings evident that phenotypic influences significantly impact to the reproductive performance of crossbred Murrah buffaloes. This highlights that in order to increase milk production and reduce calving intervals, better breeding management and the selection of superior buffaloes are required.

Keywords: Crossbred Murrah Buffalo; Phenotypic influences; Reproduction traits; Parity and Season; Breeding management

INTRODUCTION

Buffaloes are significant in global agriculture since they offer milk, meat, and draft power to millions of people, particularly in developing countries (FAO, 2020). The Murrah breed of buffalo is famous for producing a lot of milk and being able to thrive in different environments. Buffaloes, especially crossbred Murrah buffaloes, are important for meeting the dairy needs of rural communities in Nepal, where agriculture is a key part of the economy (Joshi & Pokharel, 2017). Nepal's gross domestic product (GDP) is

derived from agriculture, which employs 66% of the population. Livestock accounts for 12% of GDP, with buffaloes accounting for 6% of the total (MoALD, 2021). Further, they state that the buffalo population of Nepal is 52.5 million, contributing to an annual milk production of 1.4 million MT and meat production of 0.19 million MT, which accounts for 57% of milk and 36% of meat production. Buffaloes are the main breed of dairy and meat production has played excellent results so this activity helps the rural economic growth of farmers. Although, Murrah buffalo is the highest milk producing and their production and reproduction efficiency varied under different management conditions (Bishnoi and Singh, 2009).

The Nepalese rural farmers face the issues of crossbred Murrah buffaloes having delayed puberty, seasonal breeding, and, ultimately, low productivity. The study behind these reasons is highly relevant for genetic improvement, management practices, and policy decisions, and buffaloes are a significant source of milk and meat in Nepal (Sah & Nakao, 2006). The innovation lies in the creation of combined methods that are adapted to local conditions, in investigating the connection between nutrition and reproduction, and in emphasizing the addressing of seasonal breeding cycles. These motivations underscore the significance of knowing the influence of phenotypic factors on Murrah buffaloes' performance.

Water buffaloes (*Bubalus bubalus*) are considered an important species that have contributed to sustainable agricultural systems and rural development for many decades through the genetic improvement of indigenous buffalo with exotic breeds attracted by rural farm bulls and imported semen. Afzal et al. (2007) and Sahoo et al. (2014) found that artificial insemination has improved buffalo productivity and genetic merit. ADS (2015) states that the economic contribution of buffalo is second only to the major staple cereal crop, rice. Neopane & Pokharel (2006) notes that 35% of the population consists of exotic Murrah buffaloes, with the remaining 65% being indigenous.

The alliance between careful management practices and strategic breeding programs can lead to improved productivity and overall success in buffalo farming businesses. Evaluating these factors provides valuable information for raising rigorous breeding programs to achieve genetic improvement of the buffalo population in the Terai and hill regions (Bhattarai & Karki, 2017). This process helps in selecting buffaloes with superior genetic merits based on their high breeding values (Fooda et al., 2010). Production and reproductive traits are factors that affect profit in farm dairy herds. However, an inverse relationship exists between milk yield and reproductive traits (Pryce et al., 2002). Climatic conditions, genotype, nutrition, and herd management influence reproductive characteristics (Perera, 2011). Genotype affects reproductive traits such as sexual maturity, breeding age, calving interval, service period, whether the estrous period has passed clearly, and feeding habits of buffaloes vary between breeds and individuals of the same breed.

The milk productivity of indigenous breeds of buffaloes is pretty low, with an average of 2.85 L per animal per day. This result is getting 869.3 L per animal in a 305-day standard lactation period. In contrast, the Murrah buffalo produces 1500 L of milk per lactation period in similar management practices (Nirmal et al. 2018). The inadequate understanding of phenotypic influence on reproductive traits in crossbred Murrah buffaloes in Nepal impedes the application of effective productivity interventions. This study was done to understand and analyze the effect of phenotypic influences on the reproductive traits of crossbred Murrah buffaloes. Furthermore, the findings guidance for the aligning breeding calendars with peak calving seasons can optimize milk yield and fertility in crossbred Murrah buffaloes.

MATERIALS AND METHODS

Study area

The study focused on the districts of Dhanusha and Kaski. Dhanusa District, which is located in the Janakpur Zone of Madhesh Province, with an altitude range of 78 meters to 3937 meters and a latitude of 26°50'31.56". At latitude 28.2622 N and longitude 84.0167 E, Kaski is a mid-hill district in the province of Gandaki. The Terai has hot summers and moderate winters and the mid-hill region enjoys a temperate environment.

Study location and duration

The data on economic traits of crossbred Murrah buffaloes were collected from animal breeding record sheet of buffaloes for the period of 2017 to 2020 maintained at the Veterinary Hospital & Livestock Specialties Center, and buffaloes rearing farmers.

Production system

Data related to production system of Murrah buffaloes were collected through direct interviews with buffalo rearing farmers in two different regions, Terai and Hill, using a prepared questionnaire sheet to be answered verbally. The study encompassed crossbred Murrah buffaloes from these two locations, totaling 16,912 animals. We gathered information on reproductive and productive traits from 16,912 lactating buffaloes. Performance records of 10,987 buffaloes were sourced from the Veterinary Hospital & Livestock Specialties Center in Terai, while 5,925 were obtained from various Palika in the hill district. Data collection included information on all buffaloes born and calved at the farm, with a focus on those rearing between one and up to 100 milking buffaloes over a period of three years.

Herd management

Buffaloes were housed on large farms, with a minimum of one to 100 animals. They grazed on locally available grasses/fodder for 4 to 6 hours daily and received commercial concentrates at the 1.5 kg and roughages twice daily. The feed varied based on the animal's condition, with a chemical composition of 16% crude protein, 3% fat, 10-12% crude fiber, and 12% moisture content. Water was available, buffaloes were artificial inseminated during estrus.

Agro-climatic seasons

Four distinct seasons were used for the study, namely, summer (June to August), autumn (September to November), winter (December to February), and spring (March to May). In this study early parity has been defined as the first and second lactation, mid-parity as the third and fourth lactation, and late-parity as five or more lactations.

Statistical analysis

We entered the collected data in MS Excel on various sheets and performed data cleaning to make it better readability. The data were also analyzed using animal models through the use of R software (Harvey's Henderson Least Squares and Maximum Likelihood, LSMML PC-2; (Harvey, 199). The model was used to evaluate the effects of parity, location, and season on reproductive attributes.

$$Y_{ijk} = \mu + B_i + P_j + (BP)_{ij} + e_{ijk}$$

Where,

Y_{ijk} is the observation on the k th parity of i th breed;

μ is the overall mean;

B_i is the fixed effect of i th breed of buffalo;

P_j is the fixed effect of j th parity;

$(BP)_{ij}$ is the effect of interaction between i th breed and j th parity; and

e_{ijk} is the random error that is assumed to be normally and independently distributed (NID).

RESULTS

Reproduction traits

Birth weight

Findings of this study reflected overall least square means of birth weight was 35.10 ± 0.56 kg (Table 2).

Effect of location on birth weight

The birth weight of buffalo calves differs significantly between the Terai and hill regions of Nepal. Calves born in the Terai region have a lower mean birth weight (34.18 kg) compared to those born in the hill region (35.50 kg). Significant differences ($p < 0.01$) were observed in the birth weight of crossbred Murrah buffalo between the two locations, Terai and Hill.

Effect of season on birth weight

Birth weight significantly ($p < 0.001$) varied across seasons. Calves born in winter had the highest mean weight (35.55 kg), followed by spring (35.01 kg) and autumn (35.47 kg), whereas the lowest was in summer (34.36 kg). This advises a seasonal effect on birth weight in crossbred Murrah buffaloes.

Table 1. LSA of Variance (ANOVA) for the effect of phenotypic influences on reproductive traits in Murrah buffaloes

S.N.	Non-genetic factors	DF	Reproductive traits and their F-value				
			BW	ACO	AFC	GL	CI
1	Location	1	201.10**	43.02**	40.87**	27.16**	170.65**
2	Year	2	139.30**	38.16**	36.86**	12.13**	215.85**
3	Season	3	65.23**	26.74**	25.86**	26.19**	29.45**
4	Parity	4	3.80*	5.17*	25.86**	0.35*	2.19*

P-value <0.05 * p-value <0.01**

Effect of parity on birth weight

The birth weight of calves significantly correlated ($p < 0.001$) with parity. Third-parity buffalo calves had a mean weight of 33.63 kg, followed by second-parity calves at 33.54 kg, and first-parity calves at 33.53 kg. This highlights a relationship between birth weight and dam parity.

Age to first conception

The results of this study revealed that the overall mean age to conception of buffalo was 975.14 ± 10.54 days (Table 2).

Effect of Location

The age at conception in crossbred Murrah buffalo was observed to be 1291.22 ± 10.52 days in Terai and 971.70 ± 10.55 days in the Hill region with significant differences ($p < 0.001$) observed between the Terai and Hill regions. Accordingly, a 24.75 percent higher value have in Terai compared to Hill.

Effect of Year

The present study showed significant differences ($p < 0.001$) were observed across different time periods (2018, 2019, 2020), seasons (summer, autumn, winter, spring), and parities.

Effect of Season

The results on the age at first conception in summer, autumn, winter, and spring were 983.77 ± 10.63 , 969.41 ± 10.56 , 973.75 ± 10.56 , and 973.65 ± 10.67 days, respectively. Comparatively, a 1.01 percent higher age was observed of summer than in spring. The age at conception varied significantly by season ($p < 0.001$), with the highest mean in summer and the lowest in spring.

Effect of Parity

The overall least square mean of crossbred Murrah buffaloes for the 1st, 2nd, and 3rd parity was 1006.06 ± 0.89 days, 1005.71 ± 0.98 days, and 1009.76 ± 1.06 days, respectively. The parity had a significant effect ($p < 0.001$), with the lowest mean observed in the 2nd parity and the highest in the 3rd parity during spring.

Age at first calving

The overall mean of age of first calving was 1287.88 ± 10.50 days observed on crossbred murrah buffalo (Table 2). It was observed that Crossbred Murrah buffalo are seasonal breeders as most of the buffalo calved from July to January month (Figure 1). There are many reasons behind the seasonal breeding of the buffalo, one of the reasons is availability of green forage and fodder during the rainy season.

Effect of Location

The Terai had a higher mean (1385.53 days) than the Hill (1284.54 days) with significant effect ($p < 0.001$).

Effect of Year

Additionally, the year had a notable effect ($p < 0.01$), with 2020 showing a higher calving age at 1285.24 ± 10.53 days compared to other years.

Effect of Season

The results on age at first calving in summer, autumn, winter, and spring were 1296.54 ± 10.59 , 1296.54 ± 10.59 , 1282.41 ± 10.51 , and 1282.41 ± 10.5 days, respectively. The age at calving and season, were considered significant effect ($p < 0.001$), with the highest mean observed in summer and the lowest in spring.

Effect of Parity

The least square mean of crossbred Murrah buffaloes for the 1st, 2nd, and 3rd parity was 1318.35 ± 0.89 days, 1317.97 ± 0.97 days, and 1322.05 ± 1.06 days, respectively. The age at calving and parity, were considered significant effect ($p < 0.001$), with the lowest mean observed in the 1st parity and the highest in the 3rd parity during spring.

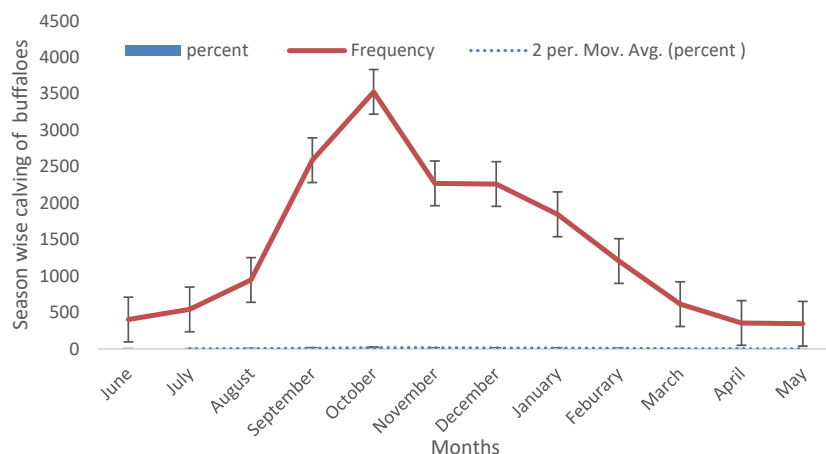


Fig. 1. Effect of season on calving of buffaloes

Gestation length

The overall mean gestation length was 312.72 ± 0.41 days (Table 2).

Effect of location

The results show a significant impact of location on the gestation length of crossbred Murrah buffaloes ($p < 0.001$). The gestation length for Terai and the hill differed slightly, with Terai being 312.62 ± 0.41 days and the hill being 312.83 ± 0.41 days. This study found that the gestation length in the hill region was 0.028% longer.

Effect of season

The results for the gestation length in summer, autumn, winter, and spring were 312.73 ± 0.41 , 313.00 ± 0.41 , 312.73 ± 0.41 , and 312.44 ± 0.39 days, respectively. The gestation length and season had a significant effect ($p < 0.001$), with the highest mean observed in autumn and the lowest in winter.

Table 2. Least Square Means and standard error for reproductive traits of the crossbred Murrah buffaloes considered in this research, Kaski and Dhanusha district, Nepal

Factors	N	BW(kg)	AC (days)	AFC (days)	GL (days)	CI (days)
Overall μ	16912	35.10 $\pm$ 0.56	975.14 $\pm$ 10.54	1287.88 $\pm$ 10.50	312.72 $\pm$ 0.41	420.00 $\pm$ 3.60
Location						
Level of Significance		*	*	*	*	
Terai	10987	34.18 $\pm$ 0. 56	1291.22 $\pm$ 10.52	1385.53 $\pm$ 10.52	312.62 $\pm$ 0. 41	422.34 $\pm$ 3.61
Hill	5925	35.50 $\pm$ 0.56	971.70 $\pm$ 10.55	1284.54 $\pm$ 10.51	312.83 $\pm$ 0.41	417.65 $\pm$ 3.61
Period						
Level of Significance			P<0.001	P<0.001	P<0.001	P<0.001
2018	4404	34.91 $\pm$ 0.56	972.47 $\pm$ 10.57	1285.24 $\pm$ 10.53	312.76 $\pm$ 0.41	423.93 $\pm$ 3.61
2019	5117	34.70 $\pm$ 0.56	971.97 $\pm$ 10.57	1284.79 $\pm$ 10.53	312.81 $\pm$ 0.41	420.61 $\pm$ 3.61
2020	7391	34.36 $\pm$ 0.57	980.99 $\pm$ 10.57	1293.61 $\pm$ 10.52	312.60 $\pm$ 0.41	415.45 $\pm$ 3.61
Season						
Level of Significance		*	*	*	*	
Summer	1964	34.36 $\pm$ 0.57	983.77 $\pm$ 10.63	1296.54 $\pm$ 10.59	312.73 $\pm$ 0.41	419.90 $\pm$ 3.63
Autumn	8316	35.47 $\pm$ 0.56	969.41 $\pm$ 10.56	1296.54 $\pm$ 10.59	313.00 $\pm$ 0.41	420.25 $\pm$ 3.61
Winter	5315	35.55 $\pm$ 0. 56	973.75 $\pm$ 10.56	1282.41 $\pm$ 10.51	312.73 $\pm$ 0.41	417.29 $\pm$ 3.61
Spring	1317	35.01 $\pm$ 0.57	973.65 $\pm$ 10.67	1286.09 $\pm$ 10.63	312.44 $\pm$ 0.39	422.55 $\pm$ 3.65
Parity						
Level of Significance		*	*	*	*	
First	6867	33.53 $\pm$ 0.04	1006.06 $\pm$ 0.89	1318.35 $\pm$ 0.89	312.26 $\pm$ 0.35	391.41 $\pm$ 0.44
Second	5709	33.54 $\pm$ 0.05	1005.71 $\pm$ 0.98	1317.97 $\pm$ 0.97	312.28 $\pm$ 0.38	396.83 $\pm$ 0.43
Third	4330	33.63 $\pm$ 0.03	1009.76 $\pm$ 1.06	1322.05 $\pm$ 1.06	312.26 $\pm$ 0.41	395.99 $\pm$ 0.43
CV		10.26	6.41	4.87	0.81	5.26
R ²		0.038	0.013	0.012	0. 007	0. 041

*Different superscripts within column indicates significant difference (p-value <0.05 % = *). ACO (Age at conception), AFC (Age of first calving), GL (Gestation length), CL (Calving length)*

Effect of parity

The least square mean of the gestation length for crossbred Murrah buffaloes in the 1st, 2nd, and 3rd parity was 312.26 ± 0.35 days, 312.28 ± 0.38 days, and 312.26 ± 0.41 days, respectively. The length and parity had a significant effect ($p > 0.001$), with the highest mean observed in the 2nd parity and the lowest in the other two parities, 1st and 3rd as well.

Calving interval

The results of this study indicate that the overall mean of calving interval was 420.00 ± 3.60 days (Table 2).

Effect of location

The overall mean of location of calving interval was observe in Terai high than Hill, with significant differences observed between the two regions ($p < 0.001$) of crossbred Murrah buffaloes. Variation in the calving interval is due to fluctuations in the service period.

Effect of year

The present study showed a significant effect ($p < 0.01$) of the year on the calving interval. The study found that in 2018, the calving interval was higher at 423.93 ± 3.61 days, and in other years, it was lower, respectively.

Effect of season

The calving interval was significantly variation ($p < 0.001$) by the seasons addressed in this study. The calving intervals for different seasons were as follows: summer 419.90 ± 3.63 days, autumn 420.25 ± 3.61 days, winter 417.29 ± 3.61 days, and spring 422.55 ± 3.65 days, respectively. The highest calving interval was observed in spring, and the lowest was in the winter season.

Effect of parity

The calving interval non-significantly varied ($p > 0.001$) with respect to parity. The calving intervals for 1st, 2nd, and 3rd parity were 391.41 ± 0.44 days, 396.83 ± 0.43 days, and 395.99 ± 0.43 days, respectively.

Effect of season on the trend of conception

Results of this study revealed that the rate of conception decreased with increased environmental temperature (Fig. 2). Accordingly, the conception rate started to increase from July when rainfall started to increase then the effect of constant maximum environmental temperature decreased from its peak point.

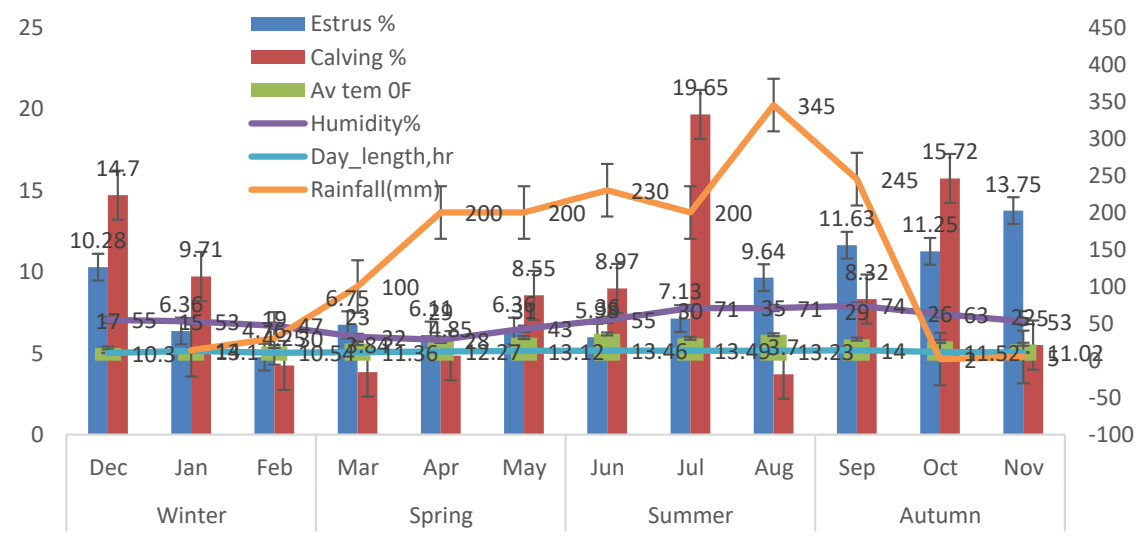


Fig. 2. Seasonal breeding pattern of crossbred Murrah buffaloes

DISCUSSION

This research aimed to explore the phenotypic influences on reproductive traits in crossbred Murrah buffaloes. The overall mean ($\pm$ S.E.) of the crossbred Murrah buffalo calves' birth weight was 35.10 ± 0.56 kg, consistent with the findings of Ahmad et al. (2002), who observed a mean birth weight of 36.3 ± 3.23 kg in Nili-Ravi buffalo calves from Punjab, Pakistan. The heavier weight observed in hill regions may be attributed to their cooler climates and higher altitudes compared to tropical regions, leading to physiological adaptations in buffaloes resulting in larger and more robust calves. Factors such as feeding management and environmental conditions may also contribute to higher birth weights, as indicated by Charlini et al. (2015) and Javed et al. (2013). However, Azmi et al. (2021), Pramod et al. (2018) reported birth weight of murrah calves to be 34.7 ± 0.50 kg and 34.76 ± 0.54 kg respectively which is in contrast to our findings. Bhoopati et al. (2020) found calves born on summer season weighs (32.57 ± 0.59) kg and was higher than other seasons and which is in contrast to our findings. Nili Ravi calves had higher average birth weight (35.86 ± 4.30 kg) than Murrah calves as per report by Akhtar et al. (2012) from Pakistan. The CV is 10.26%, showed the variation of birth weight around the mean. The coefficient of determination (R^2) is 0.038, suggesting that approximately 3.8% of the variability in birth weight can be clarified by the non-genetic factors examined in this study.

The overall mean age at conception for crossbred Murrah buffaloes, as determined by our data, was 975.14 ± 10.54 days. Adhikari et al. (2017) reported that the age at first conception for crossbred Murrah buffaloes was 2.9 ± 0.7 years greater than the age at conception in our current study. Sarwar et al. (2009) conducted a study on buffaloes' growth and reproductive efficiency in relation to feed supplies. Balamurugan et al. (2016) indicated that genetics, a healthy diet, stress, and environment are primary factors influencing puberty onset, with

hormones, particularly gonadotropins and sex steroids, playing a crucial role. According to Borghese (2005), river buffaloes typically reach puberty between the ages of 15 and 18 months, while swamp buffaloes reach puberty between the ages of 21 and 24 months.

The range of the average age at first calving in Murrah buffalo has been documented by Gupta et al. (2012) and Thiruvankadan et al. (2015), and it is significantly higher than our results, ranging from 1307.18 ± 12.39 days to 1578.7 ± 20.3 days, respectively. AFC is crucial for reproductive traits and an economic characteristic in Murrah buffaloes by Singh and Ghuman (2016). There is ample opportunity for improvement through focused selection and the implementation of appropriate breeding programs. The reproductive performance of Murrah buffaloes is significantly affected by the timing and season of calving, as reported by Gupta et al. (2012) and Kour et al. (2020). Additionally, Zicarelli (2006) stated that age at first calving can be reduced through proper nutritional and health management of growing heifers. The decrease in AFC resulting in a decrease in the cost of raising the animals to reach a productive life and an increase in the annual genetic gain (Sakar et al. 2006).

The average gestation period of Graded Murrah buffaloes maintained under field conditions in India was 308.68 ± 0.27 days (Bhave, et al. 2018) and it is comparatively higher in present study. According to Harun-Or-Rasid et al. (2019), the average gestation period of crossbred, Nilli, and Murrah buffalo in the Bangladesh was revealed that 313.40 ± 4.97 , 313.65 ± 5.16 , and 311.59 ± 6.17 days, respectively, aligning with our finding. In Bangladeshi water buffaloes, gestation length longer at Mathbaria, Pirojpur (319.56 ± 5.93 days) and Patharghata, Barguna (319.12 ± 4.69 days) (MR, et al. 2013), closed to our findings. According to Kataria et al. (2017) and Patel et al. (2018), emphasize the significance of environmental factors like temperature, humidity, and seasonal variations, coupled with proper nutrition and management practices, in maintaining optimal gestation lengths among buffaloes. These factors play a crucial role in ensuring reproductive success and herd productivity. Proper breeding techniques, including timing and method of insemination, can influence the length of gestation in crossbred Murrah buffaloes (Sahoo et al., 2014). Furthermore, ensuring proper hygiene, vaccination, and disease prevention measures can contribute to maintaining healthy pregnancies and normal gestation lengths in buffaloes (Kumari et al., 2019). Adhikari et al. (2017) found that the calving interval of crossbred Murrah buffaloes was 14.0 ± 3.4 months, aligning with our study (Table 2). Our findings corroborate previous research indicating that the calving interval in buffaloes is influenced by a multitude of factors, both genetic and non-genetic, as well as environmental (Sharma et al., 2015; Gautam & Kumar, 2017).

Moreover, management practices play a pivotal role in determining calving intervals. Access to quality nutrition, timely healthcare, and proper reproductive management practices can all contribute to shorter calving intervals (Gautam & Kumar, 2017). Genetic selection for improved fertility traits and reproductive efficiency is essential for reducing calving intervals and enhancing overall productivity (Shrestha et al., 2018). Sanker et al. (2014) reported a calving interval of 470 days longest in the 1st parity and the shortest, i.e.,

451 days, in the 5th parity (Jamal et al., 2018) (Table 2). The coefficient of variation (CV) was lowest for age at conception and gestation period, and moderate for age at first calving and calving interval, signifying varying degrees of variability in the reproductive traits of crossbred Murrah buffaloes. This moderate CV suggests that there exists a moderate level of variability within these traits. Thus, implementing selection strategies alongside enhanced management practices may lead to improvements in these reproductive traits. According to Sharma et al. (2020), a low CV observed for age at conception in Murrah buffaloes highlights the influence of factors such as nutrition, health management, and environmental conditions in maintaining consistency across reproductive parameters.

Lower rate of conception during the spring and winter season observed in buffaloes in this study might be associated to the increasing day light and the environmental temperature during these seasons. Effects of dry weather resulting shortage of feed and fodder availability during winter and spring months might have played a major role in causing lower conception rate in the following months as suggested by Devkota et al. (2012). The Terai region experiences a hot and humid climate during the summer months. High temperatures and humidity can lead to heat stress in buffaloes, which is known to affect their reproductive performance (Fig.2). Conception rates may be lower during the hottest months due to reduced estrus activity and fertility. The Terai region generally has more consistent access to forage and water throughout the year due to its lowland location. However, monsoon rains can cause flooding, affecting grazing and forage quality. Seasonal variations in forage quality and quantity can impact buffalo nutrition, potentially affecting conception rates. Day length remains relatively consistent in the Terai throughout the year, so photoperiod may have a lesser effect on buffalo reproductive patterns compared to regions with more pronounced day length variations.

CONCLUSION

Phenotypic influences like location, season, and parity significantly influenced the reproductive traits of crossbred buffaloes. The differences in parameters like age at conception, gestation length, and calving interval between Terai and mid-hill regions necessitates region-specific buffalo management strategies. For crossbred Murrah buffaloes, location impacts reproductive and productive traits like milk production, obliging customized management approaches. Understanding environmental and seasonal dynamics is crucial for developing optimized management practices, and improving reproductive performance and milk production capabilities.

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ETHICAL STATEMENT

All the procedures have been conducted in accordance with the guidelines laid down by the Institutional Ethics Committee.

Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Adhikari, P., Barsila, S. R., Devkota, B., & Gautam, G. (2017). Husbandry practice and reproductive performance of buffaloes in Chitwan and Kaski districts of Nepal. In *Proceedings of the International Buffalo Symposium, Agriculture and Forestry University* (pp. 157–160). Agriculture and Forestry University.
2. Afzal, M., Anwar, M., & Mirza, M. A. (2007). Some factors affecting milk yield and lactation length in Nili-Ravi buffaloes. *Pakistan Veterinary Journal*, 27, 113–117.
3. Ahmad, K., Javed, A., & Rehman, A. (2002). Environmental factors affecting some growth traits in Nili-Ravi buffalo calves. In *7th World Congress on Genetics Applied to Livestock Production*, Montpellier, France.
4. Akhtar, P., Kalsoom, U., Ali, S., Yaqoob, M., Javed, K., Babar, M. E., Mustafa, M. I., & Sultan, J. I. (2012). Genetic and phenotypic parameters for growth traits of Nili-Ravi buffalo heifers in Pakistan. *Journal of Animal and Plant Sciences*, 22(3), 347–352.
5. Balamurugan, B., Katiyar, R., Kharayat, N. S., Rautela, R., Chaudhary, G. R., Kumar, P. M., & Mishra, G. K. (2016). Heat stress and its negative impact on reproductive events in female animals. *The North East Veterinarian*, 16(3), 35–37.
6. Banerjee, G. C. (2009). *A textbook of animal husbandry* (6th ed.). Reprint.
7. Bhattarai, U. P., & Karki, A. (2017). Status and strategies for enhancing buffalo productivity in Nepal. In A. K. Chakravarty, A. Kumar, & R. K. Singh (Eds.), *Proceedings of the World Buffalo Congress, 2017*. <https://doi.org/10.30954/2394-8159.01.2017.1>
8. Bhawe, K. G., Khadse, J. R., Mangurkar, B. R., Gaundare, Y. S., Phadke, N. L., & Potdar, V. V. (2018). Effect of genetic and environmental factors on gestation length of graded Murrah buffaloes in field condition. *International Journal of Pure and Applied Bioscience*, 6(6), 449–454.
9. Bishnoi, D. K., & Singh, R. (2009). Comparative feeding practices of milking animals in Haryana. *Indian Journal of Animal Nutrition*, 26, 243–246.
10. Boopathi, V., Prasad, S., Kumaresan, A., Manimaran, & Prakash, M. A. (2020). Effect of environmental factors on growth performance of Murrah buffalo heifers. *International Journal of Current Microbiology and Applied Sciences*, 9(7), 2676–2683. <https://doi.org/10.20546/ijcmas.2020.907.315>
11. Borghese, A. (2005). *Technical Series 67*. Food and Agriculture Organization.
12. Charlini, B. C., & Sinniah, J. (2015). Performance of Murrah, Surti, Nili-Ravi buffaloes and their crosses in the intermediate zone of Sri Lanka. *Development*, 27, 1–7.
13. Food and Agriculture Organization of the United Nations. (2020). *World food and agriculture - Statistical yearbook 2020*. <https://www.fao.org>
14. Fooda, T., Mourad, K. A., & Gebreel, I. (2010). Phenotypic and genetic trends for milk production in Egyptian buffaloes. *Journal of American Science*, 6, 143–147.
15. Gautam, G., & Kumar, R. (2017). Factors affecting the reproductive performance of dairy buffaloes in Chitwan district of Nepal. *Journal of Agriculture and Natural Resources*, 1(1), 1–10.
16. Government of Nepal, Ministry of Agricultural Development. (2015). *Agriculture development strategy (ADS)*.
17. Gupta, J. P., Sachdeva, G. K., Gandhi, R. S., & Chakaravarty, A. K. (2012). Non-genetic factors influencing growth and production performance in Murrah buffaloes. *Indian Journal of Dairy Science*, 65(3), 45–48.

18. Harun-Or-Rashid, M., Sarkar, A. K., Hasan, M. M. I., Hasan, M., & Juyena, N. S. (2019). Productive, reproductive, and estrus characteristics of different breeds of buffalo cows in Bangladesh. *Journal of Advanced Veterinary and Animal Research*, 6(4), 553–560. <https://doi.org/10.5455/javar.2019.f382>
19. Jamal, I., Yousuf, S., Naik, M. A., & Baba, N. A. (2018). Effect of non-genetic factors on various reproduction traits in Murrah buffaloes. In *National Symposium on Biodynamic Animal Farming for the Management of Livestock Diversity Under Changing Global Scenario*, Thrissur, Kerala, India (p. 149).
20. Javed, K., Mirza, R. H., Abdullah, M., & Akhtar, M. (2013). Environmental factors affecting live weight and morphological traits in Nili Ravi buffaloes of Pakistan. *Editor Board*, 32, 1161–1164.
21. Joshi, B. K., & Pokharel, D. (2017). Challenges and prospects of buffalo research and development in Nepal. *Buffalo Bulletin*, 36(3), 343–352.
22. Kataria, A., Gupta, A. K., Yadav, P., Kaur, P., Singh, S., & Tomar, S. (2017). Environmental factors affecting reproductive performance in Murrah buffaloes: A review. *Indian Journal of Animal Sciences*, 87(6), 661–668.
23. Kour, G., Narang, R., & Singh, A. (2020). Effect of season and age at first calving on early performance traits in Murrah buffaloes. *Journal of Agri Search*, 7(2), 107–110.
24. Kumari, S., Singh, S. K., Ramesha, K. P., Kumar, A., & Gupta, S. K. (2019). Hygiene, vaccination, and disease prevention measures contribute to maintaining healthy pregnancies and normal gestation lengths in buffaloes. *Veterinary Medicine International*, 5678147. <https://doi.org/10.1155/2019/5678147>
25. Ministry of Agriculture and Livestock Development. (2021). *Statistical information on Nepalese agriculture*. Singhdurbar, Kathmandu, Nepal.
26. Neopane, L. P., & Pokharel, D. M. (2006). *Indigenous cattle of Nepal*. Animal Breeding Division, Nepal Agricultural Research Council.
27. Nirmal, B. K., Devkota, B., Shrestha, S., & Subedi, S. (2018). Country report on buffalo production systems in Nepal. In *Book of Abstracts of the IX Asian Buffalo Congress (ABC; ICAR-Central Institute for Research on Buffaloes: Hisar, India)*, 6.
28. Patel, R. K., Chauhan, H. C., Bhat, P. N., & Chandel, B. S. (2018). Effect of environmental factors on reproductive performance of buffaloes. *Indian Journal of Dairy Science*, 71(2), 157–161.
29. Perera, B. M. (2011). Reproductive cycles of buffalo. *Animal Reproduction Science*, 124(3–4), 194–199.
30. Pramod, S., Lasna Sahib, B., Becha, B. B., & Venkatachalapathy, T. (2018). Growth performance of Murrah buffalo calves under humid tropical conditions of Kerala. *Journal of Animal Research*, 8(6), 1125–1128. <https://doi.org/10.30954/2277-940X.12.2018.30>
31. Pryce, J. E., Coffey, M. P., & Brotherstone, S. H. (2002). Genetic relationships between calving interval and body condition score conditional on milk yield. *Journal of Dairy Science*.
32. Sah, S. K., & Nakao, T. (2006). Characteristics of repeat breeding buffaloes in Nepal. *Journal of Reproduction and Development*, 52, 335–341.
33. Sahoo, A., Gupta, A. K., Gupta, A., Gupta, S. K., & Kumaresan, A. (2014). Proper breeding techniques, including timing and method of insemination, can influence the length of gestation in crossbred Murrah buffaloes. *Journal of Buffalo Science*, 3(2), 45–52.
34. Sanker, S., Kumar, D., Mandal, K. G., Taggar, R. K., & Das, A. K. (2014). Factors influencing the dry period and calving interval in different grades of buffaloes. *Buffalo Bulletin*, 33(1), 120–126.
35. Sarkar, U., Gupta, A., Mohanty, T., Raina, V., & Prasad, S. (2006). Genetic and non-genetic factors affecting milk yield and milk constituents in Murrah buffaloes. *Journal of Dairy Food and Home Science*, 25, 125–128.
36. Sharma, R. K., Katiyar, A. K., & Verma, M. R. (2020). Production and reproductive performance of crossbred Murrah buffaloes in Chhattisgarh, India. *Journal of Livestock Science*, 11(1), 38–42.

37. Sharma, R., Sirohi, S. K., Arora, D., & Katara, A. (2015). Factors affecting milk production and effect of environmental factors on milk yield of Murrah buffaloes. *Buffalo Bulletin*, 34(4), 357–364.
38. Singh, B., & Ghuman, S. P. S. (2016). Status of seasonal pattern of reproductive activity in buffalo. *Indian Veterinary Journal*, 93(8), 44–47.
39. Thiruvankadan, A. K., Panneerselvam, S., & Murali, N. (2015). Study on ages at first mating and calving of Murrah buffaloes in hot and humid climate of Tamil Nadu, India. *Indian Journal of Animal Research*, 49(5), 591–594.
40. Zicarelli, L. (2006). Buffalo calf weaning and production. In *III Simposio Bufalos de las Americas - 2do Simposio de Bufalos Europa-America* (pp. 80–86). Medellin, Colombia.

Prevalence of Hemoparasites in Dogs Brought to the Neutering Camp and Associated Biochemical Parameters

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ABSTRACT

This study was conducted to investigate the prevalence of hemoparasites in mongrel dogs and their biochemical study, brought to the Neutering camp conducted by Bharatpur sub-metropolitan city of Bagmati Pradesh. Blood samples from 23 male and 10 female dogs were collected, prepared thin and thick smears, stained with Giemsa stain, and then examined under a light microscope. The age and sex of the dogs were also recorded. The serum samples were also collected for biochemical study and compared between infected and non-infected groups. The prevalence rate of haemoparasite was 24% (33/8) which includes Theileria spp (6.06%), Anaplasma spp (6.06%), Trypanosoma spp (3.03%), Ehrlichia canis (6.06%), and Mycoplasma spp (3.03%). The age-wise and sex-wise prevalence of haemoparasite were 1-5 years 6(18.18%), 6-10 years 2(6.06%), 11-15 years 0(0%), male (62.5%), female (37.5%), respectively. The Serum Glutamic Pyruvic Transaminase (SGPT), Alkaline Phosphatase (ALP), Albumin, Total Protein, blood Urea nitrogen, and Creatinine values between the infected and non-infected groups were 27.34±7.34, 93.53±14.21, 1.24±0.31, 9.02±0.69, 14.46±2.23, 0.81±0.13 and 3.16±5.07, 84.31±20.62, 2.02±0.23, 10.30±0.51, 31.04±3.93, 0.82±0.06 respectively. The significantly lower BUN, as well as lower levels of albumin and total protein, indicate malnutrition. The study showed the prevalence of hemoparasite in sub-clinical states among mongrel dogs.

Keywords: Prevalence; Hemoparasites; Blood; Biochemical Parameters; Canines

INTRODUCTION

Hemoparasites, which reside within the bloodstream of their host, encompass a diverse range of microorganisms, including bacteria, protozoa, nematodes, and rickettsiae. Typically, these parasites are transmitted between hosts through vectors such as ticks, flies, or other insects. In case of dogs, they are susceptible to various hemoparasites, including *Babesia* spp, *Trypanosoma* spp, *Leishmania* spp, *Ehrlichia* spp, *Anaplasma* spp, *Mycoplasma* spp,

and *Dirofilaria spp.*, transmitted by various arthropod vectors, influenced by the climatic changes and movements of human together with their pets enhance the susceptibility of dogs to infection (Otranto et al., 2009; Gray et al., 2009). These hemoparasites can induce detrimental effects on dogs, including fever, anemia, weakness, lethargy, pale gums, and organ damage. As a result, timely diagnosis and treatment are imperative to mitigate the impact of these infections on dogs' health. Furthermore, some hemoparasites of dogs can pose public health risks, such as *Trypanosoma*. Notably, *Ehrlichia canis* stands out as a significant health concern for dogs, with symptoms ranging from mild to severe tropical pancytopenia. (Bhattacharjee & Sarmah, 2013). Therefore, this study aimed to evaluate the common haemoparasites in dogs brought to a neutering camp and its associated blood biochemical parameters. Biochemical measurements in mongrel dogs reveal nutrition, liver, and kidney function, which aids in the early detection of future health issues and enhances their quality of life, allowing veterinarians to better plan therapy for the hemoparasite effect.

MATERIALS AND METHODS

Materials such as sterile syringes, EDTA vials, clot activators vials, glass slides, cotton, sprits, medical ice box, Giemsa stain (Qualigens), methanol, Test Kit, and Biochemical Analyzer were used in this study. The biochemical test of urea, and creatinine. ALP (alkaline phosphatase), SGPT (serum glutamate pyruvate transaminase), albumin, and total protein were performed using a commercially available test kit (Coral Company). All tests were conducted on a chem5 v³ of the biochemical analyzer (Erba Mannheim) following the manufacturer's instructions for each parameter.

Selection of dogs

Blood samples were taken from thirty -three mongrel dogs of different ages and sexes in the Bharatpur sub-metropolitan city of 16, 21, and 22 wards brought to the neutering camp. The samples were purposively selected for a cross-sectional study. The study's generalizability is limited due to its specific geography and demographics, potentially affecting statistical power and applicability. Future research should include a diverse demographic for better results.

Samples collection

5 ml of blood samples were aseptically collected from the cephalic vein of 23 male and 10 female dogs. A separate 23 gauge, 1.2 inches sterile needle, and 5 ml syringe were used to withdraw blood samples from each dog. These collected blood samples were quickly transferred into both EDTA tubes and clot activator tubes. All collected samples were labeled and then sent to the microbiology and diagnostic laboratory of Nepal Polytechnic Institute (NPI) for further analysis. The tube was kept under refrigeration at -4 °C until further laboratory works were performed.

Laboratory techniques

All collected labeled blood samples were subjected to laboratory analysis within six hours of collection. Thin smear methods were used in the examination of the blood samples.

Smear preparation and interpretation

A thin film of the blood sample was created by placing a drop of well-mixed blood on a clean, grease-free slide, approximately 2 cm from the right end. The drop of blood was then touched with the edge of another slide and pushed in a quick but gentle manner at an angle of 45 degrees, resulting in the formation of a thin blood smear with a head, body, and tail. Blood smears were created from each blood sample, air dried, and subjected to fixed in methyl alcohol for 2-3 minutes. The fixed blood smear was placed into a Coplin jar and incubated the smear in Giemsa stain for 30 minutes at room temperature. Carefully removed the stained slide from the Giemsa stain solution and rinsed the slide gently with distilled water to remove excess stain. Allowed the slide to air dry completely without blotting or wiping, as this could damage the stained cells. The stained slide was examined with the help binocular compound microscope (Olympus CX23) and photographed with Bestscope digital photomicrographic camera mounted on microscope. Scanning started with the lowest magnification objective (usually 10x) to locate the area of interest and gradually increased the magnification (100x with oil immersion) for the identification of both intracellular and extracellular hemoparasites. The periphery of the smear was primarily examined for these parasites, as they are more concentrated near the blood film edges. This method of examination enhances the likelihood of detecting these pathogens, which can often be overlooked in the central areas of the smear. Accurate identification of these protozoans is crucial for diagnosing the associated diseases and implementing appropriate treatment strategies. The findings and observations have been documented.

Sample preparation for biochemical study

Serum extraction: The clot activator tube was centrifuged at 5000 Rpm for 5 minutes to separate the serum sample then the sample was collected in an Eppendorf tube with the help of a micropipette which was stored in a deep freezer (-20°C) until biochemical analysis. Reagents for each biochemical test were provided by the Coral Company, and calibration standards were used according to the specific protocols outlined by the manufacturer. All reagents were stored at 2-8°C and used within the recommended timeframe. Serum biochemical tests for Liver function test and renal function test (albumin, SGPT, total protein, ALP, urea, creatinine) were done with the help of a biochemical analyzer. The biochemical tests were performed using the Erba Mannheim chem5 v3 biochemical analyzer, with the following procedures for each parameter. The urea concentration was quantified Brethelot method at 570 nm using the analyzer's calibration curve. Creatinine was tested in Jaffe's kinetic method at 520 nm with the Coral kit. ALP activity was measured at 406 nm using the p-nitrophenyl phosphate kinetic method and expressed in IU/L. SGPT levels were determined IFCC method by measuring the reaction at 340 nm. Albumin concentration was determined using the bromocresol green (BCG) dye-binding method at 630 nm, and total protein was quantified using the biuret method at 550 nm, all on the biochemical analyzer. The results of these assays provided crucial insights into the biochemical profile of the samples.

Statistical analysis

The obtained biochemical data were subjected to the statistical analysis described by Snedecor and Cochran. The independent t-test having means with unequal variances was carried out with the help of statistical software (Dotmatics Graphpad, <https://www.graphpad.com/quickcalcs/ttest1/>). Variables with $p < 0.05$ were considered statistically "significant," variables with $p < 0.01$ were considered statistically "highly significant" and variables with $p > 0.05$ were considered statistically "non-significant."

RESULTS

The result of prevalence of hemoparasites is given in Table 1. Out of 33 dogs eight dogs tested positive for infection. Detected infections included *Theileria spp.* (Fig. 1 and 4) in two dogs (one male and one female), *Anaplasma spp.* (Fig. 2) in two dogs (one male and one female), *Trypanosoma spp.* (Fig. 3) in one female dog, *Ehrlichia canis* (Fig. 5) in two male dogs, and *Mycoplasma spp.* (Fig. 6) in one male dog. Identified through microscopic examination (Fig.1-6). The remaining 25 dogs exhibited normal, uninfected red blood cells. The number of non-infected and infected with different haemoparasites is given graphically also in Fig. 9. The age distribution of 33 dogs included in the study is given in Fig. 7. The distribution of infected dogs in relation to their ages is given in fig. 8. Most of the infected dogs are found in lower age groups (1-5 years).

Table 1. Prevalence of the hemoparasites in mongrel dogs brought to the neutering camp.

Sex	No. of dogs	Age (year)			No. of dogs infected	Prevalence (%)	No. of hemoparasites in dogs (male and female)				
		1-5	6-10	11-15			<i>Thei. spp.</i>	<i>Ana. spp.</i>	<i>Tryp. spp.</i>	<i>E.canis</i>	<i>Myco. spp.</i>
Male	23	22	1	0	5	62.5	1	1	0	2	1
Female	10	8	1	1	3	37.5	1	1	1	0	0

Key: Nil- no hemoparasites were seen; *Thei. spp.* -*Theileria species*; *Ana. spp.* -*Anaplasma species*; *Tryp. spp.* -*Trypanosoma species*; *E. canis*-*Ehrlichia canis*; *Myco. spp.* -*Mycoplasma species*

Table 1, shows that *Anaplasma spp.* *Theileria spp* and *Ehrlichia canis* appear to be more prevalent than other hemoparasites. The dogs negative for hemoparasites are 25 (75.75%).

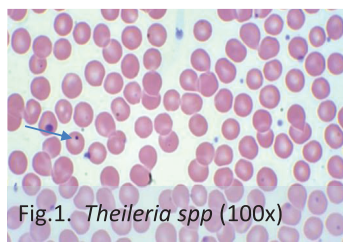


Fig.1. *Theileria spp* (100x)

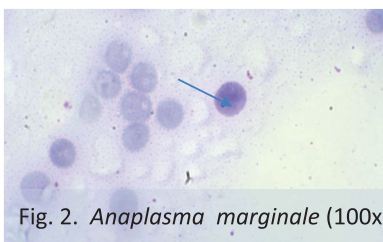


Fig. 2. *Anaplasma marginale* (100x)

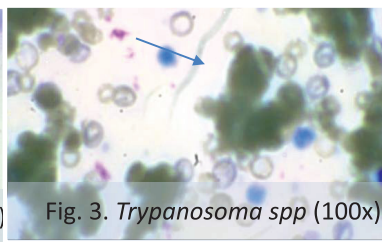


Fig. 3. *Trypanosoma spp* (100x)

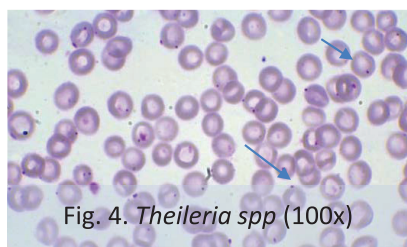


Fig. 4. *Theileria spp* (100x)

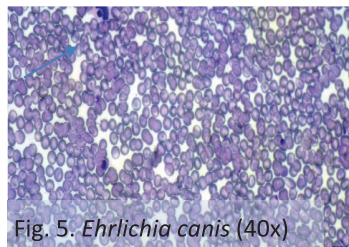


Fig. 5. *Ehrlichia canis* (40x)

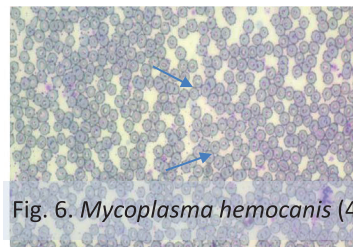


Fig. 6. *Mycoplasma hemocanis* (40x)

Fig. 1-6. Hemoparasites identified through microscopic examination

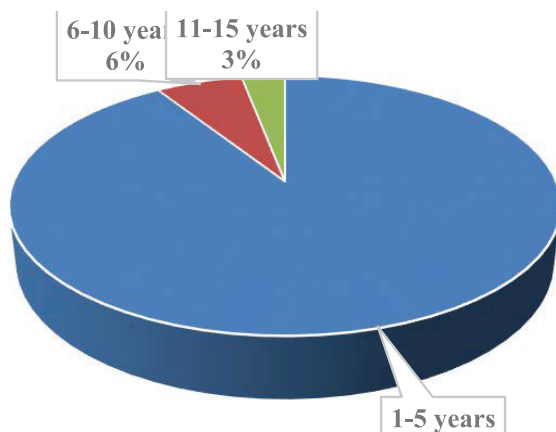


Fig. 7. Age distribution among the mongrel breed of dog

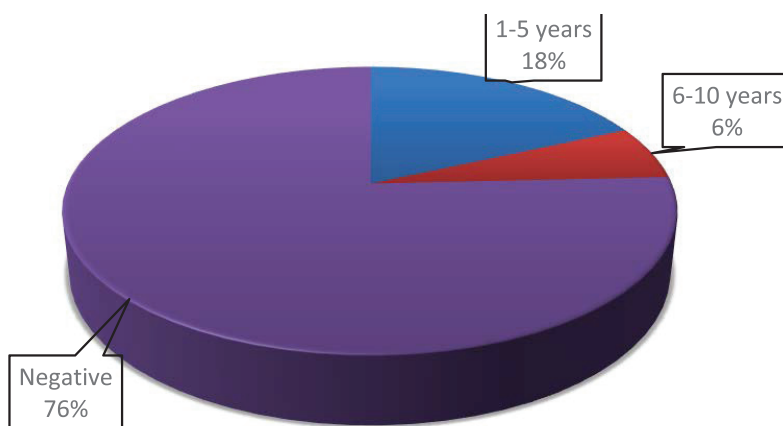


Fig. 8. Distribution of infected dogs in relation to their ages

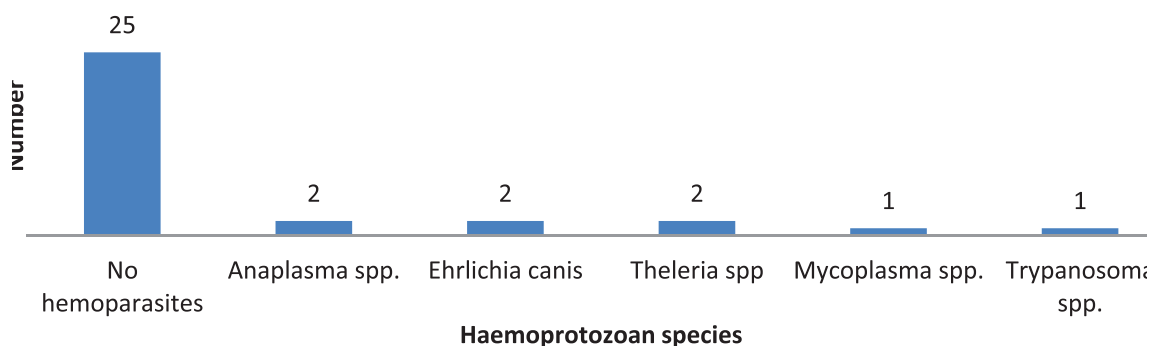


Fig. 9. Comparison of the total number of dogs infected with hemoparasites with those not infected with any hemoparasites

Table 2 shows the results of biochemical parameters for infected and uninfected dogs. Dogs infected with haemoparasites showed considerably higher urea activity than uninfected dogs ($P = 0.026$). Although not considerably, dogs infected with haemoparasites had lower ALT, albumin, and total protein activity than uninfected individuals but higher ALP activity.

Table 2. Biochemical parameters of infected and non-infected groups.

Group	Units	Standard Value	Infected	Non infected	CI	P value
UREA	(mg/dl)	7-27	14.5±2.2	31.04±3.93	-31.12 to 2.04	0.0267
CREATININE	(mg/dl)	0.5-1.8	0.8±0.13	0.82±0.1	-0.27 to 0.28	0.9900
SGPT(ALT)	(U/L)	15-40	27.34±7.35	37.16±5.07	-30.05 to 10.41	0.3301
ALP	(U/L)	50-150	93.53±14.21	84.32± 20.62	-65.88 to 47.44	0.7423
ALBUMIN	(g/dl)	2.3-3.1	1.24±0.32	2.03±0.23	-1.69 to 0.126	0.0889
TOTAL PROTEIN	(g/dl)	5.4-7.5	9.03±0.69	10.29±0.51	-3.30 to 0.76	0.2117

CI=confidence interval, P value less than 0.05 is statistically significant.

DISCUSSION

Out of 33 canines analyzed, 8 tested positive for hemoparasites. Three of the positive cases were female, and five were male. A higher prevalence (24.24%) was found in the local breed 'Mongrel' which harmonizes with the findings reported by Ifeoma., 2013. The study found that urea levels were significantly different (p -value = 0.0267) in dogs infected with hemoparasites, aligning with previous research by Zygnier et al., 2014. The increase of the serum urea to creatinine ratio was observed in infected dogs with *Babesia canis*. When it came to dogs infected with *Babesia canis*, there was no significant increase in urea levels. This could be due to factors like the absence of azotemia, a relatively small sample size, environmental influences, or the possibility of milder infections by *Babesia spp*. Increased creatinine levels in mongrel dogs may be due to kidney dysfunction, which is common in circumstances such as acute or chronic kidney disease. Creatinine is a waste product of muscle metabolism filtered by the kidneys, and reduced renal function reduces excretion, resulting in higher amounts in the blood. One study indicated that renal insufficiency or failure can have a considerable impact on creatinine clearance, resulting in creatinine buildup in the serum (Gowda et al., 2017). Furthermore, dehydration or decreased renal perfusion owing to shock or systemic illnesses might contribute to high creatinine levels. Although alkaline phosphatase (ALP) and albumin were not statistically significantly different however ALP levels were elevated in the infected group, and albumin levels were slightly lower in comparison to the non-infected dogs. The difference was not statistically significant ($p=0.74$) but it approached the threshold for significance. Serum urea levels may fall during haemoparasitic infections for a variety of reasons, including decreased protein intake due to appetite loss, nausea, and vomiting, which lower protein consumption and urea generation because urea is a consequence of protein metabolism (Kesteloot & Joossens, 1993).

Furthermore, haemoparasite induced liver dysfunction can disrupt the urea cycle, preventing the conversion of ammonia to urea (Johnson & Sherding, 2006). SGPT was

slightly elevated in hemoparasites dogs which may be due to cholecystitis, hepatitis Myocardial infarction, and other hepatic diseases but in the case of the liver (dysfunction improvement) can recover and regenerate, leading to a decrease in the release of SGPT into the bloodstream and lower serum SGPT levels (Cohen & Kaplan, 1979). In some cases, increased water consumption may be behavioral or psychogenic, with no underlying medical cause. Certain liver conditions, and urinary tract infections, can lead to increased water intake due to changes in the dog's metabolism and electrolyte balance may be the cause of a decrease in SGPT in serum (Robinson and Verbalis, 2016). Elevated ALP levels during haemoparasitic infections can be caused by a variety of factors, including liver injury, which causes increased ALP release into the circulation (Fielder & Brandao, 2022). Inflammation caused by an immunological response to haemoparasites can also encourage the liver to produce more ALP (Chen et al., 2018). Furthermore, certain hemoparasites can clog the bile ducts, inhibiting the normal flow of ALP-containing bile and causing ALP to gradually accumulate in the blood (Ethan, 2022). Blood albumin levels can drop during hemoparasitic illnesses (such as Babesiosis, Theileriosis, Anaplasmosis, Ehrlichiosis, Mycoplasmosis, and Trypanosis) for a variety of reasons. Malnutrition, characterized by appetite loss and vomiting, reduces hepatic albumin synthesis (Chapman et al., 2020). Liver disease caused by direct parasite invasion, poisons, or immunological responses inhibits albumin synthesis (Wilde & Katsounas, 2019). Some parasites generate increased vascular permeability, which permits albumin to leak into adjacent tissues (Nagy et al., 2008).

The inflammatory response to infection can shift albumin to injured tissues, further lowering blood levels (Soeters et al., 2019). Furthermore, hemolysis produced by parasites such as Plasmodium produces chemicals that contribute to inflammation and change albumin concentrations (Dey et al., 2012). Hemoparasites infections can cause a decrease in total protein levels through various mechanisms. First, certain hemoparasites, especially those causing hemolytic anemia (Pandey., 2023), release hemoglobin during red blood cell destruction, which binds to haptoglobin (a protein made by the liver) and reduces the availability of proteins for other functions. Additionally, these infections often trigger inflammation, leading to the production of acute-phase proteins by the liver. While these proteins increase in the blood, they may become less effective in their normal roles, potentially decreasing overall protein levels. Moreover, hemoparasites-induced malnutrition, appetite reduction, and impaired liver function can further contribute to reduced protein intake and synthesis. Finally, severe infections may lead to internal bleeding, causing a loss of blood proteins and a further drop in total protein levels in the bloodstream. (Assinger, 2014)

CONCLUSION

The current data imply that healthy dogs are infected with hemoparasites and are generally in a subclinical form. These parasite diseases change metabolic markers. Blood parasites were more common in dogs aged 1-5 years, particularly in male dogs; thus, efforts should be made to regulate male mongrel dogs. Additional research should be conducted to determine the effects on hematological parameters with a bigger sample size. Furthermore, investigating potential environmental factors contributing to the prevalence of these parasites could enhance prevention strategies and improve overall canine health in the region.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Assinger, A. (2014). Platelets and infection—An emerging role of platelets in viral infection. *Frontiers in Immunology*, 5, 649. <https://doi.org/10.3389/fimmu.2014.00649>
2. Baldwin C, Pandey J, Olarewaju O. Hemolytic Anemia. [Updated 2023 Jul 24]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK558904/>
3. Bhattacharjee, K., & Sarmah, P. C. (2013). Prevalence of hemoparasites in pet, working and stray dogs of Assam and North-East India: A hospital based study. *Veterinary World*, 6(11), 874–878. <https://doi.org/10.14202/vetworld.2013.874-878>
4. Chapman, B., Sinclair, M., Gow, P. J., & Testro, A. G. (2020). Malnutrition in cirrhosis: More food for thought. *World Journal of Hepatology*, 12(11), 883–896. <https://doi.org/10.4254/wjh.v12.i11.883>
5. Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., & Zhao, L. (2018). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*, 9(6), 7204–7218. <https://doi.org/10.18632/oncotarget.23208>
6. Cohen, J. A., & Kaplan, M. M. (1979). The SGOT/SGPT ratio--an indicator of alcoholic liver disease. *Digestive Diseases and Sciences*, 24(11), 835–838. <https://doi.org/10.1007/BF01324898>
7. Coucke EM, Akbar H, Kahloon A, et al. Biliary Obstruction. [Updated 2022 Nov 26]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK539698/>
8. Dey, S., Bindu, S., Goyal, M., Pal, C., Alam, A., Iqbal, M. S., Kumar, R., Sarkar, S., & Bandyopadhyay, U. (2012). Impact of intravascular hemolysis in malaria on liver dysfunction: involvement of hepatic free heme overload, NF- κ B activation, and neutrophil infiltration. *The Journal of Biological Chemistry*, 287(32), 26630–26646. <https://doi.org/10.1074/jbc.M112.341255>
9. Fielder, S., & Brandão, J. (2022). Endocrine Diagnostics for Exotic Animals. *Veterinary Clinics of North America: Exotic Animal Practice*, 25(3), 631–661. <https://doi.org/10.1016/j.cvex.2022.06.003>
10. Gowda, V. A., Shenoy, S., & Suresh, M. P. (2017). Renal dysfunction in dogs: A study on clinical and biochemical changes in acute kidney injury. *Journal of Veterinary Science and Technology*, 8(3), 1-6.
11. Hundt M, Basit H, John S. Physiology, Bile Secretion. [Updated 2022 Sep 26]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2025 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK470209/>
12. Ifeoma, N. M. (2013). The Prevalence of Haemoparasitic Infection in Dogs Attending ECWA Veterinary Clinic, Bukuru, Jos South Local Government Area, Plateau State. 2013(July), 302–308.
13. Johnson, S. E., & Sherding, R. G. (2006). Diseases of the Liver and Biliary Tract. In *Saunders Manual of Small Animal Practice* (pp. 747–809). <https://doi.org/10.1016/B0-72-160422-6/50073-5>
14. Kesteloot, H. E., & Joossens, J. V. (1993). Relationship between dietary protein intake and serum urea, uric acid and creatinine, and 24-hour urinary creatinine excretion: the BIRNH Study. *Journal of the American College of Nutrition*, 12(1), 42–46. <https://doi.org/10.1080/07315724.1993.10718281>

15. McMahan, C. S., Wang, D., Beall, M. J., Bowman, D. D., Little, S. E., Pithua, P. O., Sharp, J. L., Stich, R. W., Yabsley, M. J., & Lund, R. B. (2016). Factors associated with *Anaplasma* spp. seroprevalence among dogs in the United States. *Parasites and Vectors*, 9(1). <https://doi.org/10.1186/s13071-016-1431-7>
16. Nagy, J. A., Benjamin, L., Zeng, H., Dvorak, A. M., & Dvorak, H. F. (2008). Vascular permeability, vascular hyper permeability and angiogenesis. *Angiogenesis*, 11(2), 109–119. <https://doi.org/10.1007/s10456-008-9099-z>
17. Prevalence of Hemoparasites in the Nigerian Breed of Dogs in Lafia Area of Nasarawa State, Nigeria. (2023). 8(July), 1–6.
18. Singh, J., Singh, R. S., Singh, H., Gupta, D. K., & Randhawa, S. S. (2021). Clinical and haemato-biochemical observations in dogs naturally infected with Canine monocytic ehrlichiosis. *Exploratory Animal and Medical Research*, 11(2), 214. <https://doi.org/10.52635/EAMR/11.2.214-219>
19. Soeters, P. B., Wolfe, R. R., & Shenkin, A. (2019). Hypoalbuminemia: Pathogenesis and Clinical Significance. *JPEN. Journal of Parenteral and Enteral Nutrition*, 43(2), 181–193. <https://doi.org/10.1002/jpen.1451>
20. Wilde, B., & Katsounas, A. (2019). Immune Dysfunction and Albumin-Related Immunity in Liver Cirrhosis. *Mediators of Inflammation*, 2019, 7537649. <https://doi.org/10.1155/2019/7537649>

Evaluation of CIDR PG Protocol of Timed Artificial Insemination During Low and Transition Breeding Season in Nepalese Buffaloes

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ABSTRACT

Seasonality is seen in breeding behaviors of Nepalese buffaloes, with July to December as active breeding season, January to March as transition, and April to June as low breeding seasons. This seasonal breeding pattern causes seasonal fluctuation in milk production, and the country suffers a severe drop in milk supply during the summer months. Thus, breeding buffaloes, particularly by applying novel hormonal protocols, during the transition and low breeding seasons, can play a huge role in sustained buffalo production. Present study was designed to evaluate Nepalese buffaloes' reproductive performance during the transition and low breeding seasons by applying the CIDR PG protocol for timed artificial insemination (TAI). Murrah cross breed buffaloes (n=17) of AFU Livestock Farm (n=11), Rampur, and National Buffalo Research Programme, Tarahara (n=6) were selected for the research. All the buffaloes received the treatment protocol of CIDR combined with PG (d0: CIDR in, d6: PG inj, d7: CIDR out and after 60 hrs TAI). Eleven buffaloes received the treatment during the low breeding season (n=5 AFU farm and n=6 NCRP farm), and six buffaloes received it during transition breeding season from AFU farm only. Contributing factors like BCS, parity, internal parasite, cyclic status, the sign of heat, vaginal discharge at TAI time, vaginal and cervical changes were recorded at day 0 of the protocol. Transrectal ultrasonography was used to confirm ovulation and recording of ovarian dynamics on 7-11 days after TAI. Pregnancy was confirmed at 90 days post-AI by rectal palpation. Data analysis was carried out using SPSS. Altogether, buffaloes from AFU farm showed a 60% (3/5) conception rate during the low breeding season and 33.33 % (2/6) during transition breeding season. Buffaloes from NBRP showed a 100% (6/6) conception rate; among them, three buffaloes had conceived in subsequent heat by mating. Buffaloes with good BCS (>2.5) had more conception rate than those with low BCS (≤2.5) (81.81 % vs 33.33 %, p>0.05). Parity, endoparasite infestation, cyclic status, signs of heat visible at TAI, and vaginal discharge at TAI did not show significant association with conception. This results indicated that CIDR PG protocol for TAI is useful to have a pregnancy in buffaloes during the transition and low breeding seasons; however, BCS is the determining factor for the protocol's successful outcome.

Keywords: Buffalo; Conception; CIDR; Seasonality

INTRODUCTION

Buffalo is the most important dairy animal in Nepal, contributing chiefly to milk, meat, draft power, fuel, and leather production. The major component of agriculture GDP is milk and milk products, which are 7.21 % and 4.33% from buffalo and cattle, respectively (MoALD, 2021). Buffalo contributes 1419412 MT (57.23%) of total milk and 188172 MT (36.13%) of the total meat production in the country (MoALD, 2021).

July to December are active breeding months, January to March are transition months, and April -June is poor breeding months for Nepalese buffalo (Devkota and Bohora, 2009). Buffaloes suffers from the cessation of ovarian activity and silent heat during dry spring and early summer. Most buffaloes exposed to scorching conditions cease ovarian activity (Nanda and Nakao, 2003; De Rensis and Lopez-Gatius, 2007). True anestrus, silent ovulation, and seasonality were reported as the predominant causes of poor reproductive performances in Nepalese buffaloes (Sha et al., 2013). Among the anestrus, cyclic anestrus or silent estrus are common in buffaloes during the active breeding season (Devkota, Bohora & Yamagisi, 2012). In silent cyclic buffalo, heat expression is not observed, causing the missing of insemination/ breeding. Several hormonal protocols can be applied in silent estrus buffaloes (Brito et al., 2013).

Estrus synchronization is mainly done by using hormonal protocols to achieve fixed time . Generally, estrus synchronization is achieved by two approaches. The first approach is by controlling the luteal phase of the cycle either through the administration of prostaglandins (Brito et al., 2002) or progesterone analog (De Rensis et al., 2005). The second and most recent approach of synchronization is by controlling the follicular development and ovulation using a combination of prostaglandins, progesterone, GnRH, and hCG (Campanile et a.,2007).

A major limiting factor for reproductive performances on many dairy farms is estrus detection, which is often low (<50%). Recent studies have shown that it is possible to precisely manipulate the follicle and luteal dynamic, eliminating the need for estrus detection for AI and embryo transfer. Considerable attention has been focused on reproductive physiology and endocrinology during the last two decades to develop models to improve reproductive efficiency, particularly using the controlled breeding technique in buffaloes (Barile, 2005). Estrus synchronization protocols, largely derived from cattle, have variable results in buffaloes (De Renis and Lopez, 2007). This study was done to evaluate effect of hormonal protocols and after affecting with estrus synchronization protocols (CIDR PG) during low and transition breeding seasons under farm conditions in Nepales buffalo.

MATERIALS AND METHODS

Study site

Livestock farm of Agriculture and Forestry University, Rampur Chitwan, and National

Buffalo Research Programme was selected for the study site. AFU lies in the western-southern part of Chitwan. NBRP is situated in the eastern terai region of Nepal in the Sunsari district.

Duration research

Duration of low breeding season protocol was from 2075/02/17 to 2075/05/17 (n=11), NBRP Tarahara, Sunsari, and AFU livestock farm Rampur, Chitwan. Similarly, duration of transition Breeding season protocol was from 2075/11/25 to 2076/02/25 (n=6), at AFU livestock farm Rampur, Chitwan

Selection of research buffaloes

Seventeen buffaloes were selected for the study. Out of seventeen selected buffaloes, six were anestrus parous buffaloes (more than 100 DIM), and eleven were heifer. Eleven buffaloes were assigned to use protocol during the low breeding season, and six were assigned to transition breeding season protocol.

Estrus synchronization protocol applied for experimental trials

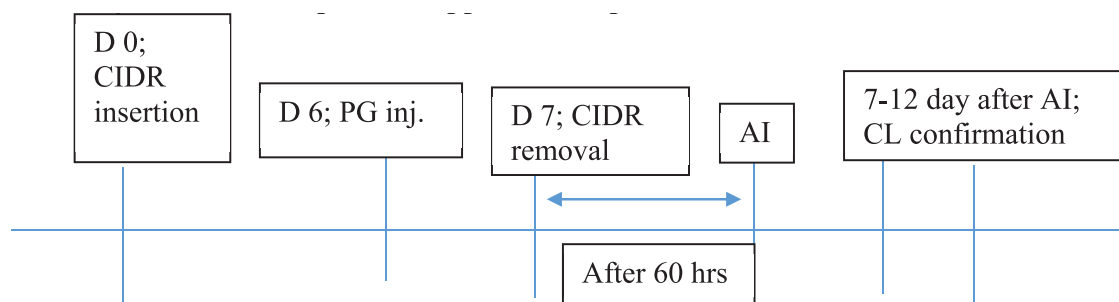


Fig. 1. Schematic representation of CIDR-PG protocol

All seventeen buffalo received CIDR-PG protocol (d 0: CIDR insert, d 6: PG injection, d 7: CIDR removal, and AI after 60 hrs. of CIDR removal) (Fig. 1). For the treatment, 1.9 gm CIDR (Eazi-Breed, Zoetis US) and 25 micrograms of PGF_{2α} (Dinoprost promethazine, Zoetis US) were used. fixed-time artificial insemination (FTAI) was done after 60 hrs. of CIDR removal. Eleven buffalo received synchronization protocol on Jestha (June) as low breeding season months (April to June), and six buffalo receive protocol on Falgun (April) as transition breeding season(January to March).

Recording of reproductive performance

Rectal palpation with transrectal ultrasonography (Farm scan L 60, BMV, China) was used, and the ovarian status of each research buffaloes was recorded. The presence of follicles and corpus luteum was recorded. The number of follicles and their size were recorded in both ovaries (Diskin et al., 2002.). Ovary with greater than 0.8 cm follicle and/or active CL is considered cyclic animals (Beg et al., 1999.) Inner vaginal membrane was categorized as pale and pink. The presence or absence of discharge of mucus from the

vagina during TAI was noted. Cervical dilation was felt by rectal palpation and obstruction to an AI gun during TAI. Uterine horns tonicity was examined by rectal palpation and sensation of tonicity of palm. The absence of tonicity is considered as the absence of tonicity (-), and the presence of tonicity is categorized as plus (+) mild tonicity, double plus (++) for good tonicity by the sensation of rectal palpation. Ovulation confirmation was done after 7 to 11 days after AI by palpating the presence of CL and recorded the size of CL by transrectal ultrasonography. Pregnancy diagnosis was done at 90 days of insemination through rectal palpation.

Others associated factors

Buffaloes were grouped into two different groups, with BCS ≤ 2.5 (Poor BCS) and with BCS > 2.5 (Good BCS). Fecal examination was done for endoparasite examination, according to Soulsby (1982).

Statistical analysis

Data entry was done in MS Office excel 2016, and statistical analysis was done by SPSS version 27. Due to small sample size, Fisher's exact test was performed to see the efficacy of protocol during different seasons on ovulation and conception rate among the different categorical factors BCS, parity, age parasite infestation during different seasons.

RESULTS

Out of the 17 animals assigned for TAI protocols across two seasons, 11 animals conceived, resulting in an overall conception rate of 64.70% (11/17). The ovulation rate observed was 88.23% (15/17). Among the conceived animals, eight conceived following estrus synchronization (TAI), while three showed heat in the subsequent cycle and conceived naturally. Conception was confirmed by rectal examination on day 90 post-insemination. The ovulation and conception rate in low breeding season was 90.91% (10/11) and 81.82% (9/11) respectively, and that in transition season was 83.33% (5/6) and 33.33% (2/6) respectively.

Reproductive parameters such as vaginal discharge, cervical dilation, uterine tonicity, ovulation, and conception rates were numerically higher during the low breeding season compared to the transition season, but statistically non-significant ($P > 0.05$) as presented in Table 1.

Table 1. Comparison between transition and low season for the reproductive performance of buffaloes at AFU Livestock Farm, Rampur after applying the TAI protocol

Parameters	Low season	Transition season	Odd ratio	P-value
Vaginal discharge (Yes)	(4/5) 80%	(4/6) 66.7%	2.0	0.62
Cervical dilatation (Yes)	(5/5) 100%	(2/6) 33.3%	10.0	0.79
Uterine tonicity (+ or more)	(4/5) 80%	(2/6) 33.3%	2.4	0.12
Ovulation (Yes)	(5/5) 100%	(5/6) 83.3 %	8.0	0.34
Conception out of TAI	(3/5) 60%	(2/6) 33.3 %	3.0	0.37
Conception out of subsequent 1 st heat and NI	-	-	-	-
Total conception on d90 of TAI	(3/5) 60%	(2/6) 33.3 %	1.7	0.87

In the low breeding season, buffaloes at AFU, Rampur, and NBRP, Tarahara showed variation in outcomes. While Rampur had a total conception rate of 60% (3/5), Tarahara recorded 100% (6/6) conception, including three animals that conceived after returning to cyclicity and were mated naturally. However, the difference was statistically non-significant (Table 2).

Table 2. Comparison between AFU Livestock Farm, Rampur and NBRP, Tarahara in low season for the reproductive performance of buffaloes after applying the TAI protocol

Parameters	AFU Rampur	NBRP Tarahara	Odd ratio	P-value
Vaginal discharge (Yes)	(4/5)80%	(5/6) 83.3%	1.3	0.89
Cervical dilatation (Yes)	(5/5) 100%	(1/6) 16.7%	1.2	0.34
Uterine tonicity (+ or more)	(4/5) 80%	(4/6) 66.7%	1.5	0.62
Ovulation (Yes)	(5/5) 100%	(5/6) 83.3 %	1.2	0.34
Conception out of TAI	(3/5) 60%	(3/6) 50.0%	3.0	0.38
Conception out of subsequent 1 st heat and NI	-	(3/3) 100.0%	-	-
Total conception on d90 of TAI	(3/5) 60%	(6/6) 100.0%	1.7	0.87

Regarding parity, parous buffaloes showed higher ovulation and conception rates, while heifer buffaloes exhibited more visible heat signs and uterine tonicity. These differences were again not statistically significant (Table 3).

Table 3. Comparison between Parous and Heifer buffalo of AFU Livestock Farm, Rampur, and National Buffalo Research Programme in low season for the reproductive performance of buffaloes after applying the TAI protocol

Parameters	Parous	Heifer	Odd ratio	P-value
Vaginal discharge (Yes)	(1/2) 50%	(7/9) 77.8%	3.5	0.43
Cervical dilatation (Yes)	(2/2) 100%	(4/9) 44.4%	1.2	0.15
Uterine tonicity (+ or more)	(1/2) 50%	(7/9) 77.8%	3.5	0.43
Ovulation (Yes)	(2/2) 100%	(8/9) 88.9 %	-	0.62
Conception out of TAI	(2/2) 100%	(4/9) 44.4%	-	0.50
Conception out of subsequent 1 st heat and NI	-	(3/3) 100.0%	-	-
Total conception on d90 of TAI	(2/2) 100%	(7/9) 77.8%	-	0.46

Factors affecting conception were analyzed (Table 4). Among these, Animals with good BCS (>2.5) had significantly higher conception rates (81.81%) compared to animals with low BCS (≤ 2.5) (33.33%), with an Odds Ratio = 9, $P = 0.046$, indicating a statistically significant difference.

There is no significant effects were observed for parity, endoparasitic infestation, cyclicity, or presence of heat signs at the time of TAI.

Table 4. Factors affecting conception after applying TAI protocols

Parameters		Conception	O d d P-value ratio
BCS	Good (>2.5)	81.8% (9/11)	9.0 0.046
	Low (≤ 2.5)	33.3 % (2/6)	
Parity	Parous	50.0% (4/8)	3.5 0.232
	Heifer	77.8% (7/9)	
Endoparasitic infestation	Positive	64.3% (9/14)	1.1 0.938
	Negative	66.7% (2/3)	
Cyclic status	Cyclic	71.4% (5/7)	1.6 0.623
	Noncyclic	60.0% (6/10)	
Sign of heat	Visible	77.8% (7/11)	3.5 0.232
	Not visible	50.0% (4/8)	
Vaginal discharge at TAI	Present	66.7% (4/6)	1.1 0.901
	Absent	63.6% (7/11)	

DISCUSSION

The results demonstrate a notable seasonal influence on the reproductive performance of buffaloes subjected to TAI protocols. The superior outcomes in the low breeding season may be attributed to favorable environmental and physiological conditions, as supported by Vale et al. (1997), who emphasized the role of environmental cues such as light and temperature in regulating reproductive function. The high ovulation rate (83.33%) but low conception rate (33.33%) during the transition season aligns with findings by Arshad et al. (2017), who also reported a 32% conception rate. Jabeen et al. (2015) recorded 30–36% conception using CIDR protocols during various breeding seasons, and Haider et al. (2015) reported a 59% rate with CIDR-GnRH protocols, indicating variability across regions and protocols.

The estrus detection rate was also better during the low breeding season, consistent with Naseer et al. (2011), who reported 80% estrus response and 60% pregnancy in anestrus Nili Ravi buffaloes. Enhanced outcomes at Tarahara could be due to better post-TAI cyclicity and mating management, even though environmental conditions were similar to Rampur. The CIDR-PG protocol demonstrated reasonable effectiveness in inducing estrus, particularly when PGF2- α was combined. PGF2- α possibly enhanced pituitary sensitivity to GnRH post-CIDR removal, which aligns with physiological insights that suggest effective stimulation of gonadotropins and improved ovulation (Vikash et al., 2014).

Although no significant differences were found between parous and heifer buffaloes, trends indicated slightly better ovulation and conception rates in parous animals, agreeing with Devkota et al. (2013), who also reported non-significant parity effects on synchronization outcomes.

The significant impact of body condition score (BCS) on conception confirms previous reports by Shah et al. (1988) and Barile (2005), highlighting the negative effects of poor nutrition and thermal stress on reproductive efficiency. Similarly, parasitic infestations, though not statistically significant here, have been shown to reduce fertility (Hammam, 2011), suggesting that parasite control before protocol application could further enhance results.

The estrus response rate of 53% in this study was lower than those reported by Yousuf et al. (2015) and Zaabel et al. (2009), who noted rates above 88% with CIDR and GnRH in Nili Ravi and Egyptian buffaloes. This discrepancy could be due to physiological or environmental variations, or differences in detection efficiency. In summary, the CIDR-PG protocol is effective in inducing estrus and improving conception in anestrus buffaloes, particularly during the low breeding season. The reproductive response is strongly influenced by body condition, and potentially by prior parasitic load, parity, and environmental conditions. Timed AI remains a useful tool to manage estrus detection issues and enhance fertility during non-ideal seasons.

CONCLUSION

This study concludes that the CIDR-PG hormonal protocol can effectively induce ovulation in anestrus buffaloes. However, the success of the protocol, which is measured by pregnancy outcomes is significantly influenced by factors such as endoparasitic load, age, and body condition score (BCS). Among these, a good BCS showed a statistically significant positive impact on conception rates. These preliminary findings suggest that while hormonal protocols can address anestrus, the overall reproductive success depends on the physiological and health status of the animals. Further research with a larger sample size is recommended to validate and expand upon these observations.

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ETHICAL APPROVAL

This research has been approved by Agriculture and Forestry University, Rampur, Chitwan, Nepal. University is in charge of overseeing this work. Samples are only taken with respected farm approval. Both of the farm are informed with the outcome and the recommendation.

Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Arshad, U., Qayyum, A., Hassan, M., Husnain, A., Sattar, A., & Ahmad, N. (2017). Effect of resynchronization with GnRH or progesterone (P4) intravaginal device (CIDR) on Day 23 after timed artificial insemination on cumulative pregnancy and embryonic losses in CIDR-GnRH synchronized Nili-Ravi buffaloes. *Theriogenology*, 103, 104–109.
2. Barile, V. L. (2005). Reproductive efficiency in female buffaloes. In *Buffalo production and research. FAO technical series* (pp. 77–108).
3. Beg, M. A., & Totey, S. M. (1999). The estrous cycle, estrous behavior and the endocrinology of the estrous cycle in the buffalo (*Bubalus bubalis*). *Animal Breeding Abstracts*, 67, 329–337.
4. Brito, L. F. C., Satrapa, R., Marson, E. P., & Kastelic, J. P. (2002). Efficacy of PGF2 α to synchronize estrus in water buffalo cows (*Bubalus bubalis*) is dependent upon plasma progesterone concentration, corpus luteum size and ovarian follicular status before treatment. *Animal Reproduction Science*, 73(1–2), 23–35.
5. Campanile, G., Di Palo, R., Neglia, G., Vecchio, D., Gasparini, B., Prandi, A., ... & D'Occhio, M. J. (2007). Corpus luteum function and embryonic mortality in buffaloes treated with a GnRH agonist, hCG and progesterone. *Theriogenology*, 67(8), 1393–1398.

6. Dash, S., Chakravarty, A. K., Sah, V., Jamuna, V., Behera, R., Kashyap, N., & Deshmukh, B. (2015). Influence of temperature and humidity on pregnancy rate of Murrah buffaloes under subtropical climate. *Asian-Australasian Journal of Animal Sciences*, 28(7), 943–949.
7. De Rensis, F., & Lopez-Gatius, F. (2007). Protocols for synchronizing estrus and ovulation in buffalo (*Bubalus bubalis*): A review. *Theriogenology*, 67(2), 209–216.
8. De Rensis, F., Ronci, G., Guarneri, P., Nguyen, B. X., Presicce, G. A., Huszenicza, G., & Scaramuzzi, R. J. (2005). Conception rate after fixed time insemination following Ovsynch protocol with and without progesterone supplementation in cyclic and non-cyclic Mediterranean Italian buffaloes (*Bubalus bubalis*). *Theriogenology*, 63(7), 1824–1831.
9. Devkota, B., Bohara, T. P., & Yamagishi, N. (2012). Seasonal variation of anestrus conditions in buffaloes (*Bubalus bubalis*) in Southern Nepal. *Asian Journal of Animal and Veterinary Advances*, 7(9), 910–914.
10. Devkota, B., Nakao, T., Kobayashi, K., Sato, H., Sah, S. K., Singh, D. K., & Yamagishi, N. (2013). Effects of treatment for anestrus in water buffaloes with PGF2 α and GnRH in comparison with vitamin-mineral supplement, and some factors influencing treatment effects. *Journal of Veterinary Medical Science*, 75(11), 1371–1375.
11. Diskin, M. G., Austin, E. J., & Roche, J. F. (2002). Exogenous hormonal manipulation of ovarian activity in cattle. *Domestic Animal Endocrinology*, 23(1–2), 211–228.
12. Ghuman, S., Honparkhe, M., & Singh, J. (2014). Comparison of Ovsynch and progesterone-based protocol for induction of synchronized ovulation and conception rate in subestrous buffalo during low-breeding season. *Iranian Journal of Veterinary Research*, 15(4), 375–379.
13. Government of Nepal (GoN), Ministry of Agriculture and Livestock Development, Department of Livestock Services. (2021). *Livestock statistics of Nepal 2020/21*. Lalitpur, Nepal.
14. Hammam, A. M., Rabab, M., El Khateeb, A., Amer, K. A., Abou-El-Dobal, S., El Shahat, K., & Scott, W. (2011). Response of Fasciola-free and infected buffaloes to CIDR Ovsynch treatment during summer season with emphasis on sex hormone and biochemical changes. *Journal of American Science*, 7(9), 810–820.
15. Jabeen, S., Anwar, M., Andrabi, S. M. H., Mehmood, A., & Shahab, M. (2015). Determination of Ovsynch and CIDR efficiency for estrus synchronization by conception rate in Nili Ravi buffaloes during low and peak breeding seasons. [Unpublished manuscript].
16. Ministry of Agriculture and Livestock Development (MOALD). (2019). *Statistical information on Nepalese agriculture*. Kathmandu, Nepal.
17. Nanda, A. S., & Nakao, T. (2003). Role of buffaloes in the socioeconomic development of rural Asia: Current status and future prospects. *Animal Science Journal*, 74(6), 443–455.
18. Naseer, Z., Ahmad, E., Singh, J., & Ahmad, N. (2011). Fertility following CIDR-based synchronization regimens in anoestrous Nili-Ravi buffaloes. *Reproduction in Domestic Animals*, 46(5), 814–817.
19. Soulsby, E. J. L. (1982). *Helminths, arthropods and protozoa of domesticated animals* (7th ed.). Bailliere Tindall.
20. Vikash, V. M., Malik, R. K., & Singh, P. (2014). Impact of CIDR in combination with different hormones for treatment of anestrus in buffaloes under field conditions of Haryana. *Haryana Veterinarian*, 53, 28–33.
21. Yousuf, M. R., Martins, J. P. N., Husnain, A., Riaz, U., Riaz, H., Sattar, A., ... & Ahmad, N. (2015). Effect of estradiol benzoate on estrus intensity and pregnancy rate in CIDR treated anestrus nulliparous and multiparous buffalo. *Animal Reproduction Science*, 159, 104–108.
22. Zaabel, S. M., Hegab, A. O., Montasser, A. E., & El-Sheikh, H. (2009). Reproductive performance of anestrus buffaloes treated with CIDR. *Animal Reproduction Science*, 110(1–2), 460–464.

Rising Antimicrobial Resistance in Avian *Escherichia coli*: Insights from One-Year Data of National Avian Diseases Investigation Laboratory Chitwan, Nepal

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ABSTRACT

Antimicrobial resistance (AMR) is a global health threat affecting both human and animal health. In poultry, *Escherichia coli* (*E. coli*), a common commensal and opportunistic pathogen, can develop resistance due to indiscriminate antimicrobial use. The emergence of multidrug-resistant (MDR) *E. coli* in poultry poses significant challenges to animal health and food safety. In Nepal, there is limited data on AMR patterns in *E. coli* from poultry. This study insights the antimicrobial resistance profile of *E. coli* isolates from poultry samples submitted to the bacteriology section of National Avian Disease Investigation laboratory, Bharatpur. A retrospective cross-sectional study was conducted from Baisakh 2080 to Chaitra 2080 BS. Total of 198 individual colonies of *E. coli* were isolated using cultural and biochemical methods that resulted 60.61% and 30.81% of *E. coli* isolates in commercial broiler and commercial layers respectively whereas the remaining 8.59% of other bird type (Giriraj, backyard & broiler parent). The resistance spectrum of *E. coli* for 7 studied antibiotics in descending order were respectively, Tetracycline (93%, 95% CI=85.9-97.49), Levofloxacin (83%, 95% CI=72.38-90.09), Trimethoprim-sulfamethoxazole (76%, 95%CI=67.82-83.76), Gentamicin (55% 95%CI= 47.59-61.91), Chloramphenicol (53%, 95% CI= 44.22-61.45), Ceftriaxone (36%, 95% CI= 27.3- 46.31), and Amikacin (18%, 95%CI=12.76-23.95). A total of 113 isolates (57.07%, 95% CI=49.86-64.07) had multi drug resistance (MDR) phenotype. In age-wise study, a greater proportion of *E. coli* isolates from broilers were found in the grower phase (16-30 days) whereas in layers, higher proportion between 1 to 18 weeks comprising about 42% was resulted. According to flock wise study, smaller-scale poultry farms may be more vulnerable to *E. coli* contamination, possibly due to less stringent biosecurity measures or management practices. These findings emphasize on prudent antibiotic use, enhanced farm biosecurity, and routine AMR monitoring.

Keywords: Antibiotic; AMR; broilers; *E. coli*; Layers

INTRODUCTION

Colibacillosis is regarded one of the most serious bacterial illnesses in avian pathology. Though *E. coli* is considered as normal inhabitant microflora in avian intestinal tract, extra intestinal pathogenic *E. coli* (ExPEC) infections generate a negative economic impact, (Tawab et al., 2016). Due to detrimental effects on growth, decline in production, the quantity of culled chickens, and the poor quality of the carcasses and eggs, colibacillosis continues to be a significant issue for the poultry industry (Chotiah & Damayanti, 2016). Avian colibacillosis is a complex syndrome, characterized by a diverse array of multiple organ lesions such as airsacculitis, pericarditis, peritonitis, salpingitis, synovitis, osteomyelitis, yolk sac infections (Ievy et al., 2020).

The primary therapeutics for the control measure of morbidity and mortality due to avian pathogenic *E. coli* is antimicrobial therapy. But excessive usage of antibiotics lowered their efficiency, eventually leading to the emergence of antibiotic resistance. In the recent decades, the overuse and abuse of antibiotics as treatments or as growth promoters has led to an exaggeration of the growing resistance against different classes of antibiotics (Agusi et al., 2024; Li et al., 2024 & Tawab et al., 2016).

MATERIALS AND METHODS

A year of registered data, from Baisakh 2080 to Chaitra 2080 BS in the registry book of microbiology unit of National Avian Disease Investigation Laboratory (NADIL) was analyzed for cross-sectional study. The collected data were entered manually in MS-Excel 2016 and preliminary data cleaning was performed. The tentative diagnosis of colibacillosis of dead or sick birds of varied age group, from areas in and around Chitwan district, brought at postmortem units of NADIL was based on clinical signs, history of flock and pathological lesions observed at the time of necropsies. Then, the liver samples of suspected cases were collected and forwarded to microbiology unit. Cultures and antibiotic sensitivity tests were routinely used for further diagnosis of suspected bacterial diseases.

All the samples positive for *E. coli* were confirmed using Gram's staining, cultural and bio-chemical examinations. Suspected lactose fermented colonies with smooth, circular pink color on MacConkey Agar (Himedia), incubated aerobically at 37 °C for 24 hours were examined for Gram's stain. Colony having similar characters with the resulted gram-negative rod was re-inoculated on Eosin Methylene Blue (EMB) agar which is selective medium for *E. coli* and incubated overnight at 37 °C. Biochemical tests viz Indole, MR, VP, Citrate, Catalase, Oxidase of isolated sample were done for the confirmation. Antibiotic susceptibilities of *E. coli* isolates were determined by the standard disk diffusion method (Tawab et al., 2016).

Antimicrobial discs

Total of seven commercially available antibiotic discs belonging to six different classes that were frequently used for AST in microbiology were considered in the study.

Table 1. Antimicrobial agents with their disc concentration

Antibiotic Disc	Size (mcg)
Gentamicin	10
Amikacin	30
Levofloxacin	5
Tetracycline	30
Trimethoprim-sulfamethoxazole	25
Chloramphenicol	30
Ceftriaxone	30

Data recording

The zones of growth inhibition of different antibiotic disc were compared with the interpretive standards zone size provided by Clinical and Laboratory Standards Institute (CLSI, 2019) (Table 2). Then, the sensitivity test results were recorded as susceptible, intermediate and resistant. The data were inserted on MS- Excel Sheet to generate the graphical presentation and descriptive analysis were done using Epi Info version 7.

Table 2. Zone diameter (in mm) interpretive standards for *E. coli*

Antibiotic Disc	Sensitive	Intermediate	Resistance
Gentamicin	≥ 15	13-14	≤ 12
Amikacin	≥ 17	15-16	≤ 14
Levofloxacin	≥ 21	17-20	≤ 16
Tetracycline	≥ 15	12-14	≤ 11
Trimethoprim-sulfamethoxazole	≥ 16	13-15	≤ 12
Chloramphenicol	≥ 18	13-17	≤ 12
Ceftriaxone	≥ 23	20-22	≤ 19

Source: CLSI, 2019

RESULTS

A total of 198 individual colonies of *E. coli* were isolated with 60.61% and 30.81% of commercial broiler and commercial layers respectively whereas the remaining 8.59% of other bird type (Giriraj, backyard & broiler parent). This study considered seven frequently used antibiotics in NADIL for antibiotic sensitivity as shown in Table 1. The resistance spectrum of *E. coli* for 7 studied antibiotics in descending order were respectively, Tetracycline (93%, 95% CI=85.9-97.49), Levofloxacin (83%, 95% CI=72.38-90.09), Trimethoprim-sulfamethoxazole (76%, 95% CI=67.82-83.76), Gentamicin (55% 95% CI= 47.59-61.91), Chloramphenicol (53%, 95% CI= 44.22-61.45), Ceftriaxone (36%, 95% CI= 27.3- 46.31), and Amikacin (18%, 95%CI=12.76-23.95). A total of 113 isolates (57.07%, 95% CI=49.86-64.07) had multi drug resistance (MDR) phenotype.

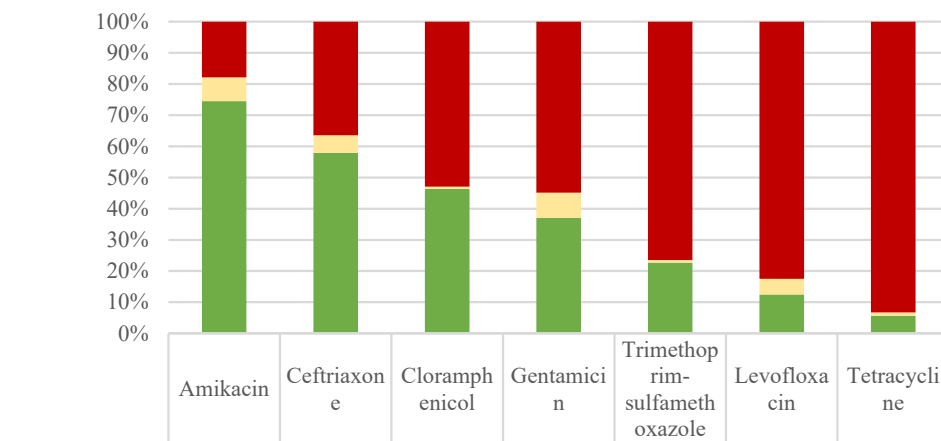


Fig. 1. Overall antibiogram profile of *E. coli* isolates

The graphical representation in Fig. 2 & 3 highlights the antibiotic resistance patterns of *E. coli* isolates from commercial broilers and layers. Notably, both groups exhibited high resistance to tetracycline, with 96% of broiler isolates and 91% of layer isolates being resistant, indicating widespread use or overuse of this antibiotic in poultry farming.

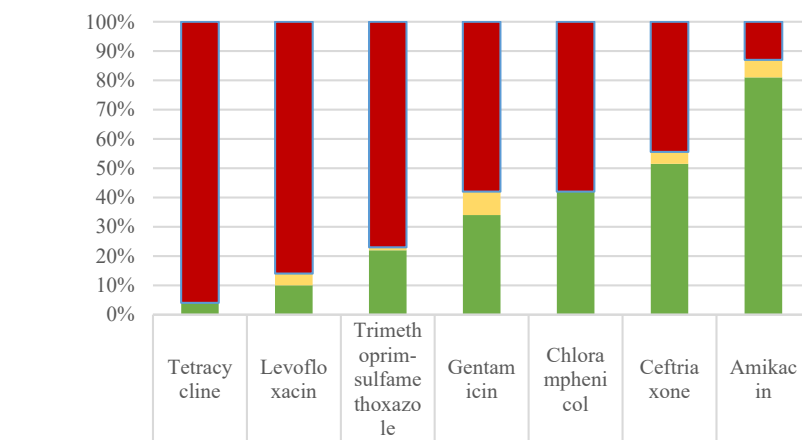


Fig. 2. Antibiogram profile of commercial broilers

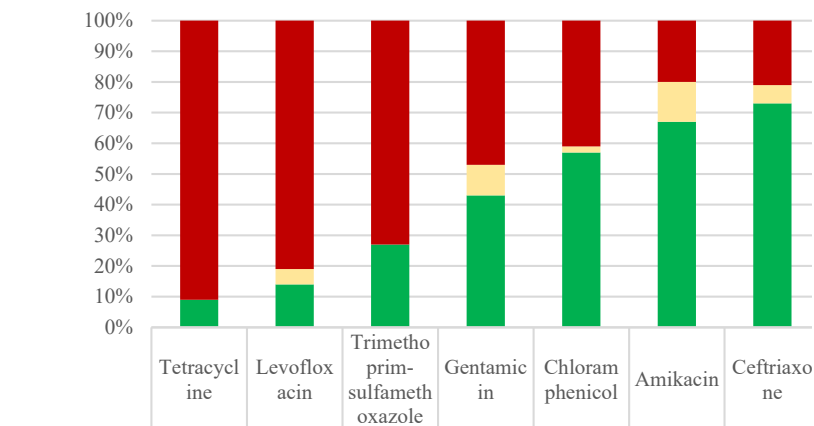


Fig. 3. Antibiogram profile of commercial layers

Age-wise distribution

In the age-wise study, a greater proportion of *E. coli* isolates from broilers were found in the grower phase (16-30 days), accounting for approximately 63%. For layers, resulted data indicates the highest frequency of *E. coli* infections from birds aged between one week and 18 weeks comprising about 42%, with a gradual decline in frequency as the layers age.

Table 3. Age-wise distribution of *E. coli* in commercial layers

CL (age in week)	Frequency	Percent	Exact 95% LCL	Exact 95% UCL
1-18	25	41.67%	29.07%	55.12%
19-36	10	16.67%	8.29%	28.52%
37-54	11	18.33%	9.52%	30.44%
55-72	10	16.67%	8.29%	28.52%
73-90	4	6.67%	1.85%	16.20%
Total	60	100.00%		

Table 4. Age-wise distribution of *E. coli* in commercial broilers

CB (age in days)	Frequency	Percent	Exact 95% LCL	Exact 95% UCL
1-15	29	24.17%	16.82%	32.83%
16-30	76	63.33%	54.05%	71.94%
31-45	15	12.50%	7.17%	19.78%
Total	120	100.00%		

Flock-wise distribution

E. coli isolates from a total of 197 samples, the majority of isolates were from flocks with fewer than 1,000 birds, which accounted for 33.50% of all isolates. The second-highest proportion was seen in flocks with 1,000–1,999 birds (31.98%), further supporting the trend of higher prevalence in smaller to mid-sized flocks.

Table 5. Flock size-wise distribution of *E. coli* isolates

Flock size	Frequency	Percent	Exact 95% LCL	Exact 95% UCL
<1000	66	33.50%	26.95%	40.56%
1000-1999	63	31.98%	25.53%	38.98%
2000-2999	27	13.71%	9.23%	19.31%
3000-5999	18	9.14%	5.51%	14.06%
6000-8999	7	3.55%	1.44%	7.18%
>8999	16	8.12%	4.71%	12.85%
Total	197	100.00%		

DISCUSSION

Among the studied antibiotics, amikacin showed higher sensitivity whereas tetracycline showed higher resistivity. In comparison to this study, Sapkota et al. (2020), the study conducted at RVL, Surkhet reported lower resistance (77%) of tetracycline. Similarly, Agusi et al. (2024) also resulted lower resistance of tetracycline (81.9%) whereas Hassan (2014) observed higher (100%) as compared to this study. The result observed for tetracycline tally with Davies et al. (2024) with studies focussing solely on phenotypic characterisation of resistance. To address this evidence gap we performed antimicrobial susceptibility testing and whole genome sequencing on *E. coli* obtained from chickens from live bird markets in Dhaka in 2018 ($n = 38$ i.e. 94%). In present study, resistance percent against Gentamicin (55%) and Chloramphenicol (53%) are nearly the same. Davies et al. (2024) with studies focussing solely on phenotypic characterisation of resistance. To address this evidence gap we performed antimicrobial susceptibility testing and whole genome sequencing on *E. coli* obtained from chickens from live bird markets in Dhaka in 2018 ($n = 38$ stated comparatively lower resistance to Gentamicin (34%) but similar finding to Chloramphenicol (55%). In contrast to this study, Tawab et al. (2016) observed higher resistance of Chloramphenicol (83%) and Trimethoprim-sulfamethoxazole (93%), whereas lower for Gentamicin (40%). Overall, the data indicates a concerning level of resistance to several commonly used antibiotics, particularly in broilers, underscoring the need for prudent antibiotic use and ongoing surveillance in poultry in production system.

The findings on age wise study in commercial broiler were on complaint with Ganaie et al. (2021), which reported higher susceptibility of *E. coli* on age of 3rd & 4th week. The age wise variation in an incidence of *E. coli* infection also have been reported by various authors. On study of age wise disease profile, Surjagade et al. (2020) stated third week being the susceptible age for *E. coli* infection followed by fourth week. Bhalerao et al. (2013) found colibacillosis in different age groups of broilers but maximum mortality was evident in birds of 3-4 weeks of age. Birds of any age can be affected, especially if their resistance is reduced by predisposing factors.

In layers, *E. coli* isolates were high from birds aged between one week and 18 weeks, with a gradual decline in frequency as the layers age. This early-life peak may reflect the increased susceptibility of pullets to *E. coli* infection due to weak & immature immune

systems (Waliaula et al., 2024), stress associated with brooding and rearing, or heightened environmental exposure. The declining trend suggests that as birds mature, they may develop increased resistance or benefit from management practices that reduce exposure risk.

According to flock wise study, higher isolates were from the flock size <1000, further supporting the trend of higher prevalence in smaller to mid-sized flocks. This suggests that smaller-scale poultry farms may be more vulnerable to *E. coli* contamination, possibly due to less stringent biosecurity measures or management practices. These results indicate that smaller poultry operations may lack the resources or infrastructure to effectively control bacterial contamination, making them more susceptible to infections like *E. coli*.

CONCLUSION

This study underscores alarming levels of antibiotic resistance in avian *E. coli* isolates. Notably, both broilers & layers group exhibited high resistance to tetracycline & levofloxacin, indicating widespread use or overuse of this antibiotic in poultry farming. Similarly, Amikacin and ceftriaxone remain relatively effective. Emphasis should be placed on prudent antibiotic use, enhanced farm biosecurity, and routine AMR monitoring.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Abd El Tawab, A., El-Hofy, F., Ammar, A., Abdel Hakeem, M., & Abdel Galil, N. (2016). Preliminary studies on *E. coli* implicated in avian colibacillosis with reference to their antibiotic resistance profiles. *Benha Veterinary Medical Journal*, 30(1), 68–77. <https://doi.org/10.21608/bvmj.2016.31348>
2. Agusi, E. R., Kabantiyok, D., Mkpuma, N., Atai, R. B., Okongwu-Ejike, C., Bakare, E. L., Budaye, J., Sule, K. G., Rindaps, R. J., James, G. K., Audu, B. J., Agada, G. O., Adegboye, O., & Meseko, C. A. (2024). Prevalence of multidrug-resistant *Escherichia coli* isolates and virulence gene expression in poultry farms in Jos, Nigeria. *Frontiers in Microbiology*, 15(June), 1–9. <https://doi.org/10.3389/fmicb.2024.1298582>
3. Bhalerao, A. K. D., Gupta, R. P., & Kumari, M. (2013). Pathological studies on natural cases of avian colibacillosis in Haryana State. *Indian Journal of Veterinary Pathology*, 52, 118–120.
4. Chotiah, S., & Damayanti, R. (2016). Colibacillosis and antibiotics resistance patterns in broiler. In

Proceedings of the International Seminar on Livestock Production and Veterinary Technology (pp. 434–440). <https://doi.org/10.14334/proc.intsem.lpvvt-2016-p.434-440>

5. Davies, A. R., Chisnall, T., Akter, S., Afrad, M. M. H., Sadekuzzaman, M., Badhy, S. C., Hasan, M. Z., Rahman, M. T., Smith, R. P., Card, R. M., Brum, E., & Chowdhury, M. G. A. (2024). Genomic characterisation of *Escherichia coli* isolated from poultry at retail through Sink Surveillance in Dhaka, Bangladesh reveals high levels of multi-drug resistance. *Frontiers in Microbiology*, 15(May). <https://doi.org/10.3389/fmicb.2024.1418476>
6. Ganaie, B. A., Bandy, M. T., Adil, S., & Mir, M. S. (2021). Age and district wise mortality due to colibacillosis in commercial broiler farms of Kashmir Valley. *SKUAST Journal of Research*, 23(1). <http://www.IndianJournals.com>
7. Hassan, M. M. (2014). Antimicrobial resistance pattern against *E. coli* and *Salmonella* in layer poultry. *Research Journal for Veterinary Practitioners*, 2(2), 30–35. <https://doi.org/10.14737/journal.rjvp/2014/2.2.30.35>
8. Levy, S., Islam, M. S., Sobur, M. A., Talukder, M., Rahman, M. B., Khan, M. F. R., & Rahman, M. T. (2020). Molecular detection of avian pathogenic *Escherichia coli* (APEC) for the first time in layer farms in Bangladesh and their antibiotic resistance patterns. *Microorganisms*, 8(7), 1–15. <https://doi.org/10.3390/microorganisms8071021>
9. Li, Z., Jia, C., Hu, Z., Jin, Y., Li, T., Zhang, X., Peng, Z., Yang, R., Chen, H., & Wang, X. (2024). Antimicrobial resistance and genomic characteristics of *Escherichia coli* strains isolated from the poultry industry in Henan Province, China. *Microorganisms*, 12(3). <https://doi.org/10.3390/microorganisms12030575>
10. Sapkota, M. M., Lamichhane, U., & Pandey, K. R. (2020). Prevalence and associated risk factor of *Escherichia coli* from the cases of poultry at regional veterinary. *Journal of Agriculture and Environment Research*, 5(3), 7–13.
11. Surjagade, S., Joshi, B. P., Mathakiya, R. A., Kapadiya, B., & Bansod, A. P. (2020). Mortality pattern and pathological study of *E. coli* infection in broiler chickens. *Journal of Entomology and Zoology Studies*, 8(6), 867–872.
12. Waliaula, P. K., Kiarie, E. G., & Diarra, M. S. (2024). Predisposition factors and control strategies of avian pathogenic *Escherichia coli* in laying hens. *Frontiers in Veterinary Science*, 11(November). <https://doi.org/10.3389/fvets.2024.1474549>

Case Study

A Veterolegal Case Report on Cow Slaughter at Rupandehi

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ABSTRACT

A case of illegal cow slaughter with a deep stab wound to the thorax was reported to the police. As part of the veterolegal investigation, veterinary officer conducted a detailed post mortem examination to determine the cause of death, estimate time of death, assess signs of trauma, and other possible aspects. The examination confirmed that the cause of death was hemorrhagic shock due to a penetrating wound to the heart. The charge sheet filed by the District Government Attorney's Office cited Section 289(2) of the National Penal Code, 2074 recommending up to three years' imprisonment. The veterinary officer also provided expert testimony in court, confirming that the wound was deliberate, fatal, and consistent with a spear, not an accidental injury or gunshot.

Keywords: Cow slaughter; Veterolegal; Penetrating wound

CASE PRESENTATION

On 2080-01-07; 6:00 am, a case of illegal bull slaughter was reported in Kotahimai Rural Municipality of Rupandehi district. A stray bull was found with a deep penetrating wound on its thorax. The incident occurred in the early hours of the morning, and initial observations suggest the animal was killed in a secluded spot at kitchen garden of suspect residence. After the act, the alleged culprit attempted to bury the carcass in a nearby river bank to conceal the evidence. However, a neighbor noticed suspicious activity and promptly alerted the local police. Upon arrival, they discovered the partially buried remains of the bull and initiated an investigation.

As part of the veterolegal investigation, a licensed veterinary officer is required to conduct a post mortem examination and provide a detailed report for evidentiary purposes.

a. Age Determination

The age was calculated via examination of its permanent dentition. Adult cattle typically develop a full set of permanent incisors by 3.5–4 years of age, replacing their deciduous teeth. The presence of fully erupted, slightly worn permanent teeth indicated the bull had reached skeletal maturity, which also aligned with the size of mature testicle.

b. Estimation of Time of Death

The time of death was approximated using post-mortem, physiological and environmental indicators. Rigor mortis has already begun but the body had not yet started to decompose which suggested death had occurred several hour priors, as rigor mortis typically manifests

within 2–6 hours and peaks at 12 hours. The absence of foul odor, advanced decomposition, or any insect activity further narrowed the timeline, as these signs typically emerge 24–48 hours after death under normal conditions. Synthesizing these findings the time of death was estimated to be 8–10 hours before the post-mortem examination.

c. Post mortem Evidence

General Condition of the Carcass: The overall condition of the carcass was assessed to be fair to good. The body condition score (BCS) was estimated between 2.5 to 3 on a 5-point scale, indicating a moderately nourished animal. However, moderate bloating of the abdomen was noted.

Head: Normal in shape and size, with no external injuries or deformities observed.

Eyes: Evidence of corneal opacity (dull and milky cornea), with enophthalmos eye ball

Nose and Mouth: Presence of water-mixed bloody discharge.

Tongue: Rolled out, with sand particles observed around the nozzle and within the buccal cavity, possibly from pre- or post-mortem environmental contact.

Neck: Positioned facing upward and slightly twisted. However, there was no dislocation of the atlanto-occipital joint or cervical vertebrae. The lymph nodes appeared soft but had normal shape and size.

Thoracic cavity: A deep stab wound approximately 1 inch wide and 1 foot deep was noted at the 3rd to 4th intercostal space on the left thoracic wall, near the base of scapula. The wound penetrated the thoracic cavity and reached the heart, with clear evidence of puncture on the outer layer of the pericardium. Signs of profuse internal bleeding from the heart were evident.

Abdominal cavity: The stomach and intestines appeared normal, with no signs of poisoning or pathological changes. The liver was of normal size, color, and consistency, showing no signs of congestion, necrosis, or disease. The gall bladder was intact, with no signs of rupture, inflammation, or biliary obstruction. No abnormal fluid accumulation or damage was noted in other abdominal organs. No disruption or trauma was noted in any other internal organs.

Conclusion of Post Mortem: Hence the cause of death was confirmed to be due to Haemorrhagic Shock

CHARGE SHEET

Following the investigation conducted by the District Police Office, Rupandehi, a charge sheet was formally submitted to the Rupandehi District Court by the District Government Attorney's Office, Rupandehi on 2080.03.26 (Bikram Sambat). The charge sheet recommended imprisonment for a term of three years for the accused, citing the legal provision under the National Penal (Code) Act, 2017, specifically:

Chapter 27: Offences Relating to Animals and Birds

Section 289 – Prohibition of killing or beating cows or oxen:

1. No person shall do, or cause to be done, any act with the intention of killing, or causing hurt to, any cow or ox.
2. Where any act referred to in sub-section (1) causes the death of any cow or ox, the offender of such offence shall be liable to a sentence of imprisonment for a term not exceeding three years.
3. A person shall be liable to a sentence of imprisonment for a term not exceeding six months if he or she causes grievous hurt to a cow or ox, and to a fine not exceeding fifty thousand rupees if other hurt is caused.

The submitted charge sheet specifically invoked sub-section (2), as the act had resulted in the death of a bull, making the offender liable under the law for a custodial sentence of up to three years.



Fig. 1. Different activities during veterolegal case; A- Site of incident as shown, red box indicates dead animal; B- Penetrating wound on animal on thoracic cavity; C- Dentition of bull showing eruption of all permanent teeth showing skeletal maturity; D, E- Postmortem examination of animal, F- Official letter of court asking for expert advice

TRIAL PROCEEDINGS

During the trial proceedings at the Rupandehi District Court, the then Veterinary Officer of the Veterinary Hospital and Livestock Expert Centre (VHLEC), Rupandehi, was summoned to appear as an expert witness. The veterinary officer (VO) was asked to respond to the following specific queries:

Cause of death: The VO confirmed that the immediate cause of death was profuse internal

bleeding resulting from a stab wound that penetrated the thoracic cavity and punctured the heart. The trauma led to exsanguination and ultimate death.

Whether stab wound caused death: Yes. The VO testified that post mortem findings, including the depth, location, and nature of the wound, clearly indicated that the bull died due to a stab wound inflicted with a sharp, pointed object.

Can such small wound cause death: Yes. The VO clarified depending on the site and depth of wound, 1-inch-wide penetrating wound is more than sufficient to cause fatal injury in a bovine animal. In this case a narrow yet pointed weapon stabbed at 3-4th intercoastal space communicated with thoracic cavity. The absence of any other injury indicated that a single shot induced death.

Other possibilities: The VO stated that it is highly unlikely for a cow to accidentally stab itself with such precision and force. The wound's trajectory was lateral, pointed backward which could have been only possible if animal could run sideways (which is impossible), thus it was deliberately inflicted by an external agent, not self-inflicted.

Weapon of injury (stabbed or gunshot): The VO explained that stab wounds and bullet wounds have distinctly different characteristics. The wound observed was linear, clean-edged, and showed no burn marks, tissue shredding, or entry/exit holes, which are commonly associated with gunshot injuries. Moreover, foreign object or projectile was neither recovered from the site nor the body, and the nature of the wound was consistent with that caused by a sharp stabbing weapon like a spear.

JUDGEMENT

The final verdict in the case was delivered on 2080-11-15 after thorough analysis of the charge sheet, scene report, post mortem report and statement of witness. While the court acknowledged that the bull had died due to a penetrating thoracic wound leading to hemorrhagic shock, the weapon used in the act could not be recovered. Furthermore, there was no direct evidence linking the accused to the act of slaughter—witnesses had only seen the suspect attempting to bury the carcass, not committing the act itself. As per Section 131 of the Muluki Faujdari Karyabidhi Sanhita, 2074, the court concluded that the burden of proof had not been met beyond reasonable doubt. Consequently, the suspected individual was acquitted and released without charges. Despite the legal closure, the question of who actually slaughtered the bull remains unanswered.

Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

Short Communications

Antibiogram of *S. aureus* and *E. coli* Isolated from Milk Samples

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ABSTRACT

Milk is a nutrient dense food consumed by all age group. However, its rich nutritional value serves as a favorable environment for pathogenic microbial growth, particularly Staphylococcus aureus and Escherichia coli. This study aimed to isolate and identify S. aureus and E. coli from raw milk samples collected across three districts of Nepal- Saptari, Morang and Sunsari- and to study antibiogram pattern of the isolates. In this study, 20 milk samples were analyzed microbiologically using selective media, biochemical characterization, and disk diffusion antibiotic susceptibility testing. The study revealed a high prevalence of S. aureus (90%) and moderate occurrence of E. coli (35%) in the samples. Antibiogram profiling indicated alarming β -lactam antibiotics resistance to Penicillin-G and Ampicillin. A notable resistance was observed by S. aureus to Cefoxitin suggesting possible methicillin-resistant strains (MRSA). In contrast, Chloramphenicol and Doxycycline hydrochloride showed highest efficacy against both isolates. These results highlight the growing threat of antimicrobial resistance (AMR) in foodborne bacteria and emphasize the necessity of strict hygiene practices in milk production, rational antibiotic usage in livestock, and regular microbial monitoring of dairy products to ensure consumer safety.

Keywords: Raw milk contamination; *Staphylococcus aureus*; *Escherichia coli*; Antibiogram profiling; Antimicrobial Resistance

INTRODUCTION

Milk, a highly nutritious food, is widely consumed by almost all age groups of people. However, its rich nutrient contents serve as an excellent medium for microbial growth. The contaminated milk, when consumed raw or improperly processed, with the presence of pathogenic bacteria can poses high public health concerns. The most frequently encountered pathogen in milk are *Escherichia coli* and staphylococcus species, particularly *Staphylococcus aureus*. These bacteria in milk can occur through within or outside udder, and from surface of equipment used for milk handling or storage (Thaker, 2013).

E. coli and *S. aureus* has been a major cause of various human and animal infections. Infection of skin and soft tissue, surgical site infection, bone and joint infections,

urinary tract infection (UTIs) in human, neonatal meningitis, enteric infection and systematic infection are the leading complication caused by *E. coli*. Likewise, bacteremia, folliculitis, carbuncle, cellulitis, arthritis, osteomyelitis, lung abscess, pericarditis, and scaled skin syndrome are some of the major difficulties caused by staphylococcus, particularly *S. aureus* (Bachir, 2015). Despite the effectiveness of cooking in inactivating most microorganisms, many secondary metabolites of staphylococcus species, such as toxins, may remain stable and biologically active after the prolonged heating resulting retainment of their pathogenic potential post-processing (Sharma, 2015).

The wide use of antimicrobial agents has played an important role in controlling various infection caused by *E. coli* and *S. aureus*. However, the emergence of antimicrobial-resistant strains has become a significant global health concern. Many resistant pathogens have been reported in both hospital and community including food, water, and livestock environments (WHO, 2023). The emergence of alarmingly antimicrobial-resistant strains signals a potential crisis in antimicrobial efficacy. Therefore, proper bacterial isolation and antibiotic sensitivity studies are crucial for effective antibiotic therapy. This helps to identify the exact bacteria causing an infection and determine the most effective antibiotics against these isolates. This ensures targeted treatment, reduce the risk of resistance development and improve patient outcomes by avoiding ineffective or unnecessary antibiotic use. (WHO, 2024)

The main aim of the study is to identify potential *S. aureus* and *E. coli* in milk sample and to determine the antimicrobial drug sensitivity pattern of various antibiotics against those isolates. This study helps to determine the microbiological quality of milk, understand the current resistance pattern of the pathogens and make proper strategies to mitigate antimicrobial resistance in food.

MATERIALS AND METHODS

Sample collection

Milk samples were collected from cattle in three districts of Nepal: Saptari, Morang and Sunsari. A total of 20 milk samples were obtained in tubes containing 0.1% sodium azide as preservative to maintain sample integrity. After the collection, the samples were placed in an ice box and transported to the laboratory at the National Animal Health Research Center (NAHRC), Lalitpur.

Isolation and identification of *Staphylococcus* and *E. coli*

The 20 different milk samples were first diluted in sterile buffer peptone water (BPW) enrichment broth. Prior to isolation, a 10 ml of homogenized milk sample was first enriched with 90ml of sterile buffer peptone water and incubated at 37°C for 24 h to promote growth. (ISO 6887-1, 2023) For the specific isolation of staphylococcus, Mannitol salt agar was used as a selective media. A loopful of inoculums from enriched broths were streaked on MSA and incubated for 48 hours at 37°C. The characteristics appearance of

yellow or golden colonies surrounded by yellow halo was considered as indication of *Staphylococcus aureus* (Bannerman, 2014).

Similarly, for the isolation of *E. coli*, a loopful of inoculums were streaked onto Eosin methylene blue (EMB) agar and incubated at 37°C for 24 hours. The appearance of green metallic sheen on EMB agar was the indication of *E. coli* strains. The isolated staphylococcus and *E. coli* was further characterized by biochemical test after the incubation for 24 h at 37°C.

Biochemical examination

The biochemical test to characterize the *S. aureus* and *E. coli* involved gram staining, SIM (sulfur, indole, motility) test, citrate utilization test and urease test.

Antibiogram pattern of the isolates to some antimicrobial agents

The antimicrobial drug sensitivity test of obtained isolates was determined by Kirby-Bauer disc diffusion method using the disk procured from HI-media laboratories (Sharma, 2022) (Fig. 3). The antimicrobial agents used involves: Ampicillin (10µg), Amikacin (30µg), Cefoxitin (30 µg), Chloramphenicol (30µg), Ciprofloxacin (5µg), Co-Trimoxazole (5µg), Doxycycline hydrochloride (30µg), Erythromycin (15µg), Gentamicin (10µg) and Penicillin-G (10µg). The diameter of the zone of Inhibition was measured in millimeters (mm) and compared with the standard zone of inhibition provided by the company's guidelines.

RESULTS

The analysis of the result revealed that out of 20 milk sample, 18 (90%) were found to be infected with *S. aureus* and 7 (35%) for *E. coli*. *E. coli* exhibited green metallic sheen on Eosin Methylene Blue (agar) and appeared as gram negative short rods (Fig. 1 and Table 1). *E. coli* tested positive for catalase, indole, and motility, but negative for hydrogen sulfide production, citrate utilization, and urease (Table 2).

S. aureus formed yellow colonies with halos on Mannitol Salt agar (MSA) and appeared as Gram-positive cocci (Fig. 2). The biochemical test resulted positive for catalase and urease, but negative for indole, motility, and hydrogen sulfide production (Table 3).

Table 1. Morphological and culture characteristics

Bacteria	Specific media	Culture characteristics	Gram staining
<i>E. coli</i>	Eosin methylene blue (EMB) Agar	Green metallic sheen	Gram negative short rods
<i>S. aureus</i>	Mannitol Salt agar (MSA)	Yellow colony with yellow halos	Gram positive cocci



Fig. 1. Isolation of *E. coli* and gram staining

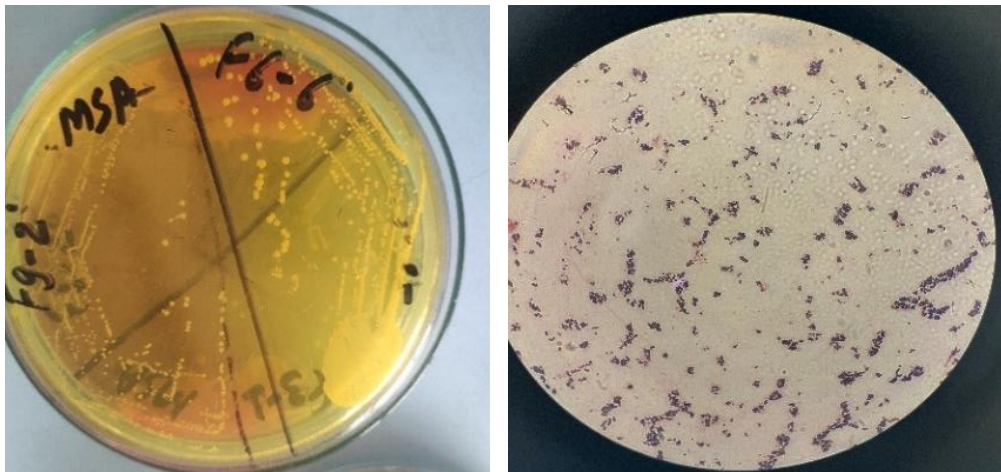


Fig. 2. Isolation of *S. aureus* and gram staining

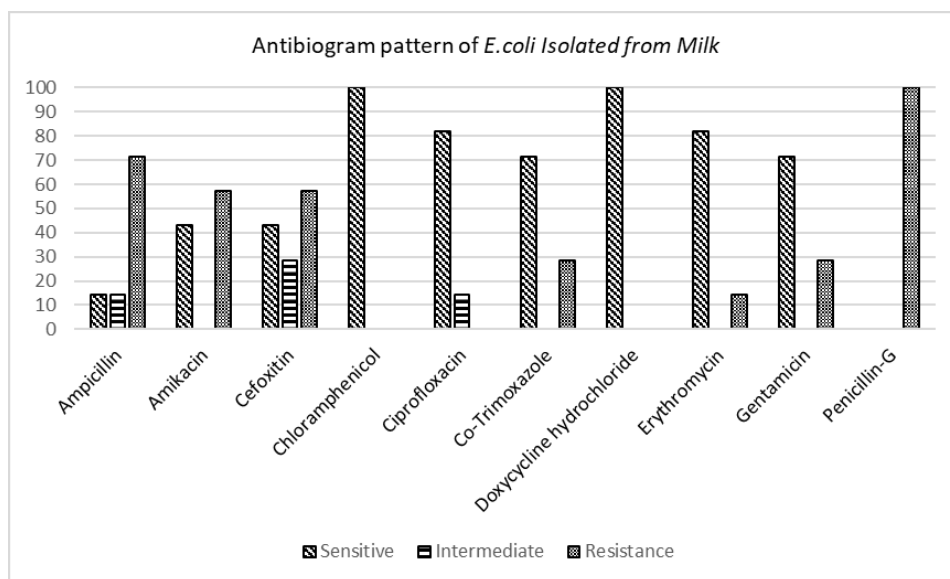
Table 2. Biochemical characterization of *E. coli*

Biochemical test	Reaction
Catalase	Positive
Indole	Positive
Motility	Motile
Hydrogen sulfide production	Negative
Citrate utilization	Negative
Urease	Negative

Table 3. Biochemical characterization of *S. aureus*

Biochemical Test	Reaction
Catalase	Positive
Indole	Negative
Motility	Negative
Hydrogen sulfide gas production	Negative
Urease	Positive

Fig. 3. Antibiotic sensitivity test using Kirby-Bauer Method

Fig. 4. Antibiogram of *E. coli* isolates

The antibiogram pattern analysis of the 7 *E. coli* revealed varied pattern of sensitivity, intermediate resistance and resistance to the tested antibiotics. All *E. coli* isolates were sensitive to Chloramphenicol (100%) and Doxycycline hydrochloride (100%) with most sensitive to Ciprofloxacin (81.71%) and Erythromycin (81.71%). The *E. coli* exhibited reduced susceptibility to Amikacin (42.86%), Cefoxitin (42.86%), Co-trimoxazole (71.43%), and Gentamicin (71.43%). The highest antibiotics resistance was marked to Penicillin-G (100%) and Ampicillin (71.43%), with intermediate resistance noted for Cefoxitin (28.57%), Ciprofloxacin (14.29%), Co-Trimoxazole (14.29%), and Ampicillin (14.29%). Additionally, the resistance was also observed for Co-Trimoxazole (28.57%), Gentamicin (28.57%), and Erythromycin (14.29%) (Fig. 4).

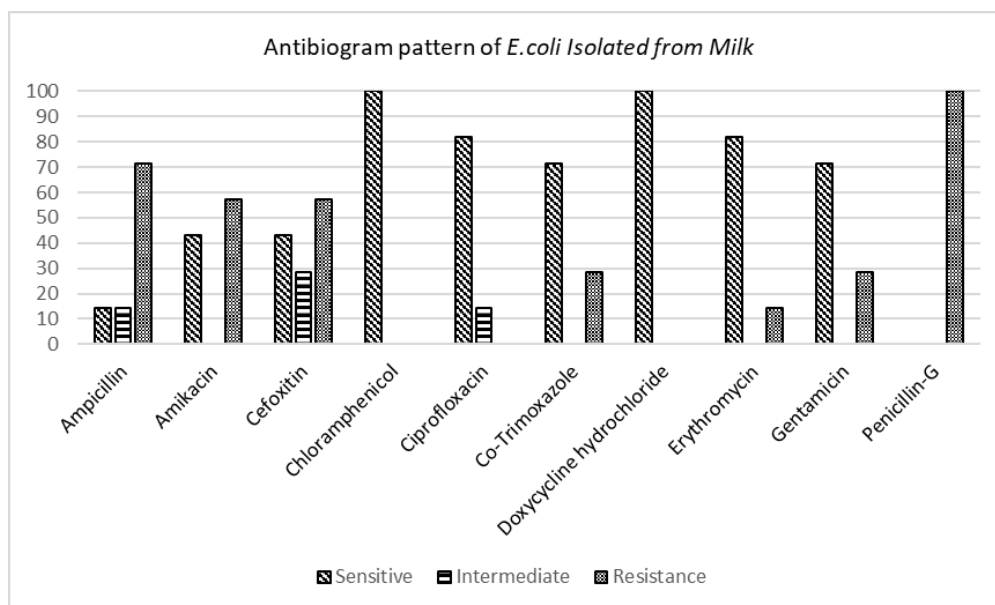


Fig. 5. Antibiogram of *S. aureus* isolates

For the 18 *Staphylococcus aureus* isolates, Amikacin (100%) and Chloramphenicol (100%) demonstrated full efficacy, with most sensitive to Erythromycin (94.44%), Ciprofloxacin (88.89%), Co-trimoxazole (88.89%), and Doxycycline hydrochloride (88.89%). A decreased sensitivity was observed to Cefoxitin (33.33%), Ampicillin (16.67%), and Gentamicin (16.67%). The highest resistance of the *Staphylococcus* was observed to Penicillin-G (100%), followed by ampicillin (83.33%), and Cefoxitin (66.67%). Doxycycline hydrochloride (61.11%) showed notable intermediate resistivity, with lower rates for Ampicillin (11.11%), Co-Trimoxazole (5.56%), and Ciprofloxacin (5.56%) (Fig. 5).

DISCUSSION

The high prevalence of *Staphylococcus aureus* (90%) and *Escherichia coli* (35%) in raw milk samples collected from Sunsari, Saptari and Morang districts of Nepal indicates the microbial vulnerability of milk. These findings align with most of the global trends and pinpoint an emerging public health concern as well as the local adaptation of antimicrobial resistant strains within milk.

The high incidence of *S. aureus* could be attributed to poor hygiene, subclinical mastitis in dairy animals, or contamination from the environment and milk handlers (Basanisi, 2015). Meanwhile, the presence of *E. coli* may be due to fecal contamination from the environment or during and after milking. Many consumers assume that boiling the raw milk can kill all the bacteria, thereby making it safe for consumption. Although the thermal processing is widely used for the elimination of pathogens, some bacterial strains such as *S. aureus* can produce thermal resistant toxins. This can ultimately harm the consumers

as the milk can still be contaminated despite the complete eradication of the pathogen (Kadariya, 2014).

A key finding of this study was the resistance of both *E. coli* and *S. aureus* isolates to Penicillin-G and Ampicillin. The finding aligns with the other studies that the bacteria are resistant to β -lactam antibiotics as these bacteria can produce enzyme called β -lactamases that are capable to destroys β -lactam antibiotics making it resistant. But the concerning finding was that all *S. aureus* and many *E. coli* were resistant to β -lactam antibiotics which might be due to horizontal gene transfer between bacteria in the milk ((Marshall, 2013).

The major reason for the failure of bacterial infection treatment may be due to the indiscriminate use of antibiotics without proper evaluation of the sensitivity of the causative organisms. Such issue has been prevalent in the livestock industry, particularly among milking animals and cattle, where antibiotics are often administered without proper diagnostic guidance. This can lead to increased economic losses and health risk for consumers and invite challenges for microbiologists in combating antimicrobial resistance (Van Boeckel, 2015).

Furthermore, several *S. aureus* isolates were resistant to Cefoxitin suggesting the possible presence of methicillin-resistant *Staphylococcus aureus* (MRSA). The notable susceptibility to chloramphenicol and doxycycline hydrochloride may be due to limited use as a first line of treatment for livestock or human infection.

This study highlights the necessity of routine microbiological monitoring along with antibiogram profiling for milk safety assessment rather than relying on traditional physicochemical parameters such as fat content and solid-not-fat (SNF) levels. Since this study is constrained by small sample size and the absence of molecular analyses, future studies can be aimed at performing genotypic characterization of resistance determinants (example: *mecA*, *blaTEM*, *tetA*) and tracking antimicrobial resistance (AMR) in milk samples over different seasons.

CONCLUSION

In conclusion, the study highlights the presence of *Staphylococcus aureus* and *Escherichia coli* in raw milk samples collected from different districts of Nepal. *E. coli* and *S. aureus* have been major causes of various infections in both humans and animals. The risk of contamination can be minimized by maintaining better hygiene during milking and milk handling. The antibiogram analysis further revealed varied resistance among the isolates to commonly used antibiotics, raising serious concerns about antimicrobial resistance (AMR) in foodborne pathogens. The identification of drug-resistant strains in milk samples emphasizes the urgent need for routine microbiological monitoring of dairy products and rational use of antibiotics in both veterinary and human medicine.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. World Health Organization. (2023, November 21). *Antimicrobial resistance*.
2. Bachir, R., & Abouni, B. (2015). *Escherichia coli and Staphylococcus aureus: Most common source of infection*. In A. Méndez-Vilas (Ed.), *The Battle Against Microbial Pathogens: Basic Science, Technological Advances and Educational Programs* (pp. 11–19). Formatex Research Center.
3. Bannerman, T. L. (2003). *Staphylococcus, Micrococcus, and other catalase-positive cocci that grow aerobically*. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, & R. H. Tenover (Eds.), *Manual of Clinical Microbiology* (8th ed., pp. 384–404). American Society for Microbiology.
4. International Organization for Standardization. (2017). *ISO 6887-1:2017: Microbiology of the food chain—Preparation of test samples, initial suspension and decimal dilutions for microbiological examination—Part 1: General rules for the preparation of the initial suspension and decimal dilutions*.
5. Kadariya, J., Smith, T. C., & Thapaliya, D. (2014). *Staphylococcus aureus and staphylococcal food-borne disease: An ongoing challenge in public health*. *BioMed Research International*, 2014, Article 827965.
6. Basanisi, M. G., La Bella, G., Nobili, G., Franconieri, I., & La Salandra, G. (2015). Molecular characterization of *Staphylococcus aureus* isolated from sheep and goat cheeses in southern Italy. *Small Ruminant Research*, 126, 68–72.
7. Marshall, B. M., & Levy, S. B. (2011). Food animals and antimicrobials: Impacts on human health. *Clinical Microbiology Reviews*, 24(4), 718–733.
8. Sharma, S., & Khajuria, A. (2015). Prevalence, identification and drug resistance pattern of *Staphylococcus aureus* and *Escherichia coli* isolated from raw milk samples of Jaipur city of Rajasthan. *Journal of Pure and Applied Microbiology*, 9(1), 611–617.
9. Sharma, R. (2022, May 18). *Kirby-Bauer disc diffusion*. Microbe Notes.
10. Thaker, B. N., Brahmabhatt, M. N., & Nayak, J. B. (2013). Isolation and identification of *Staphylococcus aureus* from milk and milk products and their drug resistance patterns in Anand, Gujarat. *Veterinary World*, 6(1), 10–13.
11. Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., ... & Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences*, 112(18), 5649–5654.

Prevalence of Brucellosis in Dairy Cattle and Buffalo in the Western Chitwan (Nepal) using Milk i-ELISA

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ABSTRACT

Brucellosis is a significant zoonotic disease affecting livestock worldwide. This study aimed to determine the prevalence of brucellosis in raw milk from cattle and buffalo in the western Chitwan of Nepal using indirect ELISA. Additionally, the study sought to calculate the prevalence of brucellosis based on factors such as age, breed, and parity, as well as identify associated risk factors. The cross-sectional study was conducted in four areas of western Chitwan, namely Saradhanagar, Bijayanagar, Rampur, and Mangalpur, within the Bharatpur municipality. A semi-structured questionnaire survey was conducted alongside milk sample collection. A total of 92 milk samples were collected, consisting of 28 buffalo and 64 cattle milk samples. The individual milk samples were stored at -20°C until testing. The milk samples underwent analysis via Milk i-ELISA (IDVet, France). This kit was utilized to detect Brucella antibodies in cattle and buffalo milk samples. The milk samples were directly tested without dilution after the elimination of the fat layer post-centrifugation. The results indicated that none of the samples tested positive for brucellosis, resulting in an overall prevalence rate of 0%. In conclusion, this study provides important insights into the brucellosis status in cattle and buffalo milk in western Chitwan, Nepal, emphasizing the absence of the disease within the examined population during the specified period.

Keywords: Brucellosis; i-ELISA; Milk; Prevalence; Dairy cattle and buffalo

INTRODUCTION

Brucellosis, a globally distributed zoonotic disease, poses significant economic, veterinary, and public health challenges, particularly in developing countries, threatening both livestock and human populations (Abdoel *et al.*, 2008). The main *Brucella* species of veterinary importance are *Brucella abortus*, *B. melitensis*, and *B. suis* (Rujeni and Mbanzamihigo, 2014), affecting bovines, sheep and goats, and pigs, respectively. This disease causes substantial economic losses due to various complications, such as delayed calving, infertility, decreased calving percentage, reduced milk production, abortions, stillbirths, and the birth of weak offspring (Adamu *et al.*, 2016). Brucellosis can also

infect humans, resulting in multisystemic disease with symptoms like intermittent fever, headache, weakness, sweating, chills, weight loss, and general discomfort (Ulu Kilic *et al.*, 2016). Transmission of *Brucella* occurs through contact with fetal fluids, placenta, fetus, and vaginal discharges of infected animals, entering the body through ingestion, broken skin, mucous membranes, and possibly intact skin. Among the different species, *B. melitensis* is particularly invasive and pathogenic to humans, causing Malta fever or undulant fever (Berhanu and Pal, 2020).

The Chitwan district of Nepal is significant in the dairy industry, with a large population of cattle (102997) and buffalo (96969) (Raut *et al.*, 2020; DLS, 2021). Consumption of unpasteurized milk and milk products from infected animals poses a risk to humans, including veterinarians, meatpacking employees, and farmers. Livestock health plays a crucial role in their performance and the overall economy. Studies in Nepal have focused on serological detection of antibodies in serum (Pyakurel and Mishra, 1977; Joshi, 1983), while recent advancements have led to the successful application of indirect enzyme-linked immunosorbent assays (i-ELISA) on milk samples. Limited research has been conducted to assess the prevalence of Brucellosis in Chitwan district, leaving a knowledge gap in understanding the true situation. This study aims to assess the epidemiology of brucellosis in cattle and buffalo in western Chitwan, a prominent milk production area, using milk i-ELISA.

MATERIALS AND METHODS

Location of the study

This cross-sectional study was carried out in four areas of western Chitwan named Saradhanagar, Bijayanagar, Rampur and Mangalpur under Bharatpur municipality considering the time and financial constraint. The milk sample collection was done from 5th February 2020 to 5th March 2020. During the sample collection procedure, a face-to-face semi-structured questionnaire survey was also performed (Fig. 1).

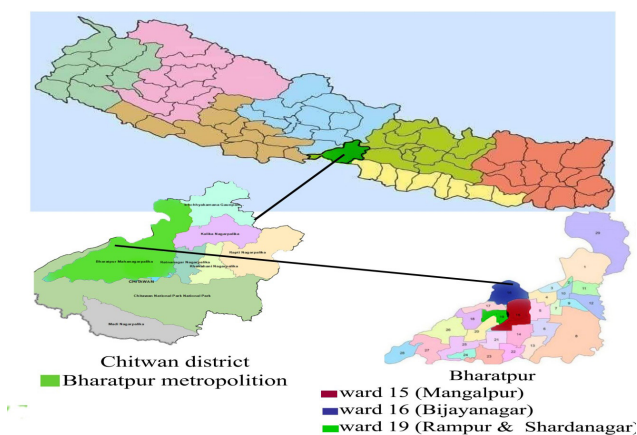


Fig 1. Political map of Chitwan district showing study sites

Sampling frame

A total of 92 milk samples (the number that fits in one ELISA kit) were taken. 5 ml individual milk samples were collected directly from the farmers (64 cattle and 28 buffalo and milk samples) and stored in refrigeration at -20°C until the day of testing. The sample size was determined using formula given by Daniel 1999 as cited in (Naing *et al.*, 2006).

i-ELISA assay

The samples collected were subjected to milk based IDvet, ELISA (IDvet, France). Milk samples were centrifuged at 5000 rpm for 5 minutes and then tested undiluted after removing the cream layer. The iELISA test involves coating antigens on a plate to capture specific antibodies from samples. Secondary antibodies linked to enzymes such as peroxidase or alkaline phosphatase are used for detection. After incubation and removal of unbound antibodies, a substrate triggers an enzymatic reaction, resulting in a measurable color change. Blue indicates the presence of antibodies, which becomes yellow with a stop solution. No color change occurs in the absence of antibodies.

RESULTS

Herd characteristics and reproductive problems

The percentage of male and female responding to the questionnaire survey was 51% (46) and 49% (44) respectively. The predominant breed among cattle in the surveyed area was Jersey, constituting 73.44% of the population, while the local breed comprised 15.63%, and mixed breeds made up 10.94% (Table 1). In terms of parity, the majority of cattle belonged to the second (34.37%) and third (37.5%) parity categories. The age distribution revealed that the highest proportion of cattle fell within the 3-5 years age group, comprising 51.56% of the population. Among buffaloes, the Murrah cross breed was dominant at 57.14%, followed by local breeds at 32.14%, with mixed breeds constituting 10.72%. Most buffaloes were from the second parity (35.37%), and none were less than three years old. The age group with the highest representation among buffaloes was 3-5 years.

Table 1. Classification based on herd characteristics

Species	Breed	Parity	Age group
Cattle (64) =69.57%	Local= 15.63%	First= 12.5%	Up to 3 years= 3.12%
	Jersey= 73.44%	Second= 34.37%	3-5 years= 51.56%
	Mixed= 10.94%	Third= 37.5%	Above 5 years=
		Fourth= 15.63%	45.31%
Buffaloes (28) =30.42%	Local= 32.14%	First= 14.28%	Up to 3 years= 0%
	Murrah cross= 57.14%	Second= 35.37%	3-5 years= 53.57%
	Mixed= 10.72	Third= 25%	Above 5 years =
		Fourth= 25%	46.42%

Indeed, the incidence of reproductive diseases was notably higher in cattle compared to buffalo (Table 2). Cattle exhibited higher rates of abortion, retained placenta, repeat breeding, and other reproductive issues compared to buffalo.

Table 2. Comparison of reproductive problems between cattle and buffalo herds

Species	Abortion	Retained placenta	Repeat breeding	Others*
Cattle (64)	5 (7.81%)	5 (7.81%)	28 (43.75%)	12 (18.75%)
Buffalo (28)	2 (7.14%)	1 (3.57%)	7 (25%)	4 (14.28%)

i-ELISA Result

None of the samples were found positive for brucellosis, indicating the overall prevalence rate to be 0%.

DISCUSSION

The analysis of milk samples from 92 randomly chosen animals using I-ELISA indicated no prevalence of brucellosis. Contrary to previous studies conducted by Khanal et al. (1997/98), Joshi et al. (2005), Poudel (2006), and Pyakurel and Mishra (1997) in various time frames, which reported the presence of *Brucella* in Chitwan and other regions of Nepal. Similar to the results from this study, the Central Veterinary Laboratory (CVL) reported no prevalence of brucellosis antibodies when tested using the ELISA method from a total of 644 serum samples from cattle (608) and buffalo (36) across various districts including Mahotari, Dhading, Makawanpur, Sindhupalchok, and Dolakha (CVL, 2012). The null prevalence in our study can be due to several factors such as the level of hygiene on the farm, age of the animals, herd size, sex, system of production, multiple livestock species within the herd, variability in diagnostic procedures, and geographical reasons. In this study, the maximum number of animals in a household was found to be six. Makita et al (2011) revealed that disease transmission is four times lower in animals with smaller herd sizes. Nearly all farmers in the study areas practiced intensive rearing methods, restricting animal movement and adhering to strict hygiene and biosecurity practices, which likely contributed to reduced disease transmission. In addition, Anka et al. (2014) found that cattle in intensive systems are 13 times less exposed to brucella compared to those in extensive systems. Moreover, the presence of an Agriculture and Forestry University in the area may have played a role in the absence of disease prevalence, as it provides various health services, trainings, workshops, and extension education to livestock farmers.

Various bacteriological, serological, and molecular methods tests are employed for diagnosis of brucellosis in dairy animals. While the isolation of organisms by culture remains the gold standard for diagnosing brucellosis, it presents challenges due to its difficulty, hazard, time consumption, limited sensitivity in chronic cases, and the necessity for highly skilled personnel and specialized biosafety cabinets (K et al., 2019). Diagnosing bovine brucellosis primarily relies on serological methods utilizing serum or milk samples. Specifically, milk reflects the IgG-based antibody response of the animal and serves as a non-invasive sampling method. Its utilization in serological detection,

instead of blood, offers a significant advantage, particularly in lactating animals. While i-ELISA offers high sensitivity, its inability to differentiate between *B. abortus* S19 vaccine antibodies and cross-reacting antibodies can slightly lower specificity in individual or bulk milk samples, leading to occasional conflicting results (Mackinnon, 1976). To avoid false negatives, other serological tests such as Complement Fixing Test (CFT), Competitive Enzyme Linked Immune Sorbet Assay (cELISA), Polymerase Chain Reaction (PCR), and isolation and culture of the organism can be employed (Viswanathan et al., 2019; Blasco et al., 1994).

CONCLUSION

The study findings indicated the absence of disease prevalence in the area, emphasizing the critical need for swift and precise assessment of brucellosis status in milk and its derivatives for public health. While IELISA offers rapid evaluation, its specificity may be reduced in individual or bulk milk. Therefore, more reliable serological tests such as CFT, cELISA, PCR, and isolation and culture of the organism should be routinely conducted from the government level.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Abdoel, T., Dias, I. T., Cardoso, R., & Smits, H. L. (2008). Simple and rapid field tests for brucellosis in livestock. *Veterinary Microbiology*, 130(3-4), 312–319. <https://doi.org/10.1016/j.vetmic.2008.01.011>
2. Adamu, S. G., Atsanda, N. N., Tijjani, A. O., Usur, A. M., Sule, A. G., & Gulani, I. A. (2016). Epidemiological study of bovine brucellosis in three senatorial zones of Bauchi State, Nigeria. *Veterinary World*, 9(1), 48–53. <https://doi.org/10.14202/vetworld.2016.48-53>
3. Anka, M. S., Hassan, L., Khairani-Bejo, S., Zainal, M. A., Mohamad, R. B., Salleh, A., & Adzhar, A. (2014). A case-control study of risk factors for bovine brucellosis seropositivity in Peninsular Malaysia. *PLOS ONE*, 9(9), e108673. <https://doi.org/10.1371/journal.pone.0108673>
4. Berhanu, G., & Pal, M. (2020). Brucellosis: A highly infectious zoonosis of public health and economic importance. *Journal of Emerging Environmental Technologies and Health Protection*, 3, 5–9.
5. Blasco, J. M., Marín, C., Jiménez de Bagüés, M., Barberán, M., Hernández, A., Molina, L., Velasco, J., Díaz, R., & Moriyón, I. (1994). Evaluation of allergic and serological tests for diagnosing *Brucella melitensis* infection in sheep. *Journal of Clinical Microbiology*, 32(8), 1835–1840. <https://doi.org/10.1128/jcm.32.8.1835-1840.1994>
6. Department of Livestock Services. (2021). *Livestock statistics of Nepal*. <https://www.dls.gov.np/>

downloadfiles/Livestock_Statistics_of_Nepal_2077_78_1659524236-1669717523.pdf

7. Joshi, D. D. (1983). A public health problem in Nepal. *Bulletin of Veterinary Science and Animal Husbandry Nepal*, 12, 15–16.
8. Joshi, D. D., Upadhyay, M., & Mishra, P. N. (2005). Brucellosis in animal and human of Chitwan. *NZFHRC Bulletin*, 37–48.
9. K, A. M., Abdel Ghani, E. A. E., Modawe, G., & Abuzeid, N. (2019). Seroprevalence of brucellosis associated with abortion among Sudanese pregnant women attending Omdurman Maternity Hospital, Sudan. *Gezira Journal of Health Sciences*, 15, 26–38.
10. Khanal, D. R., & Jha, V. C. (1997/1998). Study on bovine abortion and repeat breeding due to brucellosis in Chitwan and Eastern Terai. *Annual Report 1997/98*, NARC, AHRD, Tripureswor, 12–17.
11. Mackinnon, D. J. (1976). Serological diagnosis of brucellosis in cattle. *Scottish Medical Journal*, 21(3), 134–135. <https://doi.org/10.1177/003693307602100306>
12. Makita, K., Fèvre, E. M., Waiswa, C., Eisler, M. C., Thrusfield, M., & Welburn, S. C. (2011). Herd prevalence of bovine brucellosis and analysis of risk factors in cattle in urban and peri-urban areas of the Kampala economic zone, Uganda. *BMC Veterinary Research*, 7, Article 60. <https://doi.org/10.1186/1746-6148-7-60>
13. Naing, L., Winn, T. B. N. R., & Rusli, B. N. (2006). Practical issues in calculating the sample size for prevalence studies. *Archives of Orofacial Sciences*, 1, 9–14.
14. Poudel, A. (2006). *Seroprevalence of brucellosis in small ruminants in Nepal* (B.V.Sc. & A.H. Internship report). Institute of Agriculture and Animal Science, Tribhuvan University, Nepal.
15. Pyakurel, S., & Mishra, U. (1977). Sero-epidemiological evidence of animal brucellosis in Nepal. *Bulletin of Veterinary Science and Animal Husbandry Nepal*, 6, 6–10.
16. Raut, R., Devkota, B. N., Gautam, G., & Shrestha, S. (2020). Assessment of management and reproductive variability among the buffalo farms in Chitwan based on the scores of production efficiency. *Nepalese Veterinary Journal*, 37, 62–70. <https://doi.org/10.3126/nvj.v37i0.41282>
17. Rujeni, N., & Mbanzamihigo, L. (2014). Prevalence of brucellosis among women presenting with abortion/stillbirth in Huye, Rwanda. *Journal of Tropical Medicine*, 2014, Article ID 740479. <https://doi.org/10.1155/2014/740479>
18. The Central Veterinary Laboratory. (2012). *Prevalence of brucellosis in Nepal*. Veterinary Epidemiology Center, Directorate of Animal Health, Tripureswor, Nepal, 120–122.
19. Ulu Kilic, A., Metan, G., & Alp, E. (2013). Clinical presentations and diagnosis of brucellosis. *Recent Patents on Anti-Infective Drug Discovery*, 8(1), 34–41. <https://doi.org/10.2174/1574891x11308010007>
20. Viswanathan, N., Mangalanathan, V., & Kannan, P. (2019). Comparative evaluation of various diagnostic tests in diagnosis of bovine brucellosis. *International Journal of Livestock Research*, 9(1), 1–8. <https://doi.org/10.5455/ijlr.20180803080500>

Seroprevalence and Pathological Study of Porcine Cysticercosis

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ABSTRACT

*Porcine cysticercosis (PC) is a significant global health issue affecting pigs. This study assessed the seroprevalence and pathological features of porcine cysticercosis in the Kaski District, Gandaki Province, Nepal. The study involved 1200 pigs raised on 45 different farms under intensive, semi-intensive, and extensive farming systems. The serum ELISA test was done on 262 randomly selected pigs, and it revealed a 6.87% prevalence of PC in the study population. Further, animals testing positive on serum ELISA were examined for clinical manifestation and morphologic changes. Such animals did not exhibit any specific clinical signs and symptoms except emaciation. Morphologically, oval and rounded nodules were found on the dorsal surface of the tongue in a few animals. The necropsies demonstrated the cysts in various tissues, viz. liver, striated muscles of hind-limbs, and sub-lingual gland. Histologically, the liver showed moderate cholestasis, cellular infiltration, numerous larval migratory tracts in hepatic channels, and fibrosis in the periportal area. Accumulation of epithelioid cells in the glandular portion of ducts in sublingual glands was also observed. Based on these clinical, morphological, and histological findings, it was concluded that the PC caused by *Taenia solium* and *Taenia hydatigena* was prevalent in the swine farms of the Kaski district. Nevertheless, only a few animals showed gross and microscopic changes in seropositive animals. Therefore, the serum ELISA test and gross and microscopic pathology techniques should be used simultaneously to confirm PC.*

Keywords: Porcine Cysticercosis; Seroprevalence; ELISA; Histology

INTRODUCTION

Metacestodes of tapeworms of the genus *Taenia* cause porcine cysticercosis (PC). The zoonotic species of this parasite are *Taenia solium*, *Taenia asiatica*, and non-zoonotic *Taenia. hydatigena* (WHO, 2015a). Pigs act as the principal intermediate hosts of *T. solium* and *T. asiatica* and *T. hydatigena* (Assana et al., 2019). Pigs become infected after ingesting *T. solium* or *T. asiatica* eggs from food or an environment contaminated with faeces of human tapeworm carriers (Braae et al., 2018), whereas dog carrier faeces in *T. hydatigena* (Dermauw et al., 2016). The infection caused by *T. hydatigena* leads to clinical signs in pigs (Dorny et al., 2004). *T. hydatigena* in pigs occurs variably worldwide. Earlier, in China, the prevalence rate was reported as high as 25.7%), whereas in Laos, it was 22.4% (Conlan et al., 2012). The larval stage of the parasite infects a wide range of intermediate hosts; however, domestic hosts most often include goats, sheep, and pigs. Globally,

around 2.56-8.30 million people are symptomatic or asymptomatic of neuro-cysticercosis (WHO, 2015a). 28000 human deaths were recorded across the globe in 2010 (WHO, 2015b). Although *T. solium* is found globally, it has a more extraordinary transmission and hyper-endemicity in the rural areas of Latin America, South and South-East Asia, and Sub-Saharan Africa (WHO, 2015a). In the past, in China, around 20,00,00,000 kg of pork was discarded due to the presence of cysticercosis (Ito et al., 2003).

Nepal has also been considered endemic to zoonotic tapeworms (WHO, 2015). The prevalence of zoonotic tapeworms ranges from 0.002- to 0.1% in the general human population in Nepal (Develeesschuwar et al., 2013). A small-scale study based on the necropsy and gross pathology techniques in the Banke district of the Western region of Nepal found 29% porcine cysticercosis (Subedi et al, 2020). Similar findings on porcine cysticercosis were also reported from other parts of the country, such as 32.5% in Syanja and 28% in Kathmandu (Develeesschuwar et al., 2013).

Recent research studies in animals and humans indicate that cysticercosis has become a burden of disease in Nepal. About 73% of pigs reared in the scavenging/free-range system are likely to be fed on human faeces, kitchen waste, brewer's residues, distillers' residues, and garbage (Poudel et al., 2019). Similarly, unhealthy rearing practices include being allowed to graze in an open area, lack of latrines, poor hygiene habits, and lack of meat inspection. Lack and allow those who lack knowledge of hygienic practices and biosecurity to prevent transmission of diseases between humans and animals and vice versa (Ngowi et al., 2014). Although the disease is considered endemic in Nepal, the studies conducted in the past to assess the various dimensions of the disease were minimal and sketchy. Therefore, this research aims to uncover the PC using standard epidemiologic, laboratory, gross and microscopic pathology tools in the swine farms of the Kaski district.

MATERIALS AND METHODS

The study was conducted in various swine farming areas, specifically in the Hemja, Ramghat, and Lekhnath regions of the Kaski district within Gandaki Province. To ensure a representative sample, a simple random sampling technique was employed, utilising the random sample size formula. The calculated sample size for this research was determined to be 262 from the overall population of 1200 pigs in the selected study areas.

Blood samples were collected from the selected pigs through either the ear vein or jugular vein. The chosen animal population underwent weekly monitoring to observe any physical and physiological changes. All 262 sampled animals underwent serological testing for Porcine Cysticercosis (PC) antigens in the serum, utilising a Sandwich ELISA test kit from BIO X Diagnostics in Belgium.

Animals that tested positive in the serologic test underwent further observation and assessment against clinical manifestations, necropsy, and histopathology. This comprehensive approach allowed for a thorough examination of PC prevalence, providing

insights into the serological, clinical, and pathological aspects of the infection in the swine population in the designated study areas.

RESULTS

Seroprevalence of cysticercosis

The serological test carried out on 262 animals from different geographical locations revealed an overall 6.87 % prevalence rate of PC in the study population. The Lekhnath area had the lowest prevalence rate (1.25%), whereas the highest was in the Ramghat area (14.63%) (Table 1).

Table 1. Seroprevalence of porcine cysticercosis in various locations of the Kaski District

S.N.	Farm Area	Herd size	Seropositive	Prevalence
1	Hemja	54	3	5.56
2	Ramghat	41	6	14.63
3	Lekhnath	80	1	1.25
4	Baghmara	87	8	9.21
	Total	262	18	6.87

The prevalence rates categorised by farming type, age group, and farm setting indicated that the highest incidence of was observed in extensive farming types and rural settings, accounting for 60% of cases. Following closely, the age group of less than one year old exhibited a prevalence rate of 50%. In contrast, lower prevalence rates were noted in intensive farming, semi-intensive farming, urban settings, and among adult pigs, as outlined in Table 2.

Table 2. Prevalence of cysticercosis in different categories

S.N.	Category	Sub-category	Sample	Positive	Prevalence
1	Farming type	Intensive	25	1	20.00%
		Extensive	8	3	60.00%
		Semi-intensive	12	1	20.00%
		Less than 1 year	114	9	50.00%
2	Age group	1-2 year	81	6	33.30%
		2 years and above	62	3	16.60%
3	Area	Urban	30	2	40.00%
		Rural	15	3	60.00%

Clinical observation

All the seropositive animals were regularly examined for the clinical manifestations of PC. There were no such noticeable clinical observations in the study population except emaciation. However, 1.17% of the seropositive animals exhibited gross changes on the dorsal surface of the tongue.

Gross pathology

Twelve animals out of 18 seropositive animals were necropsied to visualize the morphologic changes in the target tissues. The necropsy examination revealed a cyst in the liver, masseter muscles, and hind limb muscles. The cysts were filled with white translucent fluid; morphologically, the cysts contained single white spots suggestive of scolex, having a size of around 1 cm (Fig. 1, 2 and 3)



Fig. 1. Cyst of *Taenia species* in the biceps femoris muscle of pig



Fig. 2. Cyst of *Taenia species* and single white nodule at the pharyngeal region



Fig. 3. Cyst of *Taenia species* in the right lobe of the pig liver.

Histopathology

The liver tissues containing PC cysts were histologically processed. The liver demonstrated haemorrhage in hepatic sinusoids, thickened capsule and blocked artery (Fig. 4), centrilobular bile obstruction (Fig. 5), periportal fibrosis and bile occlusion (Fig. 6), and migratory tracts between the hepatic cords (Fig. 7).

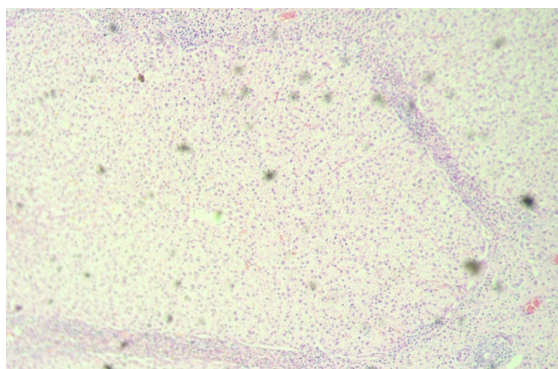


Fig. 4. Liver-haemorrhage, thickened capsule 40x

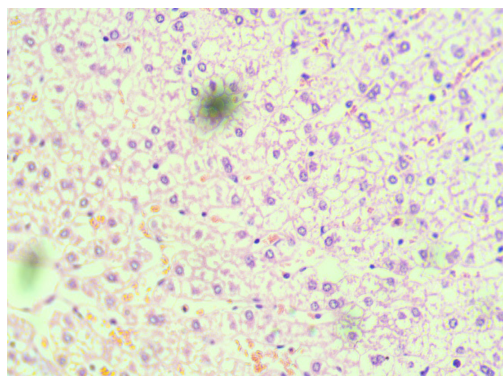


Fig. 5 Liver-centri lobar bile occlusion 40x

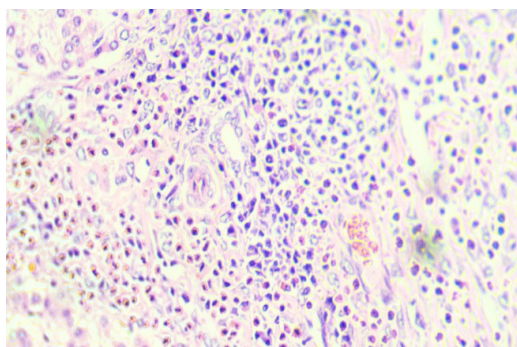


Fig. 6. Periportal fibrosis (short arrow) and bile occlusion, 40x

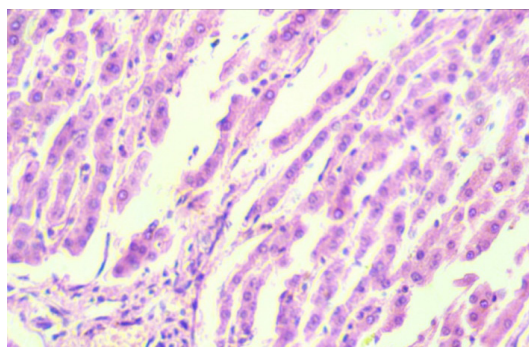


Fig. 7. Migratory tract in the liver, 40x

DISCUSSION

We found the prevalence of PC in the different farming systems and age groups in the study area. The highest prevalence (14.63%) was found in the Ramghat area. The possible reason could be that most of the pig farms in this area adopted extensive farming. In our study, the highest infection rate in the above age group might be because most of the samples were taken from pigs reared for meat purposes. Most farmers never administered deworming medication to the young pigs. In rural and urban husbandry practices, rural areas (60%) had a higher prevalence than urban areas (40%). The result could be attributed to the relatively unhygienic management practice, extensive management systems, and lack of knowledge about deworming medication among the farmers of rural areas. Urban farmers used deworming practices and had access to veterinary facilities. Depending on the features of their cells, lesions were categorised into seven grades (De Aluja & Vargas, 1988). In the destruction process, macrophages and epithelioid cells become highly active (Grades 4 and 5). Due to the migratory cyst, periportal fibrosis, haemorrhage, thickening in the capsule, and a blocked artery occurs in the liver parenchyma

CONCLUSION

Based on the serologic findings, the overall seroprevalence of PC in the study area was found to be 6.87%. Clinically, seropositive animals did not show any specific signs except emaciation. The gross pathology suggested that the cysts of *T. solium* and *T. hydatigena* were present in the sublingual gland and liver tissues. The histologic findings revealed various changes, such as haemorrhage, thickened capsule, blocked artery, periportal fibrosis, and centrilobular bile occlusion in the liver. The histologic findings, especially bile cholestasis in the liver and a migratory tract in and between the hepatic channels, suggested the presence of *T. hydatigena*, which had been misdiagnosed and misinterpreted as *T. solium* infestation or a PC in veterinary practice. Therefore, the study strongly suggests considering the infestation of *T. hydatigena* as well. Based on the findings of this study, *T. solium* and *T. hydatigena* showed a magnificent level of cross-reactivity in serum biochemistry assessment, leading to the misdiagnosis of PC. *T. hydatigena* itself is a non-zoonotic species, although it causes PC. Therefore, we recommend using gross and histopathological techniques to distinguish *T. solium* and *T. hydatigena*. In addition, the documentation on *T. hydatigena* prevalence is vital for further research.

Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Assana, E., Amadou, F., Thys, E., Lightowlers, M. W., Zoli, A. P., Dorny, P., & Geerts, S. (2019). Pig-farming systems and porcine cysticercosis in the north of Cameroon. *Journal of Helminthology*, 84(4). <https://doi.org/10.1017/S0022149X10000167>
2. Braae, U. C., Devleesschauwer, B., Gabriël, S., Dorny, P., Speybroeck, N., Magnussen, P., Torgerson, P., & Johansen, M. V. (2016). CystiSim – An agent-based model for *Taenia solium* transmission and control. *PLOS Neglected Tropical Diseases*, 10(12), e0005184. <https://doi.org/10.1371/journal.pntd.0005184>
3. Conlan, J. V., Vongxay, K., Khamlome, B., Dorny, P., Sripa, B., Elliot, A., Blacksell, S. D., Fenwick, S., & Thompson, R. C. A. (2012). A cross-sectional study of *Taenia solium* in a multiple taeniid-endemic region reveals that competition may be protective. *American Journal of Tropical Medicine and Hygiene*, 87(2), 281–291.
4. De Aluja, A. S., & Vargas, G. (1988). The histopathology of porcine cysticercosis. *Veterinary Parasitology*, 28(1–2), 65–77.
5. Dermauw, V., Ganaba, R., Cissé, A., Ouedraogo, B., Millogo, A., Tarnagda, Z., Van Hul, A., Gabriël, S., Carabin, H., & Dorny, P. (2016). *Taenia hydatigena* in pigs in Burkina Faso: A cross-sectional abattoir study. *Veterinary Parasitology*, 230, 9–13. <https://doi.org/10.1016/j.vetpar.2016.10.022>
6. Devleesschauwer, B., Pruvot, M., Joshi, D. D., De Craeye, S., Jennes, M., Ale, A., Welinski, A., Lama, S., Aryal, A., Victor, B., Duchateau, L., Speybroeck, N., Vercruysse, J., & Dorny, P. (2013). Seroprevalence of zoonotic parasites in pigs slaughtered in the Kathmandu valley of Nepal. *Vector-Borne and Zoonotic Diseases*, 13(12), 822–828. <https://doi.org/10.1089/vbz.2013.1313>
7. Dorny, P., Somers, R., Dang, T. C. T., Nguyen, V. K., & Vercruysse, J. (2004). Cysticercosis in

- Cambodia, Lao PDR and Vietnam. *Southeast Asian Journal of Tropical Medicine and Public Health*, 35(Suppl. 1), 223–226.
8. Ito, A., Nakao, M., & Wandra, T. (2003). Human taeniasis and cysticercosis in Asia. *The Lancet*, 362(9399), 1918–1920. [https://doi.org/10.1016/S0140-6736\(03\)14965-3](https://doi.org/10.1016/S0140-6736(03)14965-3)
9. Ngowi, H. A., Chenyambuga, S., Sambuta, A., Mkupasi, E., & Chibunda, R. (2014). Co-endemicity of cysticercosis and gastrointestinal parasites in rural pigs: A need for integrated control measures for porcine cysticercosis. *Scientific Parasitology*, 15(4), 123–129.
10. Nguyen, M. T. T., Gabriël, S., Abatih, E. N., & Dorny, P. (2016). A systematic review on the global occurrence of *Taenia hydatigena* in pigs and cattle. *Veterinary Parasitology*, 226, 97–103. <https://doi.org/10.1016/j.vetpar.2016.06.034>
11. Poudel, I., Sah, K., Subedi, S., Kumar Singh, D., Kushwaha, P., Colston, A., & Lightowlers, M. W. (2019). Implementation of a practical and effective pilot intervention against transmission of *Taenia solium* by pigs in the Banke district of Nepal. *PLOS Neglected Tropical Diseases*, 13(2), e0006838. <https://doi.org/10.1371/journal.pntd.0006838>
12. Subedi, D., Subedi, S., Gautam, A., Bhandari, S., & Kandel, M. (2020). Cysticercosis in Nepal: A review. *Nepalese Veterinary Journal*, 37, 148–162.
13. Thapa, B. (2017, June 21). Demand for pork meat surges 30pc in Pokhara. *The Kathmandu Post*. <https://kathmandupost.com/money/2017/06/21/demand-for-pork-meat-surges-30pc-in-pokhara>
14. World Health Organization. (2015a). *Investing to overcome the global impact of neglected tropical diseases: Third WHO report on neglected diseases 2015*. <https://apps.who.int/iris/handle/10665/152781>
15. World Health Organization. (2015b). *WHO estimates of the global burden of foodborne diseases: Foodborne disease burden epidemiology reference group 2007–2015*. <https://www.who.int/publications/i/item/9789241565165>

Seroprevalence of Influenza A virus in Pigs of Nepal

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ABSTRACT

Influenza A virus causing respiratory disease in animal and human is a negative sense, single stranded RNA virus belonging to the Orthomyxoviridae family. This virus has a wide host range and pigs are considered as an important intermediate host and possible 'mixing vessels' for genetic reassortment into novel viruses that can cause serious epidemics and pandemics. Pigs are considered to play an important role in the ecology and emergence of influenza viruses. In Nepal, only limited number of studies have been conducted on Influenza A virus. This study was aimed to estimate the seroprevalence of Influenza A virus in pigs of selected districts of Nepal. A total of 330 sera samples were collected from Jhapa, Sunsari, Kaski, Chitwan, Kathmandu, Surkhet, Banke, and Dhankuta during July 2016 to June 2017. The sera samples were tested by commercially available competitive ELISA kit and the result indicated the seroprevalence of 8.18% Influenza A antibody in pigs showing that the pigs are exposed to Influenza A viruses.

Keywords: *Influenza A; Pigs; ELISA; Seroprevalence*

INTRODUCTION

Influenza is a single stranded, helically shaped, RNA virus of the Orthomyxoviridae family consisting of three different influenza virus genera: A, B and C. Among these, Influenza A viruses are the most common which can infect a wide variety of animal species and humans including pigs (CDC, 2021). Swine influenza in pigs is caused by Type A influenza viruses distributed worldwide and became the matter of great interest since the emergence of the pandemic virus (A(H1N1)pdm09) in 2009 (Davidson et al., 2014). Influenza A viruses of swine (IAV-S) infection causes respiratory diseases in pigs characterized by coughing, sneezing, nasal discharge and fever which usually lasts for 5-7 days (Janke, 2013). This virus targets epithelial cells of entire respiratory tract and replicate in the lungs. Therefore,

the transmission of the virus mainly occurs through respiratory route. Though the infection is usually mild and rarely causes mortality, it affects in the economic productivity of the animals due to fever, weight loss and reproductive failure in sows. In addition, Influenza A virus infection contribute to Porcine Respiratory disease complex along with Porcine Reproductive and Respiratory Syndrome (PRRS), Porcine circovirus Type 2 (PCV2), *Mycoplasma hyopneumoniae* and *Actinobaccillus pleuropneumoniae*. Among different Influenza A virus subtypes, globally three subtypes i.e H1N1, H3N2, and H1N2 are found to be widely circulated in swine (Lewis et al., 2016).

Though the natural reservoir for Influenza A viruses is wild waterfowl, it has a wide host range such as swine, birds, horses, dogs, cat and sea mammals and pose a significant risk to human health as the virus can reassort into novel viruses that can cause serious epidemics or pandemics (Knipe et al., 2001). Scholtissek et al., 1978 and Kawaoka et al., 1989 reported that reassortment between human and avian influenza (AI) viruses produced the 1957 and 1968 pandemic strains (Kawaoka, Krauss, & Webster, 1989; Scholtissek et al., 1978). The study done by Claas et al., 1994 suggested that influenza cases among children in Netherland were caused by human –avian reassortment viruses similar to those circulating viruses in pigs (Claas et al., 1994). Many surveillance studies have discovered that some avian viruses can apparently cross the species barrier directly to horses, pigs and pigs are infected through contact with influenza infected humans (Nelson et al., 2012), turkeys (Yassine, Lee, & Saif, 2013) and other birds. These viruses are in a continuous course of genetic change. Pigs may serve as a “mixing vessel” of the viruses to generate a new reassorted virus (Scholtissek et al., 1985). The 2009 flu pandemic (Influenza A H1N1) causing 150,000 – 570,000 deaths was reported to be the result of reassortment of circulating human influenza and avian influenza strains with pigs suspected as mixing vessel (Dawood et al., 2012). Studies have found pig- to- human and human- to -pig (reverse zoonosis) virus transmission in North America, Europe, Asia and Africa (Gray et al., 2007; Gregory et al., 2003; Ma et al., 2015). This reverse zoonosis of influenza viruses is an important source of swine influenza virus (SIV) diversity which reduces the efficacy of vaccines to SIV in pigs (Nelson & Vincent, 2015).

In Nepal, pig farming is becoming more popular in commercialized scale in peri-urban areas and are reared under integrated farming systems with poultry, fish and swine together thereby increasing the risk of zoonotic transmission of Influenza A virus from pigs to human and human to pigs. Therefore, influenza viruses are suspected to circulate actively increasing the risk of generation and dissemination of new variants in Nepal. This study aims to determine the seropositivity of Influenza A virus in pigs in selected districts of Nepal.

MATERIALS AND METHODS

A descriptive cross-sectional study was conducted to find out the seroprevalence of antibodies against Influenza A virus in pigs. Blood samples were collected from pigs using 3 ml sterile syringe from ear vein. The syringe was kept undisturbed with the needle

-holding end positioned until the blood clots and the clear serum was separated. A total of 330 pig sera were purposively collected from eight districts of Nepal namely Jhapa (15), Sunsari (20), Kaski (16), Chitwan (26), Kathmandu (93), Surkhet (29), Banke (93), and Dhankuta (38) with the approval of the farmers (Fig. 1). The samples were then stored at -80°C for future test regardless of their age, sex and breed.

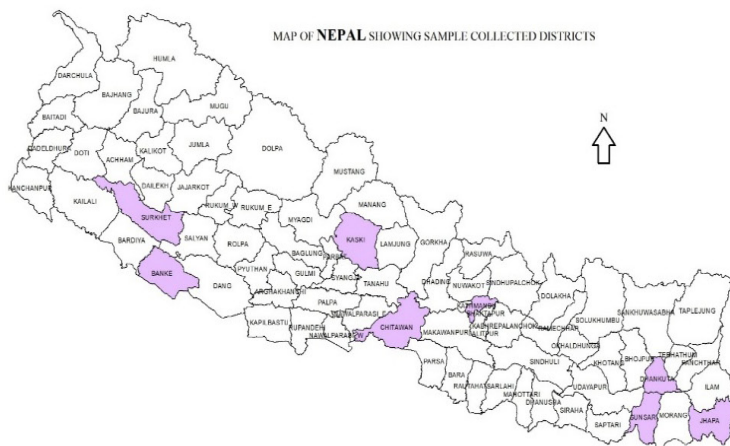


Fig. 1. Schematic representation of sample collected districts of Nepal

Then, a competitive enzyme-linked immunosorbent assay (c-ELISA) was performed for the detection of Influenza A antibodies in the collected sera samples using ID screen Influenza A Antibody competition MS Multispecies (ID Vet Innovative Diagnostics) following the manufacturer's instruction. The prevalence rate was calculated as:

$$S/N\% = (OD_{\text{sample}} / OD_{\text{NC}}) \times 100$$

Where, OD_{sample} is OD value of sample and

OD_{NC} is OD value of negative control

Samples presenting a S/N%: greater than or equal to 50% are considered negative, between 45% and 50% are considered doubtful and less than or equal to 45% are considered positive.

RESULTS

Of the total 330 sera samples, 27 samples were found positive for antibodies to Influenza A (Table 1). Overall, the seroprevalence was found to be 8.18%. In Jhapa, Sunsari, Kaski, Kathmandu, Surkhet and Banke, the seroprevalence rate was found as 26.6%, 45%, 37.5%, 5.37%, and 3.22% respectively. In Chitwan, Surkhet and Dhankuta, the antibody prevalence was found null in the collected sera samples.

Table 1. Sero-prevalence of Influenza A antibodies according to location in pigs of Nepal

Location	Positive (%)	Negative (%)	Total	CI (positive)	CI (negative)
Jhapa	4 (26.6)	7 (46.67)	15	(8.81-44.51)	(28.81-64.51)
Sunsari	9 (45)	11 (55)	20	(23.19 - 66.80)	(33.19-76.80)
Kaski	6 (37.5)	9 (56.25)	16	(14.99-60)	(33.74-78.75)
Chitwan	0	25 (96.15)	26	-	-
Kathmandu	5 (5.37)	87 (93.54)	93	(0.81-9.93)	(88.99-98.10)
Surkhet	0	29 (100)	29	-	-
Banke	3(3.22)	90 (96.77)	93	(-0.36-6.81)	(93 . 18 - 100.36)
Dhankuta	0	38 (100)	38	-	-

DISCUSSION

Influenza A viruses of swine not only can affect swine, but also can affect human health. They are the potential source for human pandemic influenza viruses with serious public health implications (Moreno et al., 2011). Studies have revealed that the first pandemic virus that happened in the twenty first century was caused by an Influenza A virus generated in swine containing genes from avian, human and swine origins, and variant viruses isolated from humans since 2010 (CDCx, 2017; Mena et al., 2016). In this study, we conducted the serological testing for antibodies to Influenza A viruses in sera of pigs. As pigs of Nepal have not been vaccinated against Swine Influenza virus, the seropositivity observed in this study suggests exposure to circulating virus. Even though the sample size is very small, the present study showed evidence that these animals were exposed to Influenza A virus via contact with infected animals or humans. Besides pigs, study should also be carried out in avian and human species. On the other hand, every year human infections of Influenza virus have been documented from Nepal. Jha et al 2019 reported that the highest number of Influenza infection in Nepalese people was caused by influenza A/H3strain (51%) followed by influenza B (40.4%) and influenza A (H1N1) pdm09 (8.6%). In pigs, our study reported the prevalence of Influenza A antibodies to be 11.21%.

Moreover, pig farming has been increasing in Nepal and we observed that the farmers did not quarantine newly introduced pigs before mixing with the herd. Furthermore, lack of biosecurity measures such as wearing separate clean clothing, lack of quarantine, uncontrolled movement of human and animals were noticed which act as risk factors for influenza transmission. Therefore, awareness against these measures should be carried out. Studies have indicated that interspecies transmission of Influenza A viruses from pigs-to-human and human-to-pigs occur in both direction which could be due to the same influenza receptors pattern in their respiratory tract (Nelson & Vincent, 2015; Van Poucke et al., 2010). Therefore, surveillance, control and prevention of the disease will

benefit both for swine health and human health. But, unfortunately, not much studies have addressed the Influenza A viruses in pigs. To the author's knowledge, only one earlier study have reported the seroprevalence of 4.76% in pig farms of Kavre, Banke and Bhaktapur which is lower than the findings of this study (Pokhrel et al., 2018). Therefore, detailed study about epidemiology and molecular characterization of Influenza A virus in pigs is highly essential.

Seroprevalence studies of Influenza A antibodies in pigs were reported by several countries. The study conducted by Suriya et al 2008 reported the seroprevalences of swine Influenza H1N1 and H3N2 among pigs of Penninsular Malaysia as 12.2% and 12.1% respectively (Suriya et al., 2008). Studies have found that influenza virus could spread more slowly and without clinically apparent signs (Loeffen et al., 2003). It is reported that swine influenza virus also contributes the porcine respiratory disease complex in finisher pigs (Thacker, 2001). In pigs, further studies on subtyping of influenza viruses and molecular characterization are very important to know the relationship of strains circulating in humans and pigs of Nepal which will help to formulate an effective control policy for Influenza viruses. In addition, effective influenza surveillance systems are essential to understand the epidemiology and seasonality of influenza and for optimizing influenza control strategies. Hence this study demonstrates that Influenza A virus is widespread in pigs of Nepal and further studies on subtyping of viruses from a wide host is very essential.

CONCLUSION

This study confirms exposure to Influenza A virus among pigs in Nepal, with a seroprevalence of 8.18%. Antibodies in unvaccinated pigs suggest natural infection, likely due to poor biosecurity and interspecies transmission—especially in Sunsari, Kaski, and Jhapa. With limited research and a growing pig population, there's an urgent need for expanded surveillance and virus characterization to guide control efforts.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Centers for Disease Control and Prevention. (2017, October 16). *Reported infections with variant influenza viruses in the United States*. <https://www.cdc.gov/flu/swineflu/variant-cases-us.htm>
2. Centers for Disease Control and Prevention. (2021, January). *Influenza virus*. <http://www.cdc.gov/vaccines/pubs/pinkbook/flu.html>

3. Claas, E. C., Kawaoka, Y., de Jong, J. C., Masurel, N., & Webster, R. G. (1994). Infection of children with avian-human reassortant influenza virus from pigs in Europe. *Virology*, 204(1), 453–457. <https://doi.org/10.1006/viro.1994.1553>
4. Davidson, I., Al-Touri, A., Raibshstein, I., Hadani, Y., Bombarov, V., Yadin, H., ... Reid, S. M. (2014). Seroepidemiology survey and isolation of swine influenza viruses from subclinical infections in Israel during the years 2009–2011. *Israel Journal of Veterinary Medicine*, 69(2), 62–67.
5. Dawood, F. S., Iuliano, A. D., Reed, C., Meltzer, M. I., Shay, D. K., Cheng, P. Y., ... Widdowson, M. A. (2012). Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: A modelling study. *The Lancet Infectious Diseases*, 12(9), 687–695. [https://doi.org/10.1016/S1473-3099\(12\)70121-4](https://doi.org/10.1016/S1473-3099(12)70121-4)
6. Gray, G. C., McCarthy, T., Capuano, A. W., Setterquist, S. F., Olsen, C. W., & Alavanja, M. C. (2007). Swine workers and swine influenza virus infections. *Emerging Infectious Diseases*, 13(12), 1871–1878. <https://doi.org/10.3201/eid1312.061323>
7. Gregory, V., Bennett, M., Thomas, Y., Kaiser, L., Wunderli, W., Matter, H., ... Lin, Y. P. (2003). Human infection by a swine influenza A (H1N1) virus in Switzerland. *Archives of Virology*, 148(4), 793–802. <https://doi.org/10.1007/s00705-002-0953-9>
8. Janke, B. H. (Ed.). (2013). *Clinicopathological features of swine influenza*. Springer.
9. Kawaoka, Y., Krauss, S., & Webster, R. G. (1989). Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *Journal of Virology*, 63(11), 4603–4608. <https://doi.org/10.1128/JVI.63.11.4603-4608.1989>
10. Knipe, D., Howley, P., Griffin, D., Lamb, R., Martin, M., & Roizman, B. (Eds.). (2001). *Orthomyxoviruses*. Lippincott Williams & Wilkins.
11. Lewis, N. S., Russell, C. A., Langat, P., Anderson, T. K., Berger, K., Bielejec, F., ... Vincent, A. L. (2016). The global antigenic diversity of swine influenza A viruses. *eLife*, 5, e12217. <https://doi.org/10.7554/eLife.12217>
12. Loeffen, W. L., Nodelijk, G., Heinen, P. P., van Leengoed, L. A., Hunneman, W. A., & Verheijden, J. H. (2003). Estimating the incidence of influenza-virus infections in Dutch weaned piglets using blood samples from a cross-sectional study. *Veterinary Microbiology*, 91(4), 295–308. [https://doi.org/10.1016/s0378-1135\(02\)00306-1](https://doi.org/10.1016/s0378-1135(02)00306-1)
13. Ma, M., Anderson, B. D., Wang, T., Chen, Y., Zhang, D., Gray, G. C., & Lu, J. (2015). Serological evidence and risk factors for swine influenza infections among Chinese swine workers in Guangdong Province. *PLoS ONE*, 10(5), e0128479. <https://doi.org/10.1371/journal.pone.0128479>
14. Mena, I., Nelson, M. I., Quezada-Monroy, F., Dutta, J., Cortes-Fernandez, R., Lara-Puente, J. H., ... Garcia-Sastre, A. (2016). Origins of the 2009 H1N1 influenza pandemic in swine in Mexico. *eLife*, 5, e16777. <https://doi.org/10.7554/eLife.16777>
15. Moreno, A., Di Trani, L., Faccini, S., Vaccari, G., Nigrelli, D., Boniotti, M. B., ... Cordioli, P. (2011). Novel H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus. *Veterinary Microbiology*, 149(3–4), 472–477. <https://doi.org/10.1016/j.vetmic.2010.12.011>
16. Nelson, M. I., Gramer, M. R., Vincent, A. L., & Holmes, E. C. (2012). Global transmission of influenza viruses from humans to swine. *Journal of General Virology*, 93(Pt 10), 2195–2203. <https://doi.org/10.1099/vir.0.044974-0>
17. Nelson, M. I., & Vincent, A. L. (2015). Reverse zoonosis of influenza to swine: New perspectives on the human-animal interface. *Trends in Microbiology*, 23(3), 142–153. <https://doi.org/10.1016/j.tim.2014.12.002>
18. Pokhrel, D., Poudel, I., Raut, R., Aryal, S., & Acharya, K. (2018). Sero-prevalence of influenza A antibodies in pigs of Bhaktapur, Kavre and Banke districts of Nepal. *International Journal of Applied*

19. Scholtissek, C., Bürger, H., Kistner, O., & Shortridge, K. F. (1985). The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. *Virology*, 147(2), 287–294. [https://doi.org/10.1016/0042-6822\(85\)90131-X](https://doi.org/10.1016/0042-6822(85)90131-X)
20. Scholtissek, C., Rohde, W., Von Hoyningen, V., & Rott, R. (1978). On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology*, 87(1), 13–20. [https://doi.org/10.1016/0042-6822\(78\)90153-8](https://doi.org/10.1016/0042-6822(78)90153-8)
21. Suriya, R., Hassan, L., Omar, A. R., Aini, I., Tan, C. G., Lim, Y. S., & Kamaruddin, M. I. (2008). Seroprevalence and risk factors for influenza A viruses in pigs in Peninsular Malaysia. *Zoonoses and Public Health*, 55(7), 342–351. <https://doi.org/10.1111/j.1863-2378.2008.01138.x>
22. Thacker, E. L. (2001). Immunology of the porcine respiratory disease complex. *Veterinary Clinics of North America: Food Animal Practice*, 17(3), 551–565. [https://doi.org/10.1016/s0749-0720\(15\)30006-2](https://doi.org/10.1016/s0749-0720(15)30006-2)
23. Van Poucke, S. G. M., Nicholls, J. M., Nauwynck, H. J., & Van Reeth, K. (2010). Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. *Virology Journal*, 7, 38. <https://doi.org/10.1186/1743-422x-7-38>
24. Yassine, H. M., Lee, C. W., & Saif, Y. M. (2013). Interspecies transmission of influenza A viruses between swine and poultry. *Current Topics in Microbiology and Immunology*, 370, 227–240. https://doi.org/10.1007/82_2011_180

Confirmatory Diagnosis of Chicken Infectious Anaemia Virus in Broiler Breeders of Nepal using Conventional Polymerase Chain Reaction

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ABSTRACT

*Chicken Infectious Anaemia (CIA) is an acute, immunosuppressive disease, primarily affecting young chickens and caused by Gyrovirus of Anelloviridae family. While the disease has been reported to have a worldwide distribution, no confirmed cases of CIA in Nepal were documented before Asar 2081 B.S. (June, 2024). This study was conducted at NAHRC, Khumaltar, Lalitpur with the aim of confirming the presence of Chicken Infectious Anemia Virus (CIAV) in Nepalese poultry flocks using the Conventional PCR technique and performing differential diagnosis in poultry birds showing chronic mortality in the absence of other suggestive clinical symptoms. Rapid diagnostic tests were done in tandem for the diagnosis of Newcastle disease (ND), Avian Influenza (AI), Infectious Bronchitis (IB), etc., which gave negative results. The conventional PCR assay was performed using the nucleic acid from organ samples of necropsied birds for the amplification of the VP2 gene of CIAV by using specific primers. It yielded the amplicon of size 419 bp, confirming the presence of CIAV DNA in the samples. Out of 66 samples, 27 (40.9%) gave positive results by PCR. Along with this, 3 positive results were found for Fowl Adenovirus and one for Avian Influenza virus. The hematocrit value was 18-41% in broiler breeders. Upon bacterial culture, positive results were found for multidrug-resistant *E. coli* and *Staphylococcus* indicating secondary infection most likely due to immunosuppression. The findings of our study confirmed the presence of CIAV in broilers and broiler breeder flocks in Nepal. Comprehensive epidemiological studies including both serological and molecular studies are necessary to assess the actual status of CIA in Nepalese poultry flocks. Effective application of vaccines in the broiler breeder flocks needs to be implemented.*

Keywords: Hematocrit; Immunosuppression; VP2 gene; Vaccine; 419 bp

INTRODUCTION

Chicken Infectious Anemia (CIA) is an acute, immunosuppressive disease, particularly of young chickens of 2-4 weeks of age. It is caused by the Gyrovirus, which is a small, circular, non-enveloped virus with icosahedral symmetry and 20-25 nm in diameter, belonging to the Anelloviridae family (Fenner et al., 2017). The virus was first isolated in Japan in

1979 (Yuasa et al., 1979). The virus contains a circular single-stranded DNA genome containing three overlapped Open Reading Frames (ORFs), which encode for three viral proteins namely, VP1 (capsid protein), VP2 (protein phosphatase), and VP3 (apoptin). VP1 is a structural protein, while VP2 and VP3 are non-structural proteins. VP1 is the major structural protein that contains ample antigenic epitopes and plays a vital role in the growth and transmission of CIAV (Shah et al., 2023). VP2 is a multifunctional protein with a role in viral replication, antigen presentation, pathology, and virus assembly (Brar et al., 2020). Apoptin (VP3) induces apoptosis of T-lymphocytes and is important in the pathogenesis of chicken infectious anaemia virus (Fenner et al., 2017). CIAV is ubiquitous and resistant to many disinfectants and chemical sterilizers (Schat & Van Santen, 2020).

The disease is characterized by anorexia, lethargy, depression, anaemia, lymphoid hypoplasia or atrophy, cutaneous, subcutaneous, and intramuscular haemorrhages, and increased mortality (Krishan et al., 2015). The virus is transmitted horizontally, vertically, and occasionally through contaminated vaccines (Zeng et al., 2023). Horizontal infection occurs through feathers, faeces, oral contamination, and from chicken to chicken, and vertical transmission through asymptomatic breeders to progeny. Although all age groups are affected, young chicks up to 3-4 weeks are most susceptible to clinical disease, and older birds show sub-clinical infection (Brar et al., 2020). Affected chicks show mortality of about 30% generally but may reach up to 60% in complicated cases (Shah et al., 2023). The young chicks have hematocrit values ranging from 6-27% (Schat & Van Santen, 2020). The hemocytoblast cells in the bone marrow and precursor lymphocytes in the thymus are important targets for virus infection due to which affected chicks are more susceptible to immunosuppression leading to secondary bacterial, viral, fungal, and parasitic infections (Adair, 2000). The disease is severe in chickens co-infected with other diseases such as Marek's, avian reovirus, reticulo-endotheliosis, and infectious bursal disease (Schat & Van Santen, 2020). The disease can be diagnosed by various methods such as viral DNA detection by PCR, propagation in cell culture, serological identification by ELISA, indirect immunofluorescence, etc. Applications of molecular tools like PCR, RE analysis, and DNA sequencing have emerged as effective confirmatory tools for diagnosing and studying the molecular epidemiology of poultry pathogens, including CIAV, which helps adapt appropriate disease prevention and control programs (Krishan et al., 2015). PCR is rapid, sensitive, specific, and diseases can be diagnosed using a small amount of tissue or biological liquid samples, so it is commonly used to monitor flock diseases (Hadimli et al., 2008).

CIAV was not confirmed in Nepal until Ashar 2081 B.S., and no vaccine had been used against it recently. Repeated vaccination and treatment failure against various diseases and chronic mortality were seen in poultry flocks but could not be confirmed as CIAV. This paper describes the method of confirmatory diagnosis of CIAV using conventional PCR along with differential diagnosis from the samples brought from different poultry farms across the country at National Animal Health Research Center (NAHRC), Khumaltar, Lalitpur.

MATERIALS AND METHODS

Study site

The study was conducted at the National Animal Health Research Center (NAHRC), NARC, Khumaltar, Lalitpur from July to November 2024.

Collection of samples

The organ samples were collected after the necropsy examination of poultry birds, including broiler breeders and broiler chickens of various age groups from Kathmandu Valley, Dang, Bhairahawa, and Dhangadi, which were brought to NAHRC, Khumaltar for confirmatory diagnosis. In the broiler birds, farmers reported constant mortality of approximately 50 birds in a flock of 5000 birds daily after the age of three weeks. The mortality did not spike or respond to any treatments prescribed by the consulting veterinarian. In the broiler breeders, the mortality was not high, the history was given as 10-15 deaths per day in a flock of 5000 breeders. The mortality did not spike or decrease even after conservative treatments that were prescribed for the breeders as well. A light decrease in egg production was reported but that too was non-significant. The birds did not have a history of vaccination against CIAV. Breeders were vaccinated against ND, IB, Salmonella, and others as used regularly in the Nepalese poultry sector.

The necropsy examination was done using standard procedures. The organ samples retained for PCR were bone marrow, spleen, thymus, liver, trachea, lungs, air sac, tendon sheath, etc. The bone marrow swabs were also taken for bacterial culture for any secondary bacterial infection. The feed samples were requested for fungal culture. Rapid diagnostic tests were done in tandem for Newcastle disease (ND), Avian Influenza (AI), and Infectious Bronchitis (IB). PCR was done for several poultry diseases namely, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, Reovirus infection, Fowl Adenovirus, Avian Influenza virus, and CIAV. The samples collected were stored at room temperature for immediate processing. In the case of broiler breeders, fresh blood was requested from the farms as well to estimate the hematocrit value, using the microhematocrit capillary tube method.

DNA extraction

The genomic DNA was extracted from the organ samples using the G-DEX™ IIc Genomic DNA Extraction kit as per the manufacturer's recommendations.

Assessment of DNA purity

The purity of the extracted DNA was assessed using a UV/Visible Nanospectrophotometer. The DNA concentration and absorbance at 260 nm, 280 nm, and 230 nm along with A260/A280 and A260/A230 ratios were measured. The A260/A280 ratio ranging from 1.9-2.1 and the A260/A230 ratio ranging from 2.0-2.2 were considered acceptable for DNA. The pure DNA was then stored at -20°C until further use.

Polymerase Chain Reaction (PCR)

After purification of extracted DNA, the conventional PCR assay was performed.

The PCR reaction was carried out in total 25 µL reaction mixture containing 12.5 µL mastermix, 0.8 µL of each forward and reverse primer, 3 µL DNA template, and 7.9 µL nuclease free water (NFW). The PCR mastermix composed of dNTPs (dATP, dTTP, dCTP, dGTP), Taq DNA polymerase, and PCR buffers (tris-HCl, KCl, MgCl₂). The primers used were: forward primer 5'-CTAAGATCTGCAACTGCGGA-3' and reverse primer 5'-CCTTGGAAGCGGATAGTCAT-3' which were reconstituted with nuclease-free water (NFW) as per the manufacturer's specification. These primers pair target the VP2 gene and amplify specific 419 bp fragments of CAV (Brar et al., 2020; Gowthaman, 2014). The PCR was performed in a thermocycler under: the precycle step of initial denaturation consisting of one cycle at 95 °C for 5 minutes followed by 35 cycles at 95°C for 30 seconds, annealing at 58°C for 1 minute, extension at 72°C for 1 minute 25 seconds, and final extension at 72°C for 10 minutes.

Agarose gel electrophoresis

The PCR products were then visualized by agarose gel electrophoresis using 1.2% agarose gel containing Red Safe DNA Dye in Tris-Acetate EDTA (TAE) buffer.

Visualization of DNA using UV transilluminator

The DNA fragments obtained by gel electrophoresis were then visualized using a UV transilluminator. The size of the product was determined by comparing it with a standard DNA ladder.

RESULTS

Postmortem findings

The primary lesions observed were erosions and ulcerations in the gizzard kaolin layer; severe, diffused hemorrhagic enteritis; and atrophy of the thymus, bone marrow, and spleen (Fig. 1). In some breeder birds, there was very faint bluish-green discoloration at the tips of the wings but these were not seen in the broilers. The necropsy findings were recorded as they were observed. The necropsy was not definitively suggestive of any specific disease.



Fig. 1. Necropsy findings observed; A- atrophy of thymus; B-hemorrhagic breast muscle; C- gizzard erosion

PCR result

The total samples brought for the diagnosis of CIAV by conventional PCR was 66 out of which 27 showed positive result, which was about 40.9%. There were a total of 6 spleen samples, 24 bone marrow samples, 2 thymus samples, 1 liver sample, 4 trachea samples, 5 lungs samples, 1 intestine sample, and remaining 23 pooled samples including liver, spleen, thymus, bone marrow, trachea, tendon sheath, air sac, lungs, and trachea. The pooled samples were included for the differential diagnosis of CIAV with other diseases. Three samples gave positive results for Fowl Adenovirus (~897bp) and one for Avian Influenza virus (~488bp) as shown in Fig. 2.

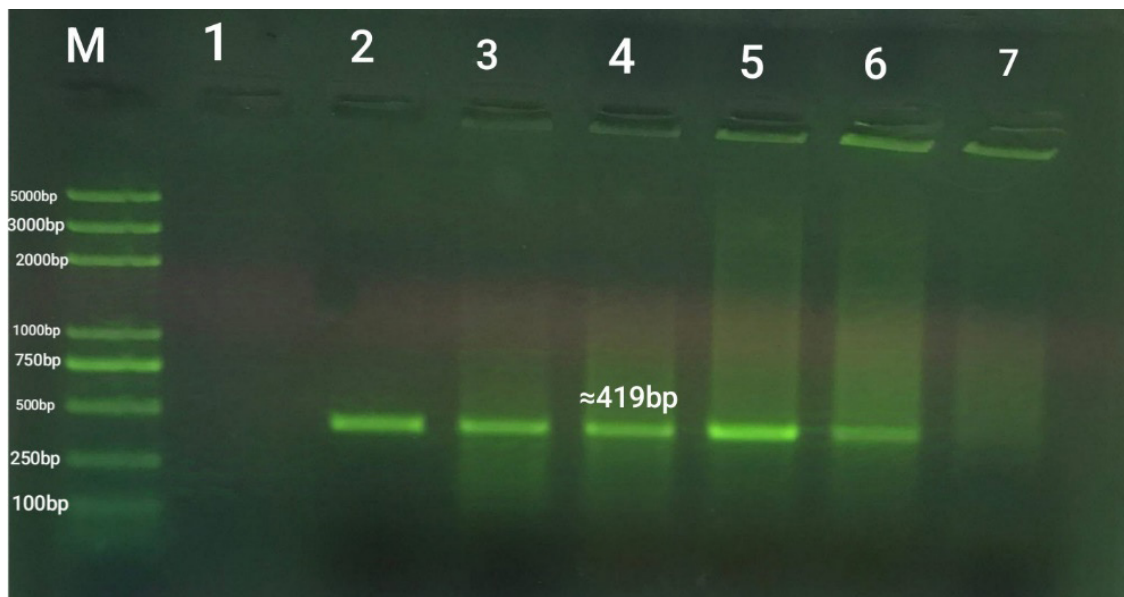


Fig. 2. Result of PCR Detection of CAV after Agarose gel electrophoresis using Trans2K® Plus DNA Marker Lane M (DNA Marker); Lane 1(Negative Control); Lane 2 (Positive Control); Lane 3,4,5,6 (Positive for CIAV); Lane 7 (Negative for CIAV).

Hematocrit findings

The hematocrit value of some broiler breeders (12) was measured using the microhematocrit capillary tube method and it ranged from 18-41%.

Other findings

Upon bacterial culture, a positive result was found for multidrug resistant *E. coli* and *Staphylococcus*. Multidrug resistant *E. coli* was found resistant to different antibiotics such as gentamicin, enrofloxacin, ciprofloxacin, azithromycin, sulfonamide, tetracycline and sensitive only to amikacin.

DISCUSSION

Chicken infectious anemia virus is an important poultry pathogen associated with anaemia, immunosuppression, and secondary microbial infection showing worldwide distribution (Fenner et al., 2017; Krishan et al., 2015). This study represents the first report of Chicken Infectious Anemia virus in Nepal, which was confirmed by conventional PCR with a positivity rate of 40.9%. The emergence of this pathogen in Nepal with significant findings augments the need for surveillance, biosecurity measures, and vaccination programs in poultry farms across the country. Weak epidemiological investigations, poor monitoring and quarantine, unavailability of effective drugs and vaccines, and inefficient biosafety and biosecurity precautions could be responsible for the outbreaks of many bacterial, viral, fungal, and parasitic diseases in Nepal (Poudel et al., 2021). These factors must have facilitated the introduction and spread of CIAV in Nepal. However, the absence of appropriate diagnostic measures has hindered its timely detection and confirmation. In our current study, the farmers reported undergoing repeated treatments for various diseases such as ND, IB, and CRD based on clinical or postmortem findings, but the mortality rate was consistent. When they transferred the samples to NAHRC where the PCR diagnostic tool was used, the infection with CIAV was confirmed. It highlights the use of confirmatory diagnostic tools such as molecular and serological techniques for effective diagnosis as well as the importance of correct diagnosis for effective disease management in poultry farms.

Broilers are an excellent source of high economic return in a short period and that's why it is a popular vocation but the broiler birds are fragile and more susceptible to different diseases than layers. Due to the genetic selection on body weight and feed conversion, the immune response (IgG, IgM, cellular response) is less or somewhat dysfunctional in broilers. If the biosecurity measures are not strict, then different health problems may arise due to the impact on the immunity of such birds (Koenen et al., 2002). The farmers reported the increased mortality of broiler birds above 3 weeks of age per day compared to broiler breeders. This may be due to the reason that the CIAV mainly causes clinical disease in younger chicks severely affecting their lymphoid organs and aplastic anemia resulting in immunosuppression. Due to immunosuppression, they are more susceptible to adenovirus and bacterial infection (Dong et al., 2022; Hoerr, 2010), which was the differential diagnosis in our study and also confirmed by PCR. Nepalese poultry farmers are mostly medium-scale poultry raisers who are not familiar with advanced poultry-raising tools and techniques, biosecurity, and management practices. This gap in knowledge and resources contributes to the frequent occurrence of *E. coli*, *Salmonella*, and mycotoxicosis infections in poultry farms across Nepal. The frequent occurrence of colibacillosis might be due to the natural occurrence of the organism in their intestine, stress-induced proliferation, or contaminated feed and water sources (Bhusal et al., 2023).

In our study, bacterial culture results confirmed the presence of multi-drug resistant *E. coli* which might indicate that it was the result of secondary infection due to CIAV-induced immunosuppression or simultaneous colibacillosis-CIAV infection due to managerial factors. The farmers relied on the use of different antimicrobials to alleviate symptoms

before CIAV was confirmed which might have contributed to the development of the multidrug-resistant *E. coli* infection as found in our study. The easy availability and over-the-counter (OTC) sales of different antimicrobials in the country are increasing their indiscriminate use against various diseases. In Kathmandu district, 90% of poultry farms especially broiler farms use antibiotics for treatment or prophylaxis (Subedi et al., 2023). About 50% of antibiotics are prescribed inappropriately and about 71% of veterinary drugs are sold by self-prescription rather than by qualified registered veterinarians (Dhakal et al., 2019). This situation has increased the risk of antimicrobial resistance, as many people are unaware that vaccination and biosecurity measures can decrease the burden of antimicrobial use in poultry.

The commonly used poultry vaccines in Nepal are against Marek's disease, Newcastle disease, Infectious Bursal Disease, Infectious Bronchitis, Fowl pox, etc (MoALD, 2081; NVPL, 2075). CIAV-induced immunosuppression is commonly associated with the failure of vaccination against these diseases as found in our study. Since it is a viral disease, no direct treatment options other than vaccination are available. Our study has created the necessity of the urgent introduction of CIAV vaccines in the Nepalese context to mitigate its impact. Maternal antibodies to CIAV completely protect young chicks against the disease for about 3 weeks of age (Karimi et al., 2010). From antibody-positive hens, vertical transmission of virus doesn't occur so the development and use of vaccines should be considered in breeder flocks. Vaccines used in the veterinary sector in Nepal are: inactivated/killed, toxoids, recombinant subunit-based vaccines, RNA/DNA vaccines, vector-based vaccines, etc (Poudel et al., 2021). The limited ability of CIAV strains to propagate to high titer levels in embryos or cell cultures, combined with the risk of reversion to virulence, presents notable challenges associated with live and killed vaccines for Chicken Infectious Anemia (Fatoba & Adeleke 2019). Thus, DNA and recombinant vaccines are considered effective against CIAV. Further molecular studies, involving DNA sequencing, to understand the genetic diversity of CIAV strains circulating in Nepal are required to introduce the CIAV vaccine in our poultry farms. Additionally, vaccine efficacy studies must be conducted from time to time to address viral mutations and for effective vaccination programs.

CONCLUSION

We conclude that Chicken Infectious Anemia has been confirmed in poultry flocks in Nepal. The PCR assay along with other diagnostic tools not only identified the presence of CIAV but also confirmed the secondary infections resulting from CIAV-induced immunosuppression. The presence of secondary infection alerts for the timely use of vaccines in breeder flocks against CIAV. Further comprehensive epidemiological studies including both molecular and serological techniques are required to accurately assess the real depth of both clinical and subclinical disease in Nepalese poultry flocks. Prompt implementation of prevention and control strategies is crucial to alleviate the economic loss caused by the disease.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Adair, B. (2000). Immunopathogenesis of chicken anemia virus infection. *Developmental & Comparative Immunology*, 24(2–3), 247–255. [https://doi.org/10.1016/S0145-305X\(99\)00078-7](https://doi.org/10.1016/S0145-305X(99)00078-7)
2. Bhusal, S. P., Gautam, G., Dhakal, R., Paudel, B., & Chapagai, D. R. (2023). Pattern of poultry diseases brought at Veterinary Teaching Hospital (VTH), Rampur and National Avian Disease Investigation Laboratory (NADIL), Bharatpur, Chitwan, Nepal during 2017/18. *Nepalese Veterinary Journal*, 40, 42–53. <https://doi.org/10.3126/nvj.v40i0.55057>
3. Brar, R., Bahnot, V., Mahajan, N. K., Tomar, P., Prakash, A., & Maan, S. (2020). Detection and molecular characterization of chicken infectious anaemia virus in poultry flocks in Haryana. *The Indian Journal of Animal Sciences*, 89(12), 21–24.
4. Dhakal, R., Joshi, B., Karn, R., Bhusal, S., & Acharya, B. (2019). A review on scenario, challenges and prospects of poultry production in Nepal. *Malaysian Journal of Sustainable Agriculture*, 3(2), 60–63. <https://doi.org/10.26480/mjsa.02.2019.60.63>
5. Dong, H. V., Tran, G. T. H., Kurokawa, A., Yamamoto, Y., Takeda, Y., Ogawa, H., & Imai, K. (2022). Genetic characterization of chicken anemia viruses newly isolated from diseased chicks in Japan in 2020. *The Journal of Veterinary Medical Science*, 84(1), 166–170. <https://doi.org/10.1292/jvms.21-0380>
6. Fatoba, A. J., & Adeleke, M. A. (2019). Chicken anemia virus: A deadly pathogen of poultry. *Acta Virologica*, 63(1), 19–25. https://doi.org/10.4149/av_2019_103
7. Fenner, F., MacLachlan, N. J., & Dubovi, E. J. (2017). *Fenner's veterinary virology* (5th ed.). Elsevier.
8. Gowthaman, V. (2014). Molecular characterization of chicken infectious anemia virus isolated from commercial poultry with respiratory disease complex in India. *Advances in Animal and Veterinary Sciences*, 2(3), 171–176. <https://doi.org/10.14737/journal.aavs/2014/2.3.171.176>
9. Hadimli, O. E., Guler, L., & Ucan, U. S. (2008). Investigation of chicken infectious anemia virus infection by PCR and ELISA in chicken flocks. *Veterinary Journal of Ankara University*, 32, 79–84.
10. Hoerr, F. J. (2010). Clinical aspects of immunosuppression in poultry. *Avian Diseases*, 54(1), 2–15. <https://doi.org/10.1637/8913-043009-Review.1>
11. Karimi, I., Mahzounieh, M., Bahadoran, S., & Azad, F. (2010). Chicken anemia virus infection in broiler chickens in Shahrekord, Iran: Serological, hematological, and histopathological findings. *Comparative Clinical Pathology*, 19(1), 63–67. <https://doi.org/10.1007/s00580-009-0904-3>
12. Koenen, M. E., Boonstra-Blom, A. G., & Jeurissen, S. H. M. (2002). Immunological differences between layer- and broiler-type chickens. *Veterinary Immunology and Immunopathology*, 89(1–2), 47–56. [https://doi.org/10.1016/S0165-2427\(02\)00180-8](https://doi.org/10.1016/S0165-2427(02)00180-8)
13. Krishan, G., Shukla, S. K., Bhatt, P., Wani, M. Y., Dhama, K., Singh Malik, Y. P., & Kumar, R. (2015). Clinical association of chicken anaemia virus with other infectious poultry diseases in North India and Nepal: Its pathological studies, molecular epidemiology and RFLP pattern of PCR amplified full length viral genome. *Advances in Animal and Veterinary Sciences*, 3(7), 395–405. <https://doi.org/10.14737/journal.aavs/2015/3.7.395.405>

14. Ministry of Agriculture and Livestock Development. (2024). Agriculture and livestock diary 2081 [Data book].
15. National Vaccine Production Laboratory. (2018). Booklet of vaccines used in livestock and poultry.
16. Poudel, U., Dahal, U., & Dhakal, S. (2021). Review of poultry production and poultry vaccine manufacture in Nepal. *Global Journal of Agricultural and Allied Sciences*, 3(1), 1–7.
17. Schat, K. A., & Van Santen, V. L. (2020). Chicken infectious anemia and circovirus infections in commercial flocks. In *Diseases of poultry* (14th ed., pp. 284–320). Wiley.
18. Shah, P. T., Bahoussi, A. N., Cui, X., Shabir, S., Wu, C., & Xing, L. (2023). Genetic diversity, distribution, and evolution of chicken anemia virus: A comparative genomic and phylogenetic analysis. *Frontiers in Microbiology*, 14, 1145225. <https://doi.org/10.3389/fmicb.2023.1145225>
19. Subedi, D., Jyoti, S., Thapa, B., Paudel, S., Shrestha, P., Sapkota, D., Bhatt, B. R., Adhikari, H., Poudel, U., Gautam, A., Nepal, R., & Al-Mustapha, A. I. (2023). Knowledge, attitude, and practice of antibiotic use and resistance among poultry farmers in Nepal. *Antibiotics*, 12(9), 1369. <https://doi.org/10.3390/antibiotics12091369>
20. Yuasa, N., Taniguchi, T., & Yoshida, I. (1979). Isolation and some characteristics of an agent inducing anemia in chicks. *Avian Diseases*, 23(2), 366–385. <https://doi.org/10.2307/1589648>
21. Zeng, Y., Zhang, H., & Zhang, H. (2023). Isolation, identification, and whole genome analysis of chicken infectious anemia virus in an outbreak of disease in adult layer hens. *Veterinary Sciences*, 10(7), 481. <https://doi.org/10.3390/vetsci10070481>

Bacteriological Quality of Milk at Different Points of Production and Supply Chain in Western Chitwan

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ABSTRACT

A study was conducted to determine the bacteriological quality of milk at different points of production and supply chain in Western Chitwan. A total of 70 milk samples collected directly from cow's teat (n=25), transport can (TC) (n=25), Dairy Co-operative milk Collection Center (DC) (n=5), Chilling Vat (CV) (n=5), Local private dairy (LD) (n=5) and pasteurized milk packet (PM) (n=5) were subjected to bacteriological quality evaluation based on Standard Plate Count (SPC) and isolation of E. coli as per the standard protocols. The mean ($\pm$ SE) SPC of milk samples from teat, TC, DC, CV, LD and PM were found to be $34.45(\pm 15.80) \times 10^6$, $57.03(\pm 18.34) \times 10^7$, $70.60(\pm 49.93) \times 10^7$, $162.20(\pm 40.50) \times 10^8$, $508.40(\pm 248.87) \times 10^7$ and $4.45(\pm 3.41) \times 10^5$ CFU/ml, respectively. The microbial load was found to be significantly ($P < 0.001$) increased from teat sample to the chilling vat. E. coli prevalence on above respective points was found to be 28%, 76%, 80%, 80%, 60% and 40%. From the present study, it can be concluded that both the raw and pasteurized milk in Western Chitwan was highly contaminated which indicates the importance of mass awareness and routine microbial quality assessment for safe milk.

Keywords: Bacteriological quality; Standard plate count; E. coli; Microbial load

INTRODUCTION

Milk is synthesized in specialized cells of the mammary gland and is virtually sterile when secreted into the alveoli of the udder (Quigley et al., 2013). But its highly perishable nature makes it an ideal media for growth and multiplication of various pathogenic microorganisms. Milk produced under hygienic conditions from healthy animals should not contain more than 5×10^5 bacterial/ml (EU, 1992). Poor pre-milking udder hygiene that fails adequately to clean dirty udders may result in the introduction of vegetation, soil, bedding material and their associated microorganisms into the milk, such foreign matters and contaminations in the milk may result high bacterial count leading to concerns regarding consumer health (FSA, 2006).

Even though milk is known to have a number of antimicrobial properties, unchilled milk will cause its bacterial population to double in less than three hours. Initial numbers and the temperature at which milk is kept both immediately after milking and subsequently will determine the rate of microbial growth (Kurwijila et al., 1992). Among many

microorganisms accessing to milk and products, *E. coli* is often used as marker organisms of fecal contamination. Most *E. coli* is harmless, but some are known to be pathogenic bacteria, causing severe intestinal and extra intestinal diseases in man (Altalhi & Hassan, 2009).

Milk passes through different handlers from the farm to the table of consumers. The safety and nutritional value of milk can, however, be significantly impacted by its quality. Several factors contribute to the degradation of milk quality along the production and supply chain. These include inadequate hygiene practices at the farm level, improper milk handling and storage, pasteurization defect, failure to maintain cold chain and suboptimal conditions during transportation (Oliver et al., 2005). Hence, understanding the bacteriological quality of milk at various stages of the production and supply chain is imperative for ensuring the safety and quality of this vital food resource.

Raw milk contamination has an impact on the dairy industry as a whole, ultimately leading to milk with poor manufacturing qualities or dairy products with lower flavor quality and shorter shelf lives (Oliver, 2010). Thus, assurance of the quality of milk and its products begins at the farms and ends in the hands of the consumers (Murphy & Boor, 2000). However, limited work has been undertaken so far on the assessment of bacteriological quality and hygiene practices of milk in the production and supply chain. Considering the importance of safe food to consumers, the present study was envisaged to determine the contamination points, microbial load and *E. coli* isolation in Western Chitwan farms.

MATERIALS AND METHODS

Five different locations of Western Chitwan (Dibyanagar, Chanauli, Gurung Chowk, Lamjung Chowk and Bhimsen Nagar) were selected for sample collection and survey. Five Dairy Co-operatives from respective places were considered. Altogether 70 purposive milk samples were collected i.e., directly from cow's teat (n=25), transport can (n=25), Dairy Co-operative (n=5), Chilling vat (n=5), Local private dairy (n=5) and pasteurized milk packet (n=5) as shown in Fig. 1 and 2. The samples in sterile screw-capped test tubes were transported to Veterinary Microbiology Laboratory, AFU, Rampur in icebox maintaining cold chain and preserved in refrigerator at 4°C temporarily. This study was completed during November to December 2023.

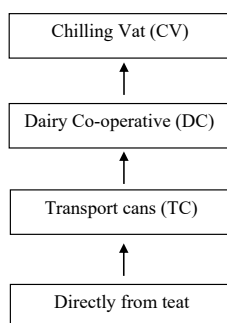


Fig. 1. Flow diagram showing the different points of milk production and supply chain

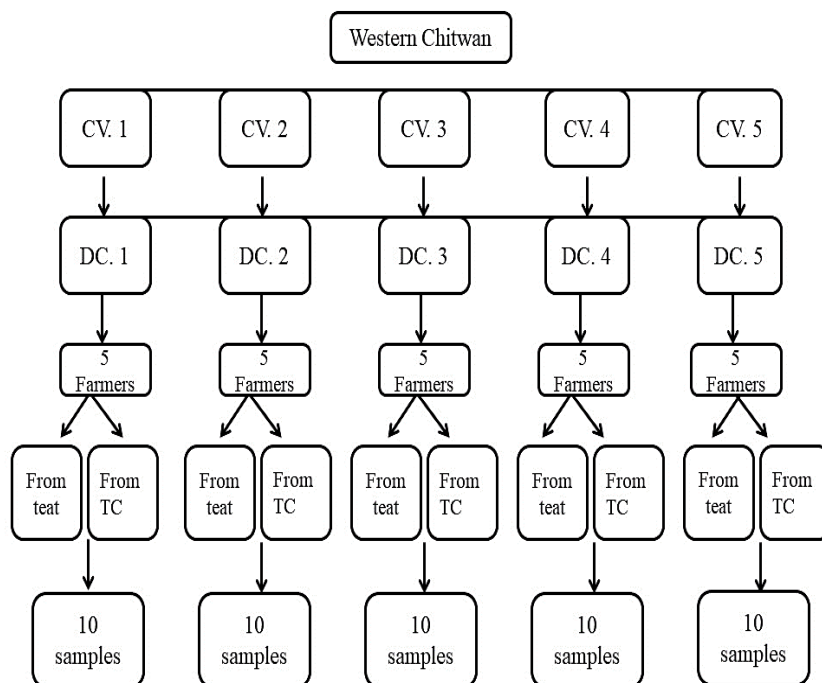


Fig. 2. Flow diagram of milk sample collection

Standard Plate Count

Serial diluted milk samples were cultured in Plate Count Agar (PCA) by pour plate method. The colonies of dishes were counted using Colony Counter. Two consecutive plates with less than 300 colonies were considered for the record. The results were expressed as colony forming units per ml using the formula given by ISO 7218:2007 E (ISO, 2007).

$$N = \frac{\Sigma C}{(V * (n_1 + 0.1n_2) * d)}$$

where,

N = No. of bacterial colonies counted

ΣC = sum of colonies counted on two consecutive dilution steps

V = Volume of inoculum in each dish/plate in ml

N1 = No. of dishes retained in first dilution.

N2 = No. of dishes retained in second dilution.

D = dilution factor corresponding to the first dilution retained.

Isolation of *E. coli*

Pre-enriched milk samples were cultured primarily on Mac Conkey Agar and subcultured on EMB Agar. Isolation and identification were done on the basis of gram staining, characteristic colony morphologies and biochemical tests (IMViC, H₂S and Motility test).

RESULTS

Survey results

A total of 25 households were visited and milk samples were collected. On observation of environmental hygiene in animal house and milking environment, some areas appeared dirty. The floor of all farmers was constructed with concrete materials where it facilitates easy cleaning. Most of farmers (80%) had manure disposal area very near by the farm. Milking was done twice a day by all farmers. Plastic containers used during milking (60%) in most farms appeared dirty in household visit. After milking, the milk was transported to Dairy Co-operative in transport can which was mostly plastic can (64%). Some farmers were found to store evening milk for morning transport due to less milk production. On daily basis, half of the farmers (50%) used detergents for cleaning transport can whereas 32% of farmers used water only. Few farmers (18%) were found to practice both occasionally. The baseline information of sampled animals with milk handling and hygiene practices of farmers is shown in Table 1.

Table 1. Baseline information of sampled animals and management practices related to hygiene

Parameters	Categories	Proportion (%)
Lactation stage	Within 100 days	24
	Beyond 100 days	76
Previous history of Mastitis	Yes	28
	No	72
Discard foremilk during milking	Yes	40
	No	60
Discard of milk during systemic illness	Yes	60
	No	40
Bedding on floor	Yes	96
	No	4
Practice of giving concerntrate while milking	Yes	16
	No	84
Washing Hand before milking	With water only	72
	With soap/Handwash	28
Washing teat and udder during milking	Before and after milking	96
	Only before milking	4
Washing teat and udder with	Water only	84
	Pov. iodine dip	16
Method of Milking	Full Hand Milking	80
	Full Hand Milking followed by striping	20

Brushing or Cleaning animal before milking	Yes	20
	No	80
Straining of Milk	Yes	88
	No	12
Manure Disposal from Shed (approx.)	Near (< 5 m)	80
	Far (> 5 m)	20

Bacteriological quality of milk samples

Total bacterial load

The mean ($\pm$ SE) SPC of different points of production and supply in Western Chitwan is presented in Table 2. The mean SPC of Direct teat milk (n=25), transport can milk (n=25), CC/ Dairy mixed milk (n=5) and chilling vat (n=5) collected from different sites of Western Chitwan were ($34.45 \pm 15.80 \times 10^6$) CFU/ml, ($57.03 \pm 18.34 \times 10^7$) CFU/ml, ($70.60 \pm 49.93 \times 10^7$) CFU/ml and ($162.20 \pm 40.50 \times 10^8$) CFU/ml respectively. There was an increasing trend of total bacterial count as the milk passed through teat, transport can, CC/ dairy and chilling vat as shown in Fig. 3. The result showed highly significant difference among the different points ($P < 0.001$).

Table 2. SPC at different points of milk production and supply chain of Western Chitwan

SPC (Mean $\pm$ SE) CFU/ml	Points of milk production and supply chain				P- value
	Teat (n=25) $\times 10^6$	TC (n=25) $\times 10^7$	DC (n=5) $\times 10^7$	CV (n=5) $\times 10^8$	
	34.45 $\pm$ 15.80	57.03 $\pm$ 18.34	70.60 $\pm$ 49.93	162.20 $\pm$ 40.50	<0.001
Log10 (CFU/ ml)	6.00 $\pm$ 0.31 ^a	7.77 $\pm$ 0.25 ^b	8.52 $\pm$ 0.23 ^{bc}	10.12 $\pm$ 0.2 ^c	

TC=Transport Can, DC= Dairy Co-operative milk collection center, CV=Chilling Vat

^{a, b, c} the values within the row not sharing common superscripts differ significantly ($P < 0.05$).

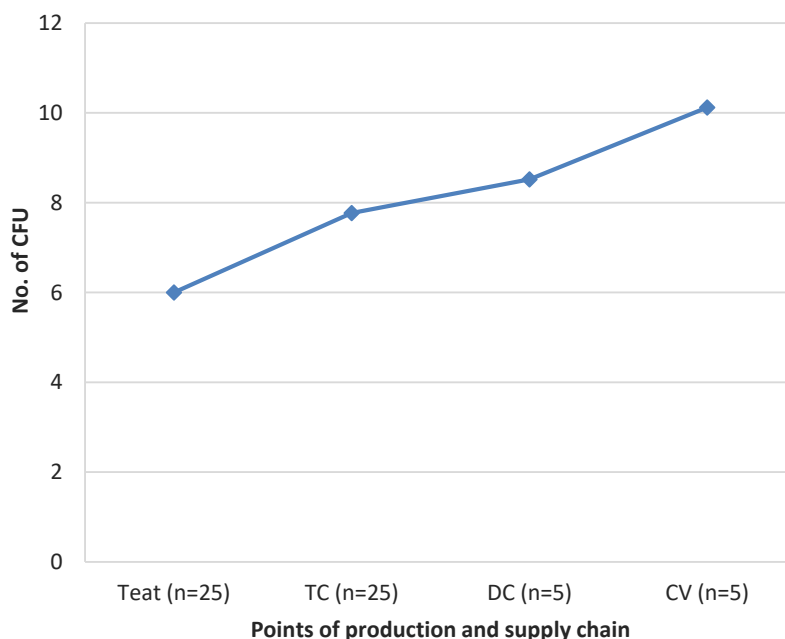


Fig. 3. Line diagram of SPC at different points of production and supply chain

Table 3 shows the SPC (CFU/ml) in cow milk samples collected directly from teat and these from transport can of producers belonging to five DC of Western Chitwan. Overall, the SPC significantly increased from teat to transport can ($P < 0.001$). On comparison of Dairy Co-operative (DC) with the Local private Dairy (LD), this study showed no significant difference in SPC count between LD ($508.40 \pm 248.86 \times 10^7$) and DC ($70.60 \pm 49.93 \times 10^7$) as shown in Table 4. Among 5 samples of pasteurized packet milk, the average SPC (Mean $\pm$ SE) was found to be $(4.50 \pm 3.41) \times 10^5$ CFU/ml. The highest count was found to be of P3 (18×10^5 CFU/ml) and the lowest count of P2 (31×10^3 CFU/ml).

Table 3. SPC in cow milk samples collected directly from teat and those from transport can of producers belonging to five Dairy Co-operatives (DC) of Western Chitwan

Location	Direct from teat (Mean $\pm$ SE)		Transport Can (Mean $\pm$ SE)		P-Value
	CFU/ml (10^5)	Log ₁₀ SPC	CFU/ml (10^6)	Log ₁₀ SPC	
DC1	100 $\pm$ 45.10	6.77 $\pm$ 0.22 ^{ab}	1312.4 $\pm$ 490.95	8.69 $\pm$ 0.44 ^{ab}	0.01
DC2	1501.0 $\pm$ 569.60	7.64 $\pm$ 0.54 ^a	1378 $\pm$ 509.39	8.96 $\pm$ 0.21 ^a	0.05
DC3	1.23 $\pm$ 0.51	4.58 $\pm$ 0.44 ^b	14.49 $\pm$ 5.80	6.78 $\pm$ 0.40 ^c	0.06
DC4	116.03 $\pm$ 47.50	5.86 $\pm$ 0.88 ^{ab}	127.83 $\pm$ 70.44	7.31 $\pm$ 0.62 ^{abc}	0.22
DC5	4.42 $\pm$ 2.95	5.17 $\pm$ 0.35 ^b	18.78 $\pm$ 5.57	7.11 $\pm$ 0.23 ^{bc}	0.01
P-value	0.005		0.003		

a, b, c the values within the column not sharing common superscripts differ significantly ($P < 0.05$).

Table 4. Comparison of SPC in cow milk (CFU/ml) between Dairy Co-operative and Local Private Dairy

	DC	LD	P-Value
CFU/ml (Mean±SE)	70.60±49.93×10 ⁷	508.4±248.9×10 ⁷	0.17
Range	14-270	12-1200	
Log ₁₀ SPC	8.52±0.23	9.22±0.40	

***E. coli* isolation**

The proportion of samples positive for *E. coli* at different points of production and supply chain is shown in Table 5. Among the 60 samples of milk samples analyzed, the *E. coli* was isolated from milk samples of all categories i.e., directly from teat 28% (7/25), transport can 76% (19/25), dairy co-operatives 80% (4/5) and chilling vat 80% (4/5). Likewise, *E. coli* was also isolated from 60% (3/5) milk samples of local private dairy milk and 40 % (2/5) milk samples of pasteurized packet milk. Thus, this study showed that proportion of *E. coli* in milk supply chain increased from farm to consumer's table.

Table 5. Proportion of samples positive for *E. coli* at different points

Source of sample	No. of samples	No. of samples with <i>E. coli</i>	<i>E. coli</i> positive (%)
Direct from teat	25	7	28 ^a
Transport Can	25	19	76 ^b
Dairy Co-operative	5	4	80 ^b
Chilling Vat	5	4	80 ^b

^{a, b} the values within the column not sharing common superscripts differ significantly (P<0.05).

Table 6 shows the factors affecting the contamination of *E. coli* in milk samples directly collected from teats. Only the stage of lactation and history of previous mastitis had effect on *E. coli* contamination. *E. coli* prevalence was higher (P=0.03) in cows within 100 days postpartum as compared to those beyond 100 days postpartum. Likewise, there was a tendency (P=0.07) that the cows with history of previous clinical mastitis had higher prevalence of *E. coli* as compared to those with no history of previous clinical mastitis.

Table 6. Factors affecting the presence of *E. coli* in milk samples directly from teat of cows

Parameters	Level	No. of sample	No. of positive sample (%)	P-value
Stage of lactation	Within 100 days	6	4 (66.67)	0.03
	Beyond 100 days	19	3(18.75)	
History of previous mastitis	Yes	7	4 (57.14)	0.07
	No	18	3 (16.67)	
Suckling by calf	Yes	4	0 (0)	0.29
	No	21	7 (33.33)	
Area of feces disposal	≤ 5 m	20	5 (25)	0.59
	≥ 5 m	5	2 (40)	

DISCUSSION

This study demonstrated that the SPC of raw milk at all points of production and supply chain was beyond the acceptable level (10^5 CFU/ml) (EU, 1992). Furthermore, the study demonstrated that the microbial load significantly increased from teat to chilling cat. While an infected animal may be the source of microbial contamination in raw cow milk, most microbes enter the milk through the handling chain (Gwandu et al., 2018). During this study, it was observed that the most milking environment, milk handling, transport and storage in overall supply was done under poor sanitary conditions. Hygiene practices and the time lag between farmers to dairy co-operative, dairy co-operative to chilling center may be the main reasons that potentiated the high microbial count.

The results underscore the importance of implementing effective hygiene practices at each contamination points to minimize microbial contamination. Addressing factors contributing to elevated microbial counts in transport cans, chilling vats, and collection vessels is crucial for maintaining milk quality. The mean ($\pm$ SE) SPC of teat samples was $34.45 (\pm 15.80) \times 10^6$. The result was found to be higher with the finding of previous studies (Ahmed et al., 2022; Limbu et al., 2020). The data was generated by interviewing 60 dairy cow owners and farm attendants using semi-structured questionnaire regarding milking area and barn hygiene, hygienic practice of milker, cleaning of the udder and milking equipments. The result showed that the majority of the interviewers (98.34%). It might indicate the prevalence of sub-clinical mastitis among the sampled animals. There was also significant increase in the SPC from teat sample to transport can. It might be due to the unclean milking and transporting equipment, inadequate sanitation or extended transport time. Also, only 40% of the farmers were found to discard foremilk while milking. That might also be possible reason for high microbial load as fore-milking practices is commonly done to remove potentially contaminated milk from the teat canal and for early mastitis detection (FSA, 2006).

The SPC of dairy co-operative and local private dairy was found to be similar in this study and also with the TPC studied by (Banik et al., 2015; Mohamed, 2018) pasteurized and UHT (Ultra High Temperature) processed. But the raw milk count highly contrast with that of previous studies (Limbu et al., 2020; Rai et al., 2020; Sah & Poudel, 2015; Suresh et al., 2021; Tassew & Seifu, 2011). High numbers of bacteria in raw milk are indicative of poor handling practices and inadequate maintenance of the cold chain. Contamination of mastitis milk with fresh clean milk and storage conditions affect the microbiological quality of the milk (Jeffrey & Wilson, 1987). The pasteurized samples ($4.5 \pm 3.41 \times 10^5$) CFU/ml showed quite higher count than that recommended by the Department of Food Technology and Quality Control of Nepal (<1000 total plate count/ml) (DFTQC, 2021). However, the result was found to be similar with the findings of previous studies in Nepal (Limbu et al., 2020; Rai et al., 2020) but lower than of Kathmandu (Acharya et al., 2017). The shelf life of pasteurized milk, which ranges from 2 to 20 days, depends on the quality of the raw milk, the processing technique, the hygienic conditions during filling, and the preservation of temperature throughout the cold chain (Rysstad & Kolstad, 2006).

The proportion of *E. coli* positive sample is found to be increasing from teat to other supply chain which supports result of (Sharma, 2016). Unclean equipments for transport and storage tank is a common cause. Coliforms can rapidly build up in the moist residues on the milking equipment which potentially serves as a source of contamination for the milk (Omoro et al., 2002). Since it is a challenge to produce a coliform free milk and its product, the existence of coliform may not always necessarily indicate direct fecal contamination of milk but also a precise indicator of poor hygiene and sanitation during milking and further handling processes.

Even pasteurized milk samples from this study were positive for *E. coli* that supports the findings of (Acharya et al., 2017; Ghimire, 2022; Limbu et al., 2020) but contrast with the possible limit of coliform zero per ml as per Nepal Gazette. The presence of coliforms in pasteurized milk sample may be due to defective pasteurization, adulteration of pasteurized milk with raw milk and unsanitary handling (Hassan et al., 2015). Post pasteurization contamination is common due to presence of some psychrotolerant spore forming bacteria and also some hard nature of thermotolerant bacteria in raw milk (Sarkar, 2015). Proper pasteurization, storage of processed milk at lower temperature and avoidance of post-pasteurization contamination are the keys to produce safe pasteurized milk (Suresh et al., 2021) the process of heating every particle of milk to at least 63°C for 30 min or 72°C for 15 sec or to any temperature-time combination which is equally efficient is one of the major thermal preservation processes adopted by the dairy industry. The microbial groups of high relevance in terms of quality and safety aspects of pasteurized milk are the pasteurization surviving thermotolerant and the low temperature growing psychrotrophic bacteria.

In this study, stage of lactation and history of previous mastitis were found to be significantly associated with *E. coli* prevalence. It is due to the significant physiological changes that a

cow goes through including hormonal imbalance, increased metabolic demands, calving and milk production stress compromising the ability to resist infections. Changes in udder health can create favourable condition for bacterial colonization and opportunistic pathogen like *E. coli* can thrive (Burvenich et al., 2003). History of previous mastitis might compromise the local immune response in udder and make it more susceptible to bacterial invasion increasing risk of recurrent mastitis or it may indicate that the cow recovered from clinical mastitis but might have harbored *E. coli* in the form of subclinical mastitis.

CONCLUSION

The SPC and *E. coli* contamination in raw milk increased at each point of production and supply chain. Earlier stage of lactation and history of previous mastitis influenced the presence of *E. coli* in milk. The bacteriological quality of both raw (dairy co-operatives as well as private dairy) and pasteurized milk was found to be in poor unhygienic condition. The results clearly highlighted the need for improved milk chain procedures, legal enforcement on the microbial guideline of marketed milk, routine monitoring of dairy industries and raw milk vendors, awareness campaign to improve the microbiological as well as keeping quality of the raw and pasteurized milk.

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Conflict of interest

All authors have read and approved the final version of this manuscript. The authors declare that they have no conflict of interest regarding the publication of the manuscript.

REFERENCES

1. Acharya, S., Bimali, N. K., Shrestha, S., & Binod, L. (2017). Bacterial analysis of different types of milk (pasteurized, unpasteurized and raw milk) consumed in Kathmandu Valley. *Tribhuvan University Journal of Microbiology*, 4, 32–38. <https://doi.org/10.3126/tujm.v4i0.21674>
2. Ahmed, Z. A., Kasim, M. H., Tegegne, B., & Salah, H. M. (2022). Assessment of hygiene practices and bacteriological quality of raw cow milk of selected dairy farm in Dessie, Ethiopia. *American Journal of Aquaculture and Animal Science*, 1(1), 27–37. <https://doi.org/10.54536/ajaas.v1i1.1071>
3. Altalhi, A. D., & Hassan, S. A. (2009). Bacterial quality of raw milk investigated by *Escherichia coli* and isolates analysis for specific virulence-gene markers. *Food Control*, 20(10), 913–917. <https://doi.org/10.1016/j.foodcont.2009.01.005>
4. Banik, S. K., Das, K. K., & Uddin, M. A. (2015). Microbiological quality analysis of raw, pasteurized, UHT milk samples collected from different locations in Bangladesh. *Stamford Journal of Microbiology*, 4(1), 5–8. <https://doi.org/10.3329/sjm.v4i1.22753>
5. Burvenich, C., Van Merris, V., Mehrzad, J., Diez-Fraile, A., & Duchateau, L. (2003). Severity of *E. coli* mastitis is mainly determined by cow factors. *Veterinary Research*, 34(5), 521–564. <https://doi.org/10.1051/vetres:2003023>

6. Department of Food Technology and Quality Control. (2021). *Annual bulletin 2078/79 (2020/21)* (Vol. 76).
7. European Union. (1992). *Council Directive 92/46/EEC*.
8. Food Standards Agency. (2006). *Milk hygiene on the dairy farm: A practical guide for milk producers to: The Food Safety and Hygiene (England) Regulations 2013, the Food Hygiene (Wales) Regulations 2006 incorporating EC regulations*.
9. Ghimire, M. (2022). *Microbiological quality and adulteration of pasteurized milk marketed in Dharan, Nepal* [Master's thesis, Tribhuvan University].
10. Gwandu, S. H., Nonga, H. E., Mdegela, R. H., Katakweba, A. S., Suleiman, T. S., & Ryoba, R. (2018). Assessment of raw cow milk quality in smallholder dairy farms in Pemba Island Zanzibar, Tanzania. *Veterinary Medicine International*, 2018, Article 1031726. <https://doi.org/10.1155/2018/1031726>
11. Hassan, G. M., Meshref, A. M. S., & Gomaa, S. (2015). Microbiological quality and safety of fluid milk marketed in Cairo and Giza Governorates. *Current Research in Dairy Sciences*, 7(1), 18–25.
12. Jeffrey, D. C., & Wilson, J. (1987). Effect of mastitis-related bacteria on total bacterial count of bulk milk supplies. *International Journal of Dairy Technology*, 40(2), 23–26. <https://doi.org/10.1111/j.1471-0307.1987.tb02829.x>
13. Kurwijila, R. L., Hansen, K. K., Macha, I. E., Abdallah, K., & Kadigi, H. J. S. (1992). The bacteriological quality of milk from hand and machine milked dairy herds in Morogoro, Tanzania. *African Livestock Research*, 1(2), 59–67.
14. Limbu, D. S., Bantawa, K., Limbu, D. K., Devkota, M., & Ghimire, M. (2020). Microbiological quality and adulteration of pasteurized and raw milk marketed in Dharan, Nepal. *Himalayan Journal of Science and Technology*, 37–44. <https://doi.org/10.3126/hijost.v4i0.33864>
15. Mohamed, F. S. (2018). Bacteriological quality assessment of milk in College of Veterinary Medicine (CVM) Dairy Farm and Kalamino Dairy Farm in Mekelle, Tigray, Ethiopia. *Journal of Dairy & Veterinary Sciences*, 8(2). <https://doi.org/10.19080/jdvs.2018.08.555734>
16. Murphy, S. C., & Boor, K. J. (2000). Trouble-shooting and causes of high bacterial counts in raw milk. *Dairy, Food and Environmental Sanitation*, 20(8), 606–611.
17. Oliver, S. P. (2010). How milk quality is assessed. *Purdue Extension, Purdue University*.
18. Oliver, S. P., Jayarao, B. M., & Almeida, R. A. (2005). Foodborne pathogens in milk and the dairy farm environment: Food safety and public health implications. *Foodborne Pathogens and Disease*, 2(2), 115–129. <https://doi.org/10.1089/fpd.2005.2.115>
19. Omore, A., Arimi, S., Kangethe, E., McDermott, J., Staal, S., Ouma, E., Odhiambo, J., Mwangi, A., Aboge, G., Koroti, E., Koech, R., & Others. (2002). *Assessing and managing milk-borne health risks for the benefit of consumers in Kenya smallholder dairy (R&D) project*.
20. Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. *FEMS Microbiology Reviews*, 37(5), 664–698. <https://doi.org/10.1111/1574-6976.12030>
21. Rai, S., Karki, B., Humagain, S., Rimal, S., Adhikari, S., Adhikari, S., & Thapa, S. (2020). Antibioqram of *Escherichia coli* and *Staphylococcus aureus* isolated from milk sold in Kathmandu district. *Nepal Journal of Biotechnology*, 8(3), 82–86. <https://doi.org/10.3126/njb.v8i3.30080>
22. Rysstad, G., & Kolstad, J. (2006). Extended shelf life milk – Advances in technology. *International Journal of Dairy Technology*, 59(2), 85–96. <https://doi.org/10.1111/j.1471-0307.2006.00247.x>
23. Sah, D., & Poudel, C. (2015). Assessment of microbial quality of milk produced by small dairies in Kathmandu. *Nepalese Veterinary Journal*, 32, 27–43.
24. Sarkar, S. (2015). Microbiological considerations: Pasteurized milk. *International Journal of Dairy*

- Science*, 10(5), 206–218. <https://doi.org/10.3923/ijds.2015.206.218>
25. Sharma, K. P. (2016). *Study on the coliform contamination in milk at various flow levels and seasonal effect on its count* [Bachelor's thesis, Himalayan College of Agricultural Sciences and Technology]. <http://www.nardf.org.np/>
26. Suresh, V., James, L., Beena, A. K., V., A. S., & M. P., D. (2021). Total viable, psychrotrophic and thermotolerant bacterial population of market samples of pasteurized milk. *Milk Science International*, 16–19. <https://doi.org/10.48435/MSI.2021.3>
27. Tassew, A., & Seifu, E. (2011). Microbial quality of raw cow's milk collected from farmers and dairy cooperatives in Bahir Dar Zuria and Mecha district, Ethiopia. *Agriculture and Biology Journal of North America*, 2(1), 29–33. <https://doi.org/10.5251/abjna.2011.2.1.29.33>

Letter to The Editor

Veterinary Training Experience in Japan: A Transformative Learning Opportunity

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To
The Editor
Nepalese Veterinary Journal
Nepal Veterinary Association
Tripureshwor, Kathmandu, Nepal

Dear Editor,

I am writing to share my thoughts on the immense benefits of “**One Year Training Journey at Nihon University of Japan**” that I felt during my recent training program on the recommendation of the Nepal Veterinary Association (NVA) and the sponsorship of the Japan Veterinary Medical Association (JVMA).

INTRODUCTION

“Dream big—but don’t just dream, walk toward it!” I once dreamed of becoming an academically accomplished woman, trained in Japan, one of the most inspiring and technologically advanced countries in the world. That dream came true when I was selected for the TPFV-II program: a one-year training (April 2024–March 2025) for Asian veterinarians, recommended by the Nepal Veterinary Association. The program was organized by the Japan Veterinary Medical Association (JVMA) and funded by the Japan Racing and Livestock Promotion Foundation. Its objective was to enhance the technical capabilities, knowledge, and practical skills of veterinarians across Asia.

The training began with Japanese language lessons delivered by expert instructors from JICE. We were also introduced to Japanese culture through experiences like the traditional tea ceremony, sword demonstrations, and earthquake disaster preparedness training.

University Life

I was placed at Nihon University, Kanagawa Prefecture, under the supervision of Professor Dr. Shibuya Hisashi. He planned a detailed laboratory rotation schedule, and I was thrilled to receive direct mentorship from such a distinguished academic leader. The training period was divided into various shifts of rotations to the multiple laboratories in the institute.

Parasitology Laboratory

My first rotation was in the Parasitology Laboratory, under the guidance of Professors Matsumoto Jun and Masudya Aya. I gained practical skills in diagnostic parasitology, including faecal microscopic examination, pepsin digestion for *Trichinella* spp., molecular identification, and sequence analysis using BioEdit. This experience strengthened my foundation in parasitology and deepened my commitment to molecular diagnostics.

Fish Pathology and Comparative Immunology

Under the mentorship of Moritomo Tadaaki and Katakura Fumihiko, I learned essential laboratory techniques, including animal handling, blood sampling, and dissection. I performed leukocyte isolation, slide preparation, SDS-PAGE, western blotting, and both RNA and DNA extraction. We conducted PCR and Sanger sequencing, and I worked on an immunological assay involving ovalbumin (OVA) antigens to monitor weekly plasma antibody levels. This rotation significantly improved my laboratory proficiency.

Veterinary Hygiene Laboratory

Supervised by Takuya Itou, I learned about cell culture using BHK and TK-ST cell lines, viral diagnostics via fluorescent antibody techniques, and PCR. I also had the opportunity to observe blood collection procedures from cattle at the university's farm. This rotation boosted both my technical confidence and practical competence.

Veterinary Public Health Laboratory

Under the mentorship of Hidenori Kabeya, I worked with fecal samples from deer and wild boars. We isolated and cultured bacteria, and identified them using whole genome sequencing (WGS). This experience emphasised the importance of precision in lab procedures and allowed me to develop hands-on experience with next-generation sequencing technologies.

Veterinary Pathology Laboratory

My final rotation was again under Prof. Shibuya Hisashi and Hirotaka Kondo. This lab focused on animal necropsies for diagnostic purposes. I learned tissue dissection, sectioning, staining, and microscopic evaluation of samples. Participation in case discussions and diagnosis of gross and microscopic lesions honed my diagnostic and analytical skills. I also appreciated the collaborative environment, which encouraged problem-solving and adaptability.

Other Activities

The professors and lab members were incredibly kind, welcoming me with traditional Japanese cuisine and warm hospitality. A memorable visit to the Meguro Parasitological Museum—with its impressive 8.8-meter tapeworm exhibit—and sightseeing in Enoshima were highlights. I was honoured with a farewell party by faculty and lab members, attended special lectures by the Miyazaki Prefectural Livestock Hygiene Centre, and delivered a

presentation on my work in Nepal. I received a certificate of completion at the closing ceremony organised by the JVMA.

Study Trip

In September 2024, we had a study trip—a refreshing break from university life. I gave an interim presentation about my country and professional experiences. We visited sites like the Animal Quarantine Service, Fukuyama Horse Club, National Institute of Animal Health, Kyoto Animal Welfare Centre, and Mizuho Dairy Farm. The cultural experiences, traditional dress, and friendships formed with international peers made this trip truly unforgettable.

My Aim

Looking ahead, I plan to apply the technical skills and knowledge gained from this training to enhance diagnostic approaches in veterinary medicine in my home country. I also aim to share what I've learned with fellow veterinarians in Nepal and continue fostering international collaborations with colleagues across Asia.

CONCLUSION

The TPFVAV-II training program in Japan was more than a professional development opportunity—it was a transformative journey that enhanced my technical expertise, broadened my global perspective, and strengthened my commitment to advancing veterinary services in Nepal. Exchange programs like this are vital for building regional cooperation, fostering innovation, and nurturing future veterinary leaders. I strongly encourage fellow veterinarians to pursue similar opportunities that contribute not only to individual growth but also to the collective advancement of veterinary science in our country.

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