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EDITORIAL

Dear Readers,

Nepal Veterinary Association (NVA) is proud to publish the 34th issue of Nepalese Veterinary Journal (NVJ) which is its official Journal. NVJ considers the articles related to various areas of Veterinary and Animal Sciences. This journal aims at disseminating the technologies and information available within the country to the readers within and abroad. This issue of NVJ anticipates these new information and technologies contribute to improve production and productivity of livestock as well as improve the quality of livestock products which ultimately reflects on better human health and also supports commitment of Nepal Government for self sufficiency in egg, milk and meat in three years.

The present issue has included wide-ranging species from pets, farm animals to fishes inclusive of different disciplines such as animal breeding, genetics and reproduction, animal health, socio-economic studies and policies to be undertaken. Some of the articles in the journal includes the work using the advancement of the scientific technologies as follows. A specific strain of Salmonella found in poultry population of Nepal has been identified using the molecular technique which helps in developing effective disease control strategy. Prevalence of zoonotically important diseases such as Avian Influenza, Tuberculosis, Brucella have been addressed. Low conception of Artificial Insemination in buffalo is the main cause of the economical losses in buffalo farming. Encouraging increase in conception rate revealed by the research using the Fixed Time Artificial Insemination (FTAI) technique will be very useful to the buffalo raisers and addresses the burning issue. Nepal has rich indigenous knowledge for treatment of different diseases which is also documented. Herbal treatment using Stinging Nettle supplement has proven effective in milk production. Economically important diseases such as Coccidiosis in poultry, policy paper on marketing and value chain are also included.

I would like to extend my heartfelt thanks to the respected reviewers and the members of the editorial team for their untiring efforts on editing and reshaping the papers for publication. In addition, the special assistance of Ms. Pramina Shrestha in type setting is also acknowledged. NVJ Editorial team anticipates papers from the professional colleagues on core as well as relevant research on livestock and poultry sector using recent advancement in science and will publish in the upcoming special issue on the occasion of the 50th Anniversary of the NVA.

Thanking you for your everlasting support!!

Neena Amatya Gorkhali (PhD, Animal Breeding, Genetics and Reproduction)
Editor-in-chief

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ISOLATION AND GENOMIC IDENTIFICATION OF *SALMONELLA PULLORUM* IN THE MAJOR POULTRY FARMS OF NEPAL

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ABSTRACT

In the present study, Salmonella pullorum bacteria were isolated from 30 liver samples of poultry collected during the postmortem examination of suspected cases of pullorum disease from five major poultry producing districts (Kathmandu, Lalitpur, Bhaktapur, Kavre and Chitwan) of central Nepal in 2014. After isolating bacterial colonies from 30 samples in Brilliant Green Agar (BGA) and Xylose Lysine Deoxycholate (XLD) agar media, biochemical tests carried out for the identification of bacteria. On carbohydrate fermentation tests, 25 isolates showed metabolic characteristics typical for Salmonella while genomic analysis performed with Polymerase Chain Reaction (PCR) by amplifying Salmonella pullorum specific rfbS gene (187 bp) targeting Adenine residue at 237 positions confirmed that 18 isolates were from poultry suffering from pullorum disease. Present result warranted the need for routine biochemical and genomic investigations for better understanding of the species of Salmonella prevalent and subsequent adoption of suitable control strategy in the poultry operation.

Keywords: Salmonella pullorum, biochemical tests, PCR, rfbS gene

INTRODUCTION

Pullorum disease in poultry is one of the important bacterial diseases causing insidious economic losses and having great implication on food security. In Nepal, the economic loss incurred by Salmonellosis in poultry operation is very high. Pullorum disease is caused by *Salmonella enteric subspecies serovar Gallinarum biovar pullorum* (*Salmonella pullorum*) (Brooks *et al.*, 2008). The sites of predilection of these Gram-negative bacteria in birds are liver, spleen and reproductive tract. The symptoms of pullorum disease are inappetance, depression, ruffled feathers, closed eyes, loud chirping, white diarrhoea, vent pasting, gasping, lameness in chicks and severe drop in egg production in layers.

Various selective and non-selective enrichment methods are used for the isolation of Salmonella bacteria (OIE 2012) while biochemical tests (WHO Global Food-borne Infections Network, 2010), classical carbohydrate fermentation tests (Nordic Committee on Food Analysis 1999) and PCR of the bacterial genomic DNA (Cha *et al.*, 2008) are used to identify the bacteria. The colonies showing typical characteristics of Salmonella are subjected to biochemical tests that describe the metabolic activity of particular microorganism; it therefore, serves as a confirmatory test for the isolated bacteria. *Salmonella pullorum* unlike other Salmonella species ferment glucose, ornithine and

lysine but not urea, lactose, citrate, indole and sorbitol (OIE, 2012). Various culture media used are Triple Sugar Iodine, Salmonella Shigella agar, McConkey agar, Motility Indole Ornithine, Simmons Citrate, Methyl red-Voges Proskauer and Eosin Methylene Blue (OIE 2012; Hossain *et al.*, 2006).

Sero-typing is done reliably through an agglutination reaction of the cellular O antigen in *Salmonella* serotypes with anti O antigen-antibody. However, it cannot differentiate between closely related biotypes such as *Salmonella pullorum* and *Salmonella gallinarum* (Hossain *et al.*, 2006; Brooks *et al.*, 2008) while classical carbohydrate fermentation tests can differentiate. *Salmonella pullorum* cannot utilize dulcitol, maltose and lactose as carbon source and uses arabinose, dextrose and lysine as energy source (OIE 2012).

Apart from classical fermentation tests, PCR is an accurate tool to detect *Salmonella* serotypes. The *Salmonella rfbS* gene, which encodes for paratose synthetase, an enzyme required in the final steps of biosynthesis pathway of O- side chain sugar is uniquely present on the *Salmonella* sero group (Devendra *et al.*, 2005) and the nucleotide sequence varied according to bacteria. Fragment of *rfbS* gene has polymorphic nucleotide sequence for *Salmonella gallinarum* (SG) and *Salmonella pullorum* (SP). SP isolates has guanine and adenine residue at position 598 and 237, respectively whereas SG has adenine and guanine residues at 598 and 237 positions, respectively (Devendra *et al.* 2005). PCR amplification done by targeting the pairing of 598th nucleotide. Because of this polymorphism, amplification is altered. The primer sequence with the forward primer, *rfbS*F(5'-GTA TGG TTA TTA GAC GTT GTT-3') complements bases 431 to 451 at this gene and the reverse primer *rfbS* R(5'-TAT TCA CGA ATT GAT ATA TCC-3') complements bases 617 to 597. The primer used amplifies SP DNA only thus confirming that the bacterial DNA is of *Salmonella pullorum* (Devendra *et al.*, 2005).

This study aimed to isolate *Salmonella pullorum* strains from different diseased samples followed by differentiation using biochemical tests and genomic investigation.

MATERIALS AND METHODS

In the present study, *Salmonella pullorum* bacteria were isolated from the liver samples during postmortem examination from chicken of Kathmandu(5), Lalitpur(5), Bhaktapur(3), Chitwan(10) and Kavre(7) districts. All bacterial cultures were performed at the Animal Health Research Division of NARC. The liver samples collected were pre-enriched in the peptone water to recover sub-lethally damaged bacteria (OIE 2012; Nordic Committee on Food Analysis 1999), and the selective enrichment media with high osmotic pressure, relatively lower pH and higher temperatures for enabling bacteria to multiply as compared with other gut bacteria. The collected samples were weighed, and 12 gm of each sample was placed in a conical flask containing 100 ml of dehydrated peptone water medium (1:9) and incubated at 37°C for 24 hours for non-selective enrichment (OIE 2012). For selective enrichment, 0.1 ml and 1 ml of peptone water were mixed in 10 ml Rappaport Vassiliadis soya peptone broth medium at 37°C and 10 ml Tetrathionate broth

at 41.4°C, respectively for 24 hours (OIE 2012). Bile salts in the media act as the selective agent to retard the growth of Gram-negative bacilli other than *Salmonellae* and inhibit Gram-positive organisms (Funk *et al.*, 2000). Bacterial culture was made with one full inoculum loop on Brilliant Green Agar (BGA) and Xylose Lysine Deoxycholate (XLD) agar and incubated at 37°C for 24 hours (OIE 2012). Then subculture was made on Nutrient agar and incubated at 37°C for 24 hours. Isolates were confirmed to be *Salmonella* by biochemical tests on Triple Sugar Iodide (TSI) agar, Motility Indole Ornithine agar, Simmons Citrate agar, Methyl Red-Voges Proskauer test, MacConkey agar, *Salmonella Shigella* agar and Eosin Methylene Blue agar. Fermentation of sugars namely lactose, sucrose, mannitol, dulcitol, acid and/or gas production on glucose, fructose, xylose and arabinose incubated at 37°C for 24 hours were carried out. Serotyping of the bacteria was done by performing slide agglutination test of the bacterial suspension and antiserum (Pullorum Antigen, Stained Antigen Polyvalent, Charles River, USA).

DNA extraction

DNA was extracted by using phenol chloroform method (Wilson 2001; Edwards *et al.*, 2004). Bacterial colonies were prepared on nutrient broth and incubated overnight. It was transferred to an Eppendorf tube and centrifuged, and the resultant bacterial pellet was re-suspended in TE buffer, cell lysis was done by SDS and proteinase K under incubation at room temperature. An equal volume of phenol chloroform was added and centrifuged to the lysate. The aqueous layer was transferred in fresh tube, to which sodium acetate and iso-propanol were added gently and DNA was allowed to precipitate. The DNA was centrifuged, washed with 70% ethanol and stored on TE buffer.

PCR amplification

For the PCR, master mix product name, primers (forward and reverse) DNA and nuclease free water was mixed in PCR tubes in the proportionate amount. The reaction cycle was carried out in a thermo-cycler, initial denaturation was done at 94°C for 10 minutes, cycle was carried out at 94°C for 1 minutes, annealing was done at 46°C for 1 minute and extension was done at 72°C for 1 minute for 30 cycles. Final extension was done at 72°C for 5 minutes and stored at 4°C. The amplified product was loaded on 2% gel and observed under UV documentation apparatus (Cha 2008; Gunaseelan *et al.*, 2012).

RESULTS AND DISCUSSION

The bacterial culture observed during the initial period of isolation was turbid on peptone water with unpleasant odor. Dirty and murky broth was observed during the enrichment on TTB and RVS medium in addition to small black and pink colonies on the XLD and BGA plates. Moreover, the biochemical tests on Triple Sugar Iodide, *Salmonella Shigella*, Methyl Red showed positive and some negative results on Voges Proskauer, urease, citrate and Motility Indole Ornithine agar. The colonies showing positive biochemical tests were further subjected to classical fermentation tests. This showed dulcitol, lactose, maltose and sucrose negative, whereas, mannitol, glucose, dextrose and lysine positive.

While serotyping, agglutination was observed confirming the isolated bacteria to be *Salmonella* that was further confirmed by PCR after amplification of *rfs* gene. In the present study, *Salmonella pullorum* bacteria isolated following standard protocols of pre-enrichment and enrichment on selective media (OIE 2012; WHO Global Foodborne Infections Network 2010). were selected for classical carbohydrate fermentation tests. Eventually those bacterial colonies which fulfilled the criteria of *Salmonella pullorum* were selected for PCR diagnosis using the *rfs* gene primer.

Eighteen out of 30 samples gave the amplified DNA band of 187bp and thus confirming that the bacteria identified in biochemical and fermentation tests are *Salmonella pullorum* (Fig. 1). The PCR result gave clear bands of 187 bp on agarose gel under UV light indicating the presence of *Salmonella pullorum* bacteria. To our knowledge, this is the first reported study of molecular characterization of *Salmonella pullorum* in Nepal using PCR.

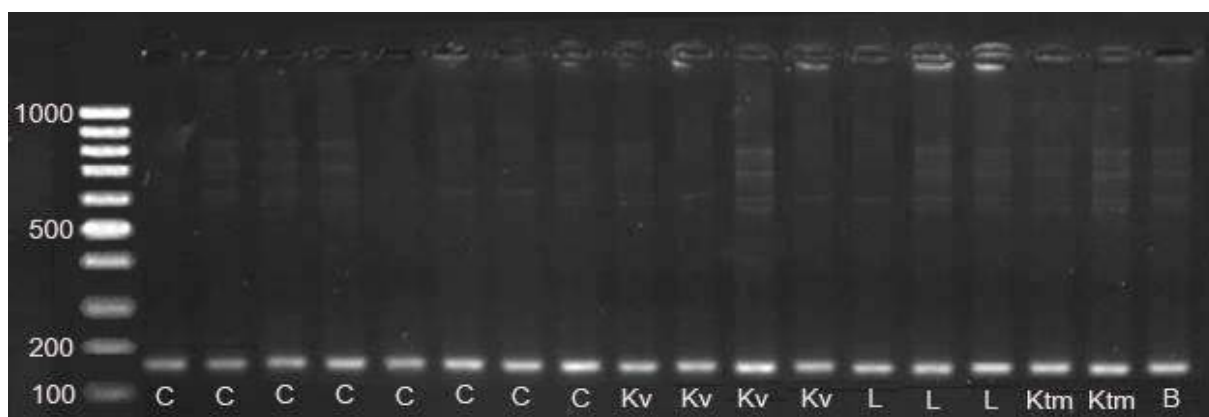


Fig1: Showing 187bp DNA bands of *Salmonella pullorum* isolated from 18 liver samples collected from Chitwan (C), Kavre (Kv), Lalitpur (L), Kathmandu (Ktm) and Bhaktapur (B).

CONCLUSION

Salmonella pullorum bacteria isolated from the infected liver samples from five different districts of Nepal were confirmed by biochemical tests and PCR amplification with demonstration of 187bp of amplified product.

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STUDY OF ANTIBIOTIC RESISTANCE ON *ESCHERICHIA COLI* IN COMMERCIAL POULTRY OF NEPAL

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ABSTRACT

The continuous use of antibiotics in compound feed at sub-therapeutic level has been an integral part of commercial poultry production in Nepal, which is one of the factors that promotes bacterial resistance. Hence, with the objective to determine antibiotic resistance in commercial poultry of Nepal, this study was designed taking Escherichia coli as a flagship bacterium. The commercial layers and broilers birds brought to veterinary teaching hospital of Agriculture and Forestry University by commercial poultry producers for disease diagnosis and treatment were considered as clinical examination of birds were carried out followed by post mortem examination (PME). Those layer/broiler birds which were not taking antibiotic orally or parenterally for last 2 weeks and diagnosed with colibacillosis on PME were included in sampling frame. Air sacculitis, fibrinous pericarditis, fibrinous perihepatitis, and coligranuloma were major criteria for presumptive diagnosis of colibacillosis on PME. The first 40 for both broiler and layer birds totaling 80 that fulfilled the criteria were selected as samples, each representing a commercial farm. All necessary information on daily management practices and previous treatments were obtained from farmer's record book or sheets or face to face interview. Avian pathogenic E. coli was isolated from aseptically collected liver samples and confirmed by biochemical tests. Antibigram of the isolates were investigated by means of Kirby-Bauer disc diffusion method. E. coli was isolated from all liver samples taken for the culture. It was found that E. coli were resistant most substantially towards Cephalixin (81.2%) and Amoxycillin (81.2%) followed by Tetracycline (78.8%), Colistin sulphate (n=50, 62.5%). Chloramphenicol (61.2%), Ciprofloxacin (55.0%), Enrofloxacin (53.8%), Levofloxacin (28.8%), however, no resistance was found against amikacin. The proportion of E. coli isolates that were resistance against Colistin sulphate ($p < 0.05$), Chloramphenicol ($p < 0.05$), Tetracycline ($p < 0.001$), Ciprofloxacin ($p < 0.01$), Enrofloxacin ($p < 0.05$) and Gentamicin ($p < 0.01$) were significantly higher in layers compared to that of broilers. In conclusion, avian pathogenic E. coli were resistant towards several antibiotic molecules commonly used in commercial poultry of Nepal, and the resistance was higher in layers compared to broilers.

Keywords: antibiotic resistance, *E. coli*, poultry

INTRODUCTION

The use of antibiotics in compound feeds has been an integral part of poultry production, not only to prevent infectious bacterial diseases but also to promote growth of host. Almost all poultry feed industry in Nepal commonly use different molecules of antibiotics as feed additives in compound feed, pelleted or mash, with the objective to enhance growth and feed efficiency. This continuous use of several types of antibiotic molecules at sub therapeutic level in feeds promotes the bacterial resistance in poultry (Aryal, 2001). The bacteria acquire resistance to particular class of antibiotic either due to de novo mutation, or due to transmission of acquired resistance from one bacterium to others, within species or between species via extra chromosomal DNA (Tenover, 2006). Resistant bacteria thwart antibiotics by interfering their mode of action such as synthesis of inactivating enzymes, alteration in configuration of cell wall or ribosome, and modification of membrane carrier systems, (Timoney *et al.*, 1988; Prescott and Dowling, 2013), inhibition of nucleic acid synthesis, inhibition of metabolic pathway (Tenover, 2006). The widespread use of antibiotics as feed additives for growth promotion or disease prevention in food animals could have negative implications for human health and the environment (Hasan *et al.*, 2011).

They also noted a common way of transmission of resistant clones and resistance plasmids of *E. coli* from poultry to human. The development of antibiotic resistance in human bacteria and its association with sub-therapeutic use of antibiotic in animal feed were discussed since early 70's (Dibner and Richards, 2005). The *E. coli* resistant isolates from poultry in a region plays important role for human *E. coli* infections in that region as Agabou *et al.* (2016) showed a clonal and epidemiological link between chicken and human ciprofloxacin-resistant *E coli* isolates in Algeria. Furthermore, the intestine of poultry consists zoonotic potential *E. coli*, which can be transferred from birds to human (Ewers *et al.*, 2009). The continuous use of antibiotics in compound feed creates a selective pressure to develop resistant *E. coli* in poultry gut that could make its way in human food chain (Diarra *et al.*, 2007). Additionally, in birds, the resistance transferred from generation to generation as pyramid. In a recent study at Sweden, scientists reported occurrence of *E. coli* resistant to nalidixic acid in grandparent birds imported to Sweden for breeding purpose and resistance transferred to parents and their broilers. Similarly, quinolones resistant *E coli* can be introduced from imported breeding birds and spread by vertical transmission (Borjesson *et al.*, 2016).

In Nepal, monitoring of antibiotic resistance in veterinary medical field started very late (Khatriwada, 2011) and only a limited number of papers have been published. Antibigram study of *E. coli* isolated in Nepal from apparently healthy and diarrheic ducks were studied by Singh *et al.*, (2013). Shrestha *et al.*, (2010) first reported a finding on antimicrobial resistance of Salmonella in poultry of Nepal. In human medical field in Nepal, a couple of studies have been conducted to understand antibiogram of *E. coli* isolated from patients of urinary tract infection (UTI). Sharma *et al.*, (2013) and Baral *et al.*, (2012) found *E. coli* as one of the major causes of UTI. Majority of *E. coli* showed a resistance towards ampicillin, cephalexin, Nalidixic acid and ciprofloxacin and are multidrug resistant.

In most of the researches, antimicrobial resistance profile of *E. coli* is studied in apparently healthy poultry where *E. coli* are isolated from faecal samples. However, in this research, the antimicrobial resistance was studied in birds which suffered from colibacillosis and *E. coli* was isolated from liver but not from faeces. In this sense, this research is unique and novel. Here, the focus is on the antimicrobial resistance pattern of *E. coli* isolated from birds infected with avian pathogenic *E. coli*. A continuous nation wise surveillance of antimicrobial resistance of *E. coli* could be a major step to understand the pattern of resistance and combat treatment failure in poultry. So, with the objective to evaluate antibiotic resistance profile in commercial broilers and layers, this study was designed taking avian pathogenic *E. coli* as a flagship bacterium.

MATERIALS AND METHODS

This research was conducted in Veterinary Teaching Hospital (VTH) and postgraduate laboratory of Department of Veterinary Medicine, Agriculture and Forestry University (AFU) of Nepal during 2014 to January 2016.

Study population

All those birds dead or live which were brought to veterinary teaching hospital for clinical and post mortem examination were defined as study population.

Clinical and PM examination, Inclusion criteria and Sample collection

First, a thorough clinical examination of the birds was conducted with special focus on clinical symptoms of colibacillosis such as swollen head, swollen eyelid, diarrhetic vent, swollen joints etc. If the birds were live, they were sacrificed by detaching atlanto-axial joint through swirling of neck. A team of registered and experienced veterinarians conducted post mortem examination (PME) of dead birds. Air sacculitis, fibrinous pericarditis, fibrinous perihepatitis, and coligranuloma were major criteria for presumptive diagnosis of colibacillosis on PME. Those layer/broiler birds which were not taking antibiotic orally or parenterally for last 2 weeks before they were brought to hospital and diagnosed with colibacillosis on PME were included in sampling frame. The first 40 birds for both broiler and layer type chicken totaling 80 birds that fulfilled the inclusion criteria were selected to collect liver samples during PME. Here, each bird in the sampling frame represented a commercial farm. All necessary information on daily management practices and previous treatments were obtained from farmer's record book, sheets or face to face interview.

The reason to take equal number of sample for both broilers and layers was to make a comparison. During postmortem examination, the liver samples were collected aseptically and dispatched to Post graduate laboratory of Department of Veterinary Medicine and Microbiology Unit of Veterinary Teaching Hospital, AFU for culture, isolation and necessary biochemical tests.

Bacteriological Examination

The surface of liver sample in petriplate was first sterilized with red hot spatula. The red-hot inoculating loop was first cooled on the sterilized surface of liver and then inserted gently into liver to take a loopful of content as inoculum. The inoculum was cultured in MacConkey agar and Eosine Methylene Blue (EMB) (HiMedia, India). The quadrant streak method, using whole plate, was employed for culture. After inoculation, the media was incubated for at least 24 hours at 37°C and colony was observed. Pink colony on MacConkey and metallic blue sheen in EMB agar were suggestive of *E. coli*. Colonies showing typical *E. coli* characteristics and morphology were transferred to nutrient agar and incubated at 37 °C for 24 hours. A single colony was used for biochemical test and confirmation. Confirmation of *E. coli* was drawn by positive Indole test, positive Methyl Red test, negative Voges Praskaur Test, and negative Citrate utilization test [IMViC test (+ + - -)] (Quinn *et al.*, 1984, and Prescott & Dowling, 2013). In indole test, pink/red color on the top of broth indicates a positive result for *E. coli*. Methyl red (MR) and Voges Proskauer (VP) test used MRVP broth. Appearance of red color in MR test and pinkish red color in VP test indicates positive for *E. coli*. Appearance of blue color in citrate agar means positive test for *E. coli*.

Antimicrobial susceptibility testing

The isolates of avian pathogenic *E. Coli* from nutrient agar were subjected for antimicrobial susceptibility test (AST) for study of resistance pattern according to protocol of National Committee for Clinical Laboratory Standards (NCCLS, 2003). The *in vitro* antimicrobial susceptibility pattern of *E. coli* was determined in Muller Hinton agar plates by Kirby-Bauer disc diffusion method. For this, at least 20 pure colonies of *E. coli* were taken directly from nutrient agar plate to make a suspension of 1 ml normal saline. The colony was mixed well in the vortex mixture and a slight turbid color was obtained. The suspension was streaked using a sterile, non-toxic swab on an applicator stick, in three directions over the entire surface of Muller-Hinton media (Hi-media, India) to obtain uniform inoculation. The antimicrobial disc (HiMedia, India) was placed onto the agar surface gently using sterile forceps and kept no closer than 24 mm (center to center) which is equivalent to 6 discs per standard 90 mm petri-dish. The medium was incubated aerobically at 37° C for 24 hours. The degree of resistance or susceptibility was studied based on zone of inhibition, and interpretation was inferred as mentioned in (Table 1).

The selection of antimicrobial molecules was based on their frequency and volume of use as additive in compound feed and while treating colibacillosis in poultry. This information was obtained from registered veterinarians working in poultry industries in Chitwan district, veterinary teaching hospital of AFU, avian laboratories, and veterinary drug centers in Chitwan district through either face to face interview or database analysis of veterinary teaching hospital of AFU. The antimicrobial molecules selected were given in (Table 1).

Table 1: Antimicrobials and their concentration tested in disc diffusion method, and their interpretation criteria

Antimicrobial disc	Abbreviation	Concentration /disc	Interpretation criteria of zone of inhibition (mm)		
			Resistance	Intermediate	Sensitive
Amoxycillin	AC	20 µg	≤13	14-17	≥18
Colistin Sulphate	CL	10 µg	≤10		≥11
Cephalexin	CFX	5 µg	≤15	16-18	≥19
Chloramphenicol	C	30 µg	≤12	13-17	≥18
Tetracycline	T	30 µg	≤14	15-18	≥19
Ciprofloxacin	CF	5 µg	≤15	16-20	≥21
Enrofloxacin	EN	10 µg	≤14	15-17	≥18
Livofloxacin	LE	5 µg	≤13	14-16	≥17
Gentamicin	G	10 µg	≤12	13-14	≥15
Amikacin	AK	30 µg	≤14	15-16	≥17

Statistical analysis

The required data were collected based on clinical signs, post mortem examination of live or dead birds, culture, isolation and antimicrobial susceptibility test. All descriptive statistics were presented as frequency and percentage. The resistance profile was described in frequency and proportion. The association of antimicrobial resistance of *E coli* with other factors was studied through Pearson's chi square test and Fisher's exact test (for the frequency less than 5). The data analysis was done using SPSS 18.0 version.

RESULTS

The result showed that most of the commercial birds brought to hospitals were Hy-line, Lohman, and H& N for laying hen types and Vancobb- 500 for broiler types. Swollen head syndrome, lameness, synovitis were the characteristic clinical signs and post mortem lesions for diagnosis of colibacillosis. The recognition of presence of these clinical signs and post mortem lesions in broilers or layers was based on the knowledge and experience of Veterinarians in the research team and standard photographs of lesions. The distribution of these clinical signs and post mortem lesions are given in (Table 2). Significantly, higher proportion of broiler birds were found to suffer from swollen head syndrome, lameness, synovitis compared to that of layer birds. Omphalitis was significantly higher in broiler types, however, coligranuloma was significantly higher in laying hen type birds (Table 2).

The association of type of bird (broilers or layers) with presence or absence of characteristics clinical signs and post mortem lesion (one by one) was studied through Pearson's chi-square test (a 2×2 contingency table for each sign and lesion). The degree of freedom was 1 [(2-1) × (2-1) =1].

Table 2: Distribution of clinical signs and postmortem lesions in birds (Broilers and Layers)

Clinical signs	Bird type		Pearson's χ^2 - test p value
	Broiler (n=40)	Layers (n=40)	
Swollen head	21 (52.5)	5 (12.5)	0.00
Lameness	17 (42.5)	3 (7.5)	0.01*
Synovitis	11 (27.5)	1 (2.5)	0.03*
Post-mortem lesions			
Omphalitis	16 (40.0)	0 (0)	NA
Peritonitis	17 (42.5)	23 (57.5)	0.26
Fibrinous pericarditis	34 (85.0)	38 (95.0)	0.63
Fibrinous perihepatitis	31 (77.5)	31 (77.5)	
Coligranuloma	0 (0)	9 (22.5)	0.02*

The value in parentheses are in percent (within the bird type). * Fischer's exact test

NA= The omphalitis was not compared between broilers and layers because it is the characteristics post mortem lesion on broilers only but not in layers. So, there were no any omphalitis in laying hens.

Antimicrobial resistance pattern in poultry

The antibiogram study showed that avian pathogenic *E. coli* isolated from layers had developed resistance to several antibiotic molecules in higher proportion compared to that of broilers. In addition, the avian pathogenic *E. coli* showed relatively higher resistance to cephalexin and amoxicillin compared to other antibiotics both in broilers and laying hen birds. However, we found that *E. coli* had not developed resistance to amikacin both in broilers and layers. The interesting finding, we got in this study was that a proportion of *E. coli* isolated from layers had shown resistance to Gentamicin but none of the *E. coli* isolated from broilers had shown resistance towards Gentamicin (Table 3).

Table 3: Comparison of resistance of *E. coli* to different antibiotic molecules in birds

Antimicrobial agent	Antimicrobial resistance in bird type		Pearson's χ^2 test p-value
	Broiler (n=40)	Layer (n=40)	
Amoxicillin	30 (75.0)	35 (87.5)	0.275
Colistin sulphate	20 (50.0)	30 (75.0)	0.021
Cephalexin	31 (77.5)	36 (90.0)	0.130
Chloramphenicol	19 (47.5)	30 (75.0)	0.012
Tetracycline	25 (62.5)	38 (95.0)	0.000
Ciprofloxacin	16 (40.0)	28 (70.0)	0.007
Enrofloxacin	15 (37.4)	28 (70.0)	0.004
Levofloxacin	7 (17.5)	16 (40.0)	0.059
Gentamicin	0 (0)	7 (17.5)	0.006*
Amikacin	0 (0)	0 (0)	

The value in parenthesis indicates percent; * Fischer's exact test

The association of antimicrobial resistance of *E. coli* with type of bird (broilers or layers) was studied (one by one for each antimicrobial) through Pearson's chi-square test. A 2×2 contingency table was formed (broilers and layer vs resistance or not resistance). Thus, the degree of freedom was 1 [(2-1) × (2-1) =1].

In our study, *E. coli* resistance to Cephalexin was highest (81%, N=80) followed by Amoxicillin (81%), Tetracycline (79%), Colistin sulphate (62%), Chloramphenicol (61%), Ciprofloxacin (55%), Enrofloxacin (54%), Levofloxacin (29%), but there was no resistance against amikacin. The *E. coli* isolate which is resistant to two or more than two antimicrobials were considered multidrug resistant isolates. Substantial proportion of *E. coli* were multidrug resistance. The frequency of multidrug resistant *E. coli* isolated from broilers and layers were highest for Amoxicillin-Cephalexin combination, and Tetracycline and Amoxicillin combination respectively. The details of multidrug antimicrobial resistance profile of *E. coli* isolates are presented in (Table 4).

Table 4: Number of *E. coli* isolates resistance to various antimicrobials

No. of antimicrobials resistant to which <i>E. coli</i>	Combination	No. of resistant isolates of <i>E. coli</i>	
		Broiler	Layers
2	AC-CFX (Broiler) AC-T (Layer)	30	34
3		22	31
4	AC-CFX-T-C	17	26
5	AC-CFX-T-C-CL	14	23
6	AC-CFX-T-C-CL-CF	9	17
7	AC-CFX-T-C-CL-CF-LE (Broiler) AC-CFX-T-C-CL-CF-EN (Layer)	2	8
8	AC-CFX-T-C-CL-CF-LE-G	0	1
9	AC-CFX-T-C-CL-CF-EN-LE-G	0	0
10	AC-CFX-T-C-CL-CF-EN-LE-G-AK	0	0

The resistance profile of *E. coli* for combination with highest frequency are mentioned only. For eg: *E. coli* had highest no of resistance for combination of two antimicrobials (Amoxicillin and Cephalexin) in broiler and that of tetracycline and Cephalexin in layers.

DISCUSSION

The avian pathogenic *E. coli* showed the highest level of resistance to cephalexin in both broiler and layer type birds. The antimicrobial resistance of *E. coli* isolated from layers showed a significant higher proportion for colistin sulphate ($p<0.05$), chloramphenicol ($p<0.05$), tetracycline ($p<0.001$), ciprofloxacin ($p<0.01$), enrofloxacin ($p<0.05$) and gentamicin ($p<0.01$) in comparison to that of broilers. All the *E. coli* isolated from broilers were sensitive for gentamicin whereas 17.5% ($n=7$) of isolates in layers had developed resistance to it. *E. coli* isolates from layers mostly showed resistant for most of the antibiotic molecules. The high resistance to several antibiotics in layers might be due to high level of selective pressure because of continuous use of antibiotics since long time in

feed. The continuous use of antimicrobial agent creates a selective pressure on bacteria to emerge as a resistant strain (Tenvor, 2006). Dibner & Richards (2005) discussed an epidemiological linkage on prophylactic use of antimicrobials in animal feed and emergence of resistance. World Health Organization (2000) suggested in the report that animal health management should be routinely practiced avoiding prophylactic use of antimicrobials to as to take precaution to prevent antibiotic resistance. All these reports and finding underpins that the continuous use of antimicrobials is one of the causes to develop resistance in bacteria. This finding coincides with the findings of Karczmarczyk, *et al.*, (2011) where resistance to Tetracycline was the highest followed by Cephalothin (cephalosporin), the Amoxicillin. This finding is also partly supported by the findings of Saidi *et al.*, (2012) where *E. coli* isolates showed moderate rates of resistance to tetracycline and chloramphenicol.

This study showed that most of *E. coli* isolates were multiple drug resistant (MDR) which is in the line of Guerra *et al.*, (2003), Miranda *et al.*, (2008) and Jiang *et al.*, (2011). Antibiotic resistance of avian bacterial pathogens is also a common problem in poultry in Bangladesh. Hasan *et al.*, (2011) noted that more than 55% (N=101) of *E. coli* isolated were resistant to at least one or more of the tested antibiotics, and 36% of the isolates showed multiple-drug-resistant phenotypes. The most common resistances observed were against Tetracycline. This supports our finding in the way that the resistance of *E. coli* was highest against tetracycline in laying birds. It also reported moderate resistance to gentamicin which is in same line to our finding.

According to Jiang *et al.*, (2011), *E. coli* strains isolated from both apparently healthy (from feces) and diseased (from liver) poultry (chicken, ducks and partridges) (n=389) from China had highest resistance to tetracycline (91%), amoxicillin (40%), and Chloramphenicol (34%). The high rate of resistance towards tetracycline, amoxicillin and chloramphenicol support our finding. They also found substantial proportion of *E. coli* isolates from poultry which were multidrug resistant. Similarly, Zhang *et al.*, (2014) from Hebei of China reported high rate of resistance toward (N=111) gentamicin (95%), amikacin (46%), which is contrast to this study. This can be because of continuous over exploitation of aminoglycosides in growing and finishing poultry in China, however the drugs antimicrobials in the group of aminoglycosides are not commonly used in poultry feed. Samanta *et al.*, (2013) from India reported prevalence of *E. coli* in healthy layers and their environment. The report showed that the resistance of the isolates was most frequently observed to chloramphenicol (87%, n= 313). In their study, none of the isolates was found to possess quinolone resistance which is contrast to our finding where substantial proportion of *E. coli* have developed resistant to quinolones (ciprofloxacin, levofloxacin). Several reports of multidrug resistant *E. coli* isolated from apparently healthy or sick chickens or from faecal and liver samples of poultry have been published emanating from African countries (Maine *et al.*, 1998; Geornaras *et al.*, 2001; Oladele *et al.*, 2008,).

One of the most striking factors for antimicrobial resistance in poultry is the haphazard use of antibiotics. The other factors which promotes this resistance might be poor sanitation of barn and hence the crowding of several bacteria in gastrointestinal tract of

poultry. The higher level of antibiotic resistance in laying hens could be because of continuous use of antibiotics for longer time compared to that of broilers. All the laying hens in the samples were more than 20 weeks of age and they have been provided with antibiotics in feed since day one old. The higher level of resistance to Amoxicillin and Tetracycline shown in the study could be because of higher use of these antimicrobial molecules in poultry either in feed or via drinking water. Amoxicillin and tetracycline are the most commonly used antibiotic molecules for treatment of poultry in Nepal. Most of the researchers Miranda *et al.*, (2008); Rosengren(2008) and Varga *et al.*, (2009) and Jiang *et al.*, (2011) agreed that there is a direct relationship between antimicrobial resistance and antimicrobial use. The layers birds have longer period of growth relative broilers which results in consumption of higher quantities of a wider range of antimicrobials by laying hens. This could be a reason for higher proportion of antimicrobial resistance of *E. coli* to various antimicrobials (Jianget *al.*, 2011).

The higher proportion of resistant bacteria towards amoxicillin and tetracycline possess a chance of spilling over to other population. These bacteria can transmit the resistance gene to other bacteria to develop the resistance for antimicrobials (Aleksun and Levy, 2007). Till date, the resistance towards amikacin has not been reported in poultry, however, if the use of antibiotic molecules increases in the increasing order, some days in future, the bacteria in poultry can develop resistance substantially even to gentamicin and amikacin. The intestinal flora of poultry can provide a reservoir of antibiotic resistant bacteria that can infect or colonize humans via the food chain.

CONCLUSION

The *E. coli* isolated from broilers were highly resistant to cephalixin followed by amoxicillin, tetracycline, and chloramphenicol. The *E. coli* isolated from layers were highly resistant to Tetracycline followed by Cephalixin, Amoxicillin, Ciprofloxacin, Enrofloxacin etc. The *E. coli* isolated from both broilers and layers were sensitive to amikacin. *E. coli* isolated from broilers were not resistant to Gentamicin, however, some proportion of *E. coli* isolated from layers were resistant to Gentamicin. The level of antimicrobial resistance was found higher in *E. coli* isolated from layers relative to broilers. A substantial proportion *E. coli* isolated from both broilers and layers suffered from colibacillosis showed a multi-drug antimicrobial resistance pattern.

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SURVEILLANCE OF AVIAN INFLUENZA IN FAECAL SAMPLES OF WILD BIRDS AT SAURAHA, CHITWAN NATIONAL PARK

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ABSTRACT

Wild birds are the carrier of Avian influenza virus as reservoir. They spread virus through faecal drops. Considering this fact, a study was carried out to detect the avian influenza virus in fresh fecal swabs in Chitwan National Park, Sauraha. A total of 40 samples were collected from Baghmara Buffer zone community forest, 30 samples from Rapti river bank and 10 samples from National trust for nature conservation areas. The faecal samples were collected during the summer season (March). The test was carried out by using a rapid test kit (synbiotic kit) USA. On rapid test, all the 80 samples were negative. All 80 samples were further inoculated in nine days embryonated eggs for haemagglutination tests for the confirmative diagnosis that showed negative result. Preliminary diagnosis revealed that wild birds were free from Avian Influenza in Chitwan National Park region.

Keywords: Avian Influenza, haemagglutination, wild birds

INTRODUCTION

Avian influenza is an infectious disease of fowls characterized by a variety of syndromes ranging from sub clinical to acute generalized fatal disease. Historically, outbreaks of avian influenza first occurred in Italy in 1878, when it was a lot of dead birds (Ligon, 2005). Then came another outbreak of Avian Influenza in Scotland in 1959 (Fauci, 2006). The virus that causes Avian Influenza in Italy and Scotland are the current strain of H5N1 virus appeared again attacked poultry and humans in various countries in Asia, including Indonesia, which caused many deaths in humans (Sims, 2006). Avian influenza refers to the disease caused by infection with Type A influenza viruses. It was first identified in Italy in the early 1900s and is now known to exist worldwide. These viruses occur naturally and have been isolated from more than 100 different species of wild aquatic birds which act as reservoir. It can infect domestic poultry and other bird and animal species (Hanson *et al.*, 2005). Influenza pandemics are associated with high morbidity, excess mortality, and social and economic disruption. Avian influenza virus can be divided into two groups, highly pathogenic avian influenza virus (HPAI) and low pathogenic avian influenza virus (LPAI) based on their difference in virulence. Infection of poultry with HPAI viruses can cause severe disease with high mortality. Both HPAI and LPAI viruses can spread rapidly through poultry flocks (Rebel *et al.*, 2011). Avian influenza refers to infection of birds with avian influenza Type A viruses. These viruses occur naturally among wild aquatic birds worldwide and can infect domestic poultry and other bird and animal species. Wild aquatic birds can be infected with avian influenza A

viruses in their intestines and respiratory tract, but usually do not get sick. However, avian influenza A viruses are very contagious among birds and some of these viruses can sicken and even kill certain domesticated bird species including chickens, ducks, and turkeys (Obenauer *et al.*, 2006). It should also be realized that our knowledge of LPAI viruses in wild birds cannot simply be extrapolated to HPAI viruses. For instance, the most important host species may be quite different (Ellis *et al.* 2004; Liu *et al.*; 2005).

On 9 January 2017, the National Health and Family Planning Commission of China reported to WHO 106 cases of H7N9 which occurred from late November through late December, including 35 deaths, 2 potential cases of human-to-human transmission, and 80 of these 106 persons stating that they have visited live poultry markets. The cases are reported from Jiangsu (52), Zhejiang (21), Anhui (14), Guangdong (14), Shanghai (2), Fujian (2) and Hunan (1). Similar sudden increases in the number of human cases of H7N9 have occurred in previous years during December and January (WHO, 2017).

AIVs replicate in the gastrointestinal tract (sampled by swabbing the cloaca or collecting droppings) and in the respiratory tract (sampled by swabbing the oropharynx). Individual mallards (*Anas platyrhynchos*) have historically shown higher detection probability from cloacal swabs. Accordingly, 61% of studies investigating contemporary infection sampled the gastrointestinal tract alone. However, the site of infection may differ between species (Brown *et al.*, 2008).

MATERIALS AND METHODS

Study area

The samples were collected from the Chitwan National Park, Sauraha. Wild birds are seasonal migratory, and study has revealed that most of the wild bird species were found in Chitwan National Park. The samples were collected from Bhagmara buffer zone community forest, Rapti river bank and National trust for nature conservation areas at the periphery of Chitwan national park.

Sample collection

The fecal swabs were collected from the birds from the green leaves of bushes and plants and transported to the media containing Transport medium with penicillin 2000IU/ml, streptomycin 2mg/ml, gentamycin 50ug/ml. Then samples were stored at cool box containing ice packs. The samples were collected in morning and evening period of the days because this is the time that birds excret faeces. A total of 80 samples were collected for the study from the wild birds of Chitwan National Park. Out of them 40 samples were collected from Bhagmara Bufferzone Community forest, 30 from Rapti River Bank and 10 from National trust for Nature Conservation.

Preparation of Phosphate buffer saline (PBS)

The following ingredients were used for the preparation of PBS solution (Table 1). The ingredients were dispensed in a beaker and the solution was mixed well. It was filtered through a muslin cloth. The pH was adjusted by adding HCl or bicarbonates drop by drop with the pipette and finally autoclaved at 121°C for 15 minutes.

Table 1: Phosphate buffer solution

Ingredients	Gram/litre
Sodium chloride(NaCl)	8g
Di-sodium hydrogen orthophosphate (Na₂HPO₄)	1.15g
Potassium chloride(KCl)	0.2g
Distilled water	1 litre

Preparation of Alsever's solution

The following ingredients were used for the preparation of Alsevier's solution (Table 2). The ingredients were dispensed into a beaker to mix well. The beaker was covered with aluminium paper and finally autoclaved at 121°C for 15 minutes at 15 pounds of pressure.

Table 2: Alsevier's solution

Ingredients	Gram/litre
Dextrose	20.5 g
Sodium citrate	8 g
Citric acid	0.55 g
Sodium chloride	4.2 g
Distilled water	100 ml

Rapid test

The collected samples were transported in a cool box to Central Veterinary Laboratory, Tripureshwar. The samples were centrifuged for 15 minutes to obtain supernatant. Then, initial screening test was performed using rapid test kit for avian influenza virus (Synbiotics from the USA). The flu detect strip is an in-vitro, rapid immune chromatographic assay designed to aid in the qualitative detection of Influenza Type A virus in tracheal and cloacal specimen from symptomatic birds or flocks. This assay detects all 15 subtypes of Influenza type a virus. First 200µl of the viral transport media containing extracted sample was transferred into the test tube provided and 3 drops of extraction buffer was added to the tube. The test strip was removed from the descant vial for each sample to be tested. Then the strip was placed directly into the test tube containing the sample. The test strip was placed so that the pink pad was submerged in the extracted sample. The test tube was inoculated in the sample for 15 minutes then test strip was removed from the test tube to read. After 15 minutes, pink/purple bands in the Centre of the test strip between the two absorptions pads would be observed in positive cases. The samples were negative on rapid test However; inoculation of samples in chickens' eggs was carried out for confirmatory diagnosis.

Inoculation of samples in chicken embryonated eggs

The inoculation of the sample was carried out in 9-day old embryonated hen eggs. For each sample 0.02 ml of the samples were inoculated through allantoic sac route of at least

four embryonated eggs. The eggs were incubated at 35-37°C for 72 hours. Eggs containing dead or dying embryos as they arose, and all eggs remaining at the end of the incubation period, were chilled at 4°C and the allantoic fluids were harvested from each egg. Then they were tested for haemagglutination (HA) activity. Detection of HA activity indicates a high probability of the presence of an influenza type A virus. The fluid giving negative reactions that were passed into another batch of eggs.

Haemagglutination (HA) Test

The reagents used for these tests are isotonic PBS (0.1M), pH 7.0-7.2 and red blood cells taken from a minimum of three SPF chickens and pooled in an equal volume of Alsever's solution. Cells were washed three times in PBS before using as a 1 % (packed cell v/v suspension). 50 µl of PBS was dispensed into each well of plastic U-bottomed microtitre plate. 50 µl of sample suspension (i.e. allantoic fluid) was poured in the first well followed by serial dilution of the sample suspension that was made across the plate. 50 µl of 1% (V/V) chicken RBCs was added to each well. The plate was gently tapped to mix and then RBCs were allowed to settle, and plate was determined at 37°C or at room temperature 27°C for 40 minutes. HA was determined by tilting the plate and observing the presence or absence of tear-shaped streaming of RBCs. The titration was read to the highest dilution giving complete HA. The fecal samples for HA tests were negative.

Haemagglutination Inhibition (HI) Test

25 µl PBS was dispensed into each well of plastic U-bottomed microtiter plate. 25 µl serum in the first well followed by the serial dilution of the serum. Again, 25 µl diluted sample in each well (i.e. one to twelve) on the respective labeled to keep single sample in one line. Then incubation was done for 45 minutes. Then 25 µl of 1 % chicken RBCs was added in all the wells. Then the wells were incubated at 4°C for 45 minutes. Finally, result was observed. (In positive case, there would be appearance of distinct button or tear shaped but in negative case there would be no appearance of distinct button, however there was agglutination of RBCs. The fecal samples for HI tests were negative.

Data Analysis

Datas of faecal samples collected from the Chitwan National Park were analyzed by One-way Anova test for every measurement using statistical package Minitab 2000, versions 13.20 (Minitab, 2000).

RESULTS

The samples were collected from Bhaghmara Buffer zone community forest, Rapti river Bank and National trust for nature conservation during the period of March to August 2007 as mentioned in below tables.

Total forty samples of wild birds (Blackish, Oriental Mag Pheasant, Black hooded oriole, Asian open billstork, Jungle babbler, Common tailor bird, Eurasian golden oriole, Large cuckoo-shrike birds) were collected from Bhaghamara Buffer zone community forest (Table 3). The faecal drops were collected at morning and evening from nine different

varieties of wild birds. The samples results were negative on Rapid and Haemagglutination test.

Table 3: Site of sample collection and result of Rapid test and HA

Site of sample collection	Sample size	Types of birds	Number of samples in each test tube	Results of Rapid and HA test
Baghmara Buffer Zone Community Forest	40	Blackbish	7	Negative
		Oriental Mag pierobin	6	Negative
		Rufous Treepie	5	Negative
		Black hooded orial	7	Negative
		Asian open billstork	8	Negative
		Jungle babler	2	Negative
		Common tailor bird	3	Negative
		Eurasian golden orial	1	Negative
		Large cuckooshire	1	Negative

Table 4:Site of sample collection and result of Rapid test and HA

Site of sample collection	Sample size	Types of birds	Number of samples in each test tube	Results of Rapid and HA test
Rapti River Bank	30	Lesser adjunct stork	6	Negative
		Chestnut tailed starling	4	Negative
		Asian open billstork	7	Negative
		Pound heron	3	Negative
		Spangled drongo	6	Negative
		Indian roller	4	Negative

Total thirty samples of wild birds (Lesser adjunct stork, Chestnut tailed starling, Asian open billstork, Pound geron, Spangled drongo, Indian roller) were collected from Rapti river Bank (Table 4). The faecal drops were collected at morning from six different varieties of wild birds. The samples results were negative on Rapid and Haemagglutination test.

Total ten samples collected of wild birds (long tailed shrike, grey crown wood pecker, Red vented bull bull, Black dronge, Indian cuckoo, wild pigeon, crow) from National Trust for nature conservation areas. The faecal drops were collected at morning from seven different varieties of wild birds.

Table 5: Site of sample collection and result of Rapid test and HA

Site of collection	sample size	Types of birds	Number samples in test tube	of Results of Rapid and HA test
National Trust for nature conservation	10	Long tailed shrike	1	Negative
		Grey crown woodpecker	2	Negative
		Red vented bullbull	1	Negative
		Black dronge	1	Negative
		Indian cuckoo	3	Negative
		Wild pigeon	1	Negative
		Crow	1	

DISCUSSION

The total numbers of samples were negative in rapid and Haemagglutination test. In the surveillance of wild birds in Europe, only one percent of sample found to be positive in a sample of 8500 taken in a period of two years. Winker *et al.* (2007) reported isolation of 22 avian influenza from a sample of 1700 from 1100 migratory birds in the state of Alaska, USA. So, in comparison to these samples our samples were very small and collected within a period of a week. Virus remains infectious for 24 hours to 48 hours on nonporous environmental surfaces and less than 12 hours on porous surfaces according to the report of (ISDA,2006). So, the samples might have given negative results. Samples might have been confused with the fresh samples due to dew in the morning. Idaho state department of Agriculture (2006) report suggests that acidic pH conditions inactivate the virus. So, the samples that were collected from the river banks might cause the virus inactive.

The prevalence of AIV infection has long been recognized to vary over time and space. Viruses have been most frequently isolated from duck populations in North America and Europe in late summer and early autumn (Suss *et al.*, 1994). Less frequent isolations from wintering populations have prompted suggestions that prevalence rapidly decreases over the course of autumn migration. So, pre-migratory staging grounds in late summer and early autumn are considered the optimal time and location for conducting surveillance among waterfowl (Brown *et al.*, 2008). This research supports the season of our sample collection too. Despite sampling >56,000 birds in the Netherlands from 1998 to 2009, only 20 of the 174 species were sampled >300 times. Moreover, prevalence in each species may vary over space and time. Although passerines have often been found negative for Avian infection, recent evidence suggests that, when sampled in or near waterfowl-rich bodies of water, a high proportion of individuals from 8 different passerine families show infection (Peterson *et al.*, 2008). The above research gives strong evidence that the large

number of samples are required for the surveillance of such type of researches. The samples were collected during the period of the summer so due to high temperature virus might be inactivated so due to this the results might have been negative. However, for proper risk assessment studies, we need better understanding of the interface between wild and domestic birds, the possible transmission of influenza viruses between these populations, bird behavior, age, structures of populations, and detailed migration routes.

CONCLUSION

The fecal swabs samples collected from the wild birds were negative on both rapid and haemagglutination test. Similarly (Adhikari *et al.*, 2006) had also carried surveillance of Avian influenza in Chitwan National park and Zoo, Jawlakhel. It was reported that collected faecal swabs were negative on HA test. The surveillance of wild birds to be performed both in summer and winter seasons. A regular active surveillance programme is essential in wild birds as many of them are acting as the reservoir of the avian influenza. The regular surveillance programme could be carried in other national parks too. These types of surveillance indicate warning signals for the introduction of HPAI, H5N1 virus and provide access to strains for characterization. However, for proper risk assessment studies, we need better understanding of the interface between wild and domestic birds, the possible transmission of influenza virus between these populations, bird behavior, age structures of populations, and detailed migration routes. The current increased interest in influenza virus surveillance in wild and domestic birds provides a unique opportunity to increase our understanding not only of HPAI epidemiology but also of ecology of Low pathogenic avian influenza viruses in their natural hosts, at the same time and cost.

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EFFECTS OF HIGHLY PATHOGENIC AVIAN INFLUENZA H5N1 OUTBREAK IN NEPAL FROM FINANCIAL AND SOCIAL PERSPECTIVES: A CASE STUDY

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ABSTRACT

The outbreaks of highly pathogenic avian influenza (HPAI) H5N1 have serious public health, economic and social implications. After the first two report of HPAI H5N1 outbreak in Nepal in Jhapa in 2009, a third outbreak was reported in Pokhara the following year where more than 11,000 birds were culled to control the disease. However, the financial and social effects of the Pokhara bird flu outbreak were never assessed. The objectives of this study were to evaluate the local financial and social effects of HPAI H5N1 outbreak in Pokhara and to understand farmers and consumers perceptions on bird flu and the control measures implemented by the government. A total of 200 people including poultry farmers, butchers, veterinary drug dealers, feed suppliers, and consumers were interviewed using the semi-structured questionnaire. The result showed that the direct loss of Pokhara bird flu outbreak from the culling of birds was more than 4.5 million Nepali rupees. The small scale commercial and backyard farmers and the butchers were the most affected from this outbreak. A large proportion of the consumers temporarily stopped to consume chicken meat and shifted to other meat sources. Most of the participants in this study had at least heard about the bird flu. The farmers were unhappy by the compensation provided by the government and perceived that the government control efforts were not satisfactory and were provided little assistance for their rehabilitation. In conclusion, there was a momentary local financial and social effect of the Pokhara bird flu outbreak which may not very important from the macroeconomic point of view but is important for the affected small-scale farmers and the butchers. It is recommended that the government review their policy and provide small scale commercial and backyard farmer a compensation that is closer to the market price and promote large-scale poultry farmers to go for insurance.

Keywords: Bio-security, Bird Flu, Economic impacts, Poultry value chain, Small-scale farmers.

INTRODUCTION

Avian influenza (AI) caused by influenza A virus is a disease of wild birds (Alexander, 2000; Stallknecht and Brown, 2007). The AI viruses (AIVs) cause sub-clinical infection in wild birds, but sometimes are responsible for outbreaks leading to massive deaths (Olsen *et al.*, 2006). Occasionally, AIVs cross the species barrier to infect domestic birds, including chicken, ducks, quails, turkeys and pigeons (Alexander, 2001; Swayne and Suarez, 2000). The AIVs have not only infected bird species, but sometimes caused diseases in mammalian species, including tigers (Thanawongnuwech *et al.*, 2005), cats

(Cardona *et al.*, 2009; Songserm *et al.*, 2006), and humans (Maines *et al.*, 2005; Peiris *et al.*, 2004; Peiris *et al.*, 2007). The AIVs can be either a highly pathogenic or low pathogenic based on their ability to cause disease (Lee and Saif, 2009). There are two proteins found on the surface of AIVs namely hemagglutinin (H) and neuraminidase (N) (Alexander, 2000). The Hemagglutinin protein varies from H1 to H9 and the neuraminidase protein range from N1 to N16 (Fouchier *et al.*, 2005). A Highly pathogenic AI (HPAI) is mostly caused by H5 and H7 subtypes though all subtypes in this group are not highly pathogenic (Alexander, 2001). Among the HPAI virus, H5N1 subtype caused the large outbreaks in poultry and sporadic human infections in early decades of 2000 (Alexander, 2007; Gilbert *et al.*, 2006; Songserm *et al.*, 2006; Tiensin *et al.*, 2005; Wan *et al.*, 2008). By February 2016, this HPAI H5N1 virus infection caused 846 human illnesses, out of which 449 people died (WHO, 2016).

In addition to the public health risks, the HPAI H5N1 outbreaks have severe economic and social implications due to high mortalities in commercial poultry and culling of birds by the governments to rapidly contain the disease (FAO, 2016). During the outbreaks of HPAI H5N1 in Southeast Asia from 2004 to 2006, millions of birds were culled by their respective governments to control the spread of the disease (Gilbert *et al.*, 2008; Tiensin *et al.*, 2007). Nearly 64 million birds in Thailand (NaRanong, 2007), 50 million birds in Vietnam (McLeod and Dolberg, 2007), were killed for an early containment of HPAI H5N1 spread. These massive culling of birds has negative macroeconomic effects on the national economy as well as a severe loss to the smallholder poultry farmers (FAO, 2016). The smallholder farmers are particularly vulnerable as their livelihoods are highly dependent on the success of their poultry farming (FAO, 2016). A backyard poultry farmer was estimated to lose nearly US\$14 which is equivalent to more than NRs 1,400 due to dead birds and foregone income in bird flu outbreak in the West Bengal India (Hinrichs, 2008).

In Nepal, the first outbreak of HPAI H5N1 was reported from the eastern region of Nepal in the Mechinagar municipality of Jhapa district in January 2009 (Karki *et al.*, 2014). The second outbreak of HPAI H5N1 was reported from the same district in Saranamati Village Development Committee in February 2009 (OIE, 2016). The Government of Nepal culled the birds within a designated area followed by disinfection as per the provision of the Bird Flu Control Order (BFCO), a legal document of Nepali government for the control and containment of HPAI outbreaks. No additional HPAI H5N1 outbreaks were reported in 2009. In January 2010, an outbreak of HPAI H5N1 was reported from the Pokhara Sub-metropolitan ward number 17 of Kaski district in the western region of Nepal (OIE, 2016). In the Pokhara HPAI H5N1 outbreak, 153 adult ducks died on the farm where the outbreak was first recorded (DLSO Kaski, 2011). More than 11,000 apparently healthy birds from the nearby areas were culled to control the disease (DLSO Kaski, 2011).

Several hundred-small scale commercial and backyard poultry farmers and other actors of poultry value chain such as the butchers, feed producers and veterinary drug entrepreneurs were possibly affected by this bird flu outbreak due to the culling of the poultry and poultry products, and a ban period of 45 days imposed by the government to re-operate their poultry businesses. However, there have been no studies being conducted to estimate

the local financial and social impacts of HPAI H5N1 outbreak that occurred in Pokhara in 2010. Further, the awareness level of the farmers and consumers of Pokhara on bird flu and their perception on the government control efforts were unknown. The objectives of this study were to (i) evaluate the local financial and social effects of HPAI H5N1 outbreak in Pokhara, and (ii) to qualitatively understand farmers and consumers perceptions on bird flu and the control measures implemented by the government.

MATERIALS AND METHODS

Study area and approach

The study area was Pokhara Sub-metropolitan ward number 17 and its vicinity in Kaski district in western Nepal (Fig. 1). Pokhara is one of the largest cities of Nepal and is a well-known tourist hub for both internal and international tourists. There are scores of lakes and natural water bodies in Pokhara making it a perfect habitat for various local and migratory bird species.

This study was conducted during May to June 2011. Qualitative and quantitative approaches were used to collect and analyze the data. Qualitative approaches included a semi-structured questionnaire survey, key informant's interview, and an observation by the researcher. Among the quantitative methods, descriptive statistics were used to analyze the collected information. Secondary data on Kaski demographics were obtained from the published technical reports of the District Livestock Services Offices (DLSO), Kaski. The questionnaires were pretested on 10% of the participants.



Fig. 1: Map of the Kaski district showing study area (indicated by lines). The red star on the map is the outbreak location. Map of Nepal with Kaski is shown in the inset.

Sample size and sampling design

In total, 200 individuals were interviewed using a semi-structured questionnaire. The major participants included in this study were key players in the value chain of poultry production that included local commercial and backyard poultry farmers, butchers, Veterinary drug suppliers, feed suppliers, and consumers. As there are different actors in the value chain of poultry production and consumption, a stratified sampling design was used to select the participants. A total of five strata namely, poultry farmers, butchers, Veterinary drug suppliers, feed suppliers and consumers were chosen. Individual participants within each stratum were selected to represent a wide geographic coverage within the study area and their willingness to participate in the study. The number of people interviewed in each category included 100 poultry farmers, 30 butchers, 10 Veterinary drug suppliers, 10 feed suppliers and 50 consumers. The Veterinary drug and feed suppliers were used as a key informant because of their connections with the farmers from their surrounding areas.

Data analysis

The data obtained from the questionnaires were processed in Microsoft Excel 2007 (Microsoft, Redmond, Washington, USA). Descriptive statistical analyses were performed in the software Epi Info version 3.5.4. (CDC, Atlanta, Georgia, USA).

RESULTS

Demographic information and knowledge on bird flu

Farmers

Most of the farmers (n=100) in this survey were middle-aged persons. More than 60 percent of the farmers were below 40 years of age, and remaining 40 percent were above 40 years of age (minimum 18 years, maximum 68 years). Fifty-seven percent of the respondents were male and 43% were female. More than 95% of the farmers were Hindus followed by Buddhist (4%) and Islam (1%).

In terms of poultry farming, 53% and 47% of the farmers were commercial and backyard poultry farmers respectively. Nearly 70% of the farmers reared only chickens, while about 20% had mixed farming and remaining 10% reared ducks. Ninety-two percent of the farmers had heard about the bird flu disease and the outbreak that occurred in Pokhara. The small fraction of the farmers who had not heard about the bird flu and outbreak in Pokhara were backyard poultry farmers. Eighty-four percent farmers said that poultry was culled from their house during the outbreak. In total, 98% of the farmers responded that they continued to consume poultry and poultry products after the bird flu outbreak. Half of the farmers responded that they improved the bio-security conditions of their farm after the outbreak, and 98% of total farmers respondent that they did not get any support from the government to rehabilitate their poultry business.

Consumers

Among the consumers (n=50), nearly half of the participants were under 30 years of age (minimum 18 years, maximum 65 years). Sixty-two percent of the total consumers

interviewed were females, and 38% of them were male. Ninety percent were Hindu followed by 10% Buddhist.

Out of the total respondents, 96 percent of the consumers had heard about the bird flu disease and the outbreak that occurred in Pokhara. Among the consumers who consume meat (84% of total consumers), 62% of the respondent left to consume poultry meat temporarily after bird flu outbreak in Pokhara while 38% said that they continued to consume the poultry meat after the bird flu outbreak. Eight percent of the consumers responded that they slaughter poultry at their house. Nearly 86% of the respondent cooked poultry meat for 30 minutes or more while 14% cooked for less than 30 minutes. Fifty-five percent of the consumer consumed egg by either boiling, making a pouch, half boiled or frying while 27% only consumed boiled eggs and other 18% only consumed fried eggs. Ninety-four percent of the consumers responded that there were no programs from the government to boost up their confidence for poultry consumption.

Financial losses due to the bird flu outbreak

In Pokhara bird flu outbreak, a total of 153 ducks died in an index farm and an additional 11,128 birds were culled during the stamping out operation to contain the outbreak. Among the total birds culled, 7,606 were broilers, 2,739 were backyard birds, 491 were ducks, 190 were layers, and 102 were other birds (quails and pigeons) (DLSO Kaski, 2011). From our key informant's interview, we found that the general market rate at the time of the outbreak was NRs 300 for a marketable broiler, NRs 500 for layers birds, NRs 1,200 for an adult duck, NRs 500 for backyard adult bird, and around NRs 200 for other birds.

The direct loss associated with the killing and culling of birds would be 153 ducks in index case* NRs 1200 + 7,606 broilers*NRs 300 + 2,739 backyard birds*NRs 500 + 491 ducks* NRs1200 + 190 layers *NRs 500 + 102 other birds * NRs 200= NRs 4,539,500. Though the direct losses farmers suffered were above NRs 4.5 million, Government of Nepal compensated the farmers with only NRs 1.1 million at the rate of NRs 100 per bird for 11,128 birds culled as per the legal provisions of Bird Flu Disease Control Order, 2064 and its amendment 2065. This means that farmers directly lost more than NRs 3.4 million due to the killing and culling of birds.

In addition to the direct losses, there were indirect losses associated with the bird flu outbreak due to the effect in the poultry market. The survey indicated that the demand for poultry and poultry products in our study area decreased by 80% for 2 weeks after bird flu, and took about two months to recover. It was found that a small scale commercial farmer rearing 500 broilers, on an average, lost nearly NRs 10,000 of their income due to the outbreak. This loss was observed mostly among the farmers who marketed their birds within one month of the outbreak. Backyard farmers on an average lost their income by NRs 1,200 to NRs 2,000 for about 1 month. Layers farmers who participated in this survey were minimally affected economically as there was no reduction in the price of eggs and sales were also comparable to pre-outbreak level.

In this survey, butchers (n=30) were affected severely for 2 weeks and partially for about 1 month during which the demand for meat was extremely low. The price of live broiler bird went down from NRs 160 per kg broiler to NRs 100 per kg broiler after the bird flu outbreak. The price of dressed chicken went down from NRs 300 per kg to NRs 190 per kg. Butchers in this study on an average used to earn NRs 10,000 to NRs 50,000 per month before the outbreak. After the outbreak, they lost 80% of their income for first two weeks and gradually recovered within a month.

Feed suppliers and veterinary drug suppliers experienced a short-term direct financial loss but recovered within a month to cover the losses of a previous month. Their sales decreased for about one month but then immediately peaked. During that time period, they took less order from the company or stocked them for some time.

Social effects of the bird flu outbreak

It was difficult to quantify the social effects of bird flu outbreak in Pokhara. However, qualitative observations during the interviews and farms visit indicated that there was some psychological panic going on among some of the farmers and consumers. The government personnel involved in the stamping out operation were wearing the personal protective equipment as per the scientific guideline which is a good practice. This led some of the people to think that this disease is dangerous and was wondering if it is safe to be around the community with a lot of chickens and ducks. Some of the farmers shared the story that their neighbour was treating them differently as they could be potential risks for them to transmit the disease.

DISCUSSION

This study evaluated the local effects of HPAI H5N1 outbreak on the actors of poultry value chain in Pokhara from economic and social perspective. Smallholder poultry farmers and butchers were comparatively most affected, while feed and veterinary drug suppliers were least affected largely due to their resiliency. The direct losses estimated was over 4.5 million Nepali rupees, which may not be a high amount from a macroeconomic perspective, but is important for the affected smallholder farmers.

All the actors of the poultry value chain included in this survey had heard about the bird flu disease and the outbreak that occurred in Pokhara with few exceptions. Less than five percent of the farmers, primarily backyard farmers, had never heard about the bird flu disease. The result is like the findings of other studies conducted in Nepal to assess the awareness level of farmers and other poultry value chain actors about the bird flu disease, specifically Rupandehi (Neupane *et al.*, 2012), Kathmandu (Paudel *et al.*, 2013), Kaski (Poudel, 2013) and Bhaktapur (Manandhar *et al.*, 2014). This indicated that the awareness campaign for HPAI in the future needs to be focused on backyard farmers.

This study indicated that the habit of poultry meat consumption was affected by the bird flu outbreak. Consumers were found to be the most sensitive as 62% of them who used to consume meat left to eat poultry meat, at least temporarily for about a month. They switched to other meat sources such as the goat meat, buffalo meat, and fish. However,

only two percent of the farmers left to consume poultry meat temporarily after the bird flu outbreak. Decrease in the poultry meat consumption during and after the bird flu outbreak was also observed in other countries such as Egypt (Yalcin, 2006) and Vietnam (Figuie and Fournier, 2008). The consumer's practices on meat cooking and egg consumption were found to be safe mostly. About 8% of the consumers were still slaughtering poultry at their home which needs to be discouraged. Enactment of the Slaughter House and Meat Inspection Act, 1999 would be an important step to minimize the risk of exposure of bird flu virus to general consumers. A large proportion of the farmers, consumers and other actors of poultry value chain were unsatisfied with the government compensation scheme and believed that the government did not do enough to support the rehabilitation process.

Smallholder farmers and butchers were most affected financially in Pokhara bird flu outbreak. The government compensated only about one-fourth of the total direct loss and affected farmers lost more than NRs 3.4 million from the government imposed culling of their apparently healthy birds. In addition, smallholder farmers lost about NRs 10,000 on an average during the Pokhara outbreak from the ban imposed on poultry farming for about a month. Butchers, who mainly sold chicken meat, lost nearly 80 percent of their income from the pre-outbreak income of NRs 10,000 to NRs 50,000 per month for the first two weeks of the outbreak and then bounced back to normal business. In Jhapa, NRs. 1,154 million per year were estimated to be lost to entire poultry value chain operating in that district from 2000/01 to 2009/10 (Ghimire *et al.*, 2012). Financial analysis at this scale was not conducted for Pokhara outbreak in this study, which made it difficult to compare it with the loss estimated for Jhapa. As the veterinary and feed suppliers were minimally affected in Pokhara bird flu outbreak, it is estimated that the loss was lower compared to that estimated in Jhapa. The other discrepancy for this difference may be associated with more poultry being culled in Jhapa outbreak and higher cost for stamping out operation as it was the first outbreak of HPAI in Nepal. Nevertheless, the scale of stamping out operation in Nepal was very low compared to Southeast Asian countries, such as Thailand where about 63.8 million birds (NaRanong, 2007), and Vietnam where 50 million birds were culled (McLeod and Dolberg, 2007). This led to much lower macroeconomic impacts in Nepal compared to those countries.

The limitation of this study was that the detailed indirect costs including the costs of stamping out operation were not considered in this study. In addition, the effects of bird flu outbreak in the tourism industry and fast food businesses were also not included. A detailed economic analysis needs to be conducted to unravel the full picture of the impacts of bird flu outbreak in Pokhara.

CONCLUSION

This study highlighted the local financial and social effects of bird flu outbreak in Pokhara in 2010 on the poultry value chain actors. Socially, middle-aged farmers were most affected which corresponds to the population structure in that area. In total, smallholder farmers directly lost more than NRs. 4.5 million due to the culling of apparently healthy birds, out of which only about NRs 1.1 million were compensated by the government. Mainly, the smallholder poultry farmers and butchers selling chicken were the most

affected. The overall effect of bird flu outbreak in Pokhara was very transient and lasted for less than two months. Farmers were highly unsatisfied with the government compensation scheme and perceived that the government did not do enough to support the rehabilitation of the poultry business. A recent study on the cost-benefit analysis of avian influenza control in Nepal indicated the continuation of the control programs or shifting to other approaches such as vaccination would be economically beneficial than doing nothing (Karki *et al.*, 2015). Therefore, the government should keep on continuing the control efforts they are doing during the outbreak time with some modifications to address the farmers concerns. It is recommended that the government increase the compensation rate to the backyard and small-scale farmers, and come up with the rehabilitation programs for the affected poultry chain actors. Enforcement and promotion of a government supported insurance scheme to the commercial poultry farmers might be an alternative for the larger poultry industries to minimize their losses. A public-private partnership approach in surveillance activities and improving bio-security in the poultry farms would be helpful to prevent or reduce the future bird flu outbreaks. Additionally, the government should continue the public awareness programs which might help to reduce the psychological trauma they go through during the bird flu outbreaks.

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STUDY OF HEMATOLOGICAL AND SERUM BIOCHEMICAL PROFILE IN PRE-PARTURIENT MURRAH CROSS BUFFALOES IN CHITWAN DISTRICT

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ABSTRACT

The aim of this study was to establish the reference values for hematology and serum biochemistry of pre-parturient Murrah crossbred buffaloes of Western Chitwan (Bharatpur Metropolitancity). The study was cross-sectional in which 31 apparently healthy pregnant Murrah cross buffaloes ranging from 5-8 years were selected randomly after confirmation by rectal palpation. Blood samples collected from jugular vein were stored in EDTA tubes for hematological analysis and for biochemical analysis centrifuged at 1500 rpm for 20 minutes to separate serum after 1 hour of collection. Hematological indices were determined manually using routine laboratory techniques and serum biochemicals were analysed using automated analyzer (Dimension RXL). Serum biochemicals like Glucose, BUN, Creatinine, Calcium, Phosphorous, Magnesium, AST, ALT, total serum Protein and albumin level were found within the normal range. Similarly, hematologic indices Hb, PCV, Platelet counts, RBC counts, WBC counts, and differential leukocyte counts were also in normal ranges. However, eosinophil (7.83 ± 0.82), and monocyte (4.4 ± 0.53) counts were higher at significant numbers which suggests gastrointestinal parasitic infestations and/or allergic diseases along with some chronic infectious diseases in the buffalo herd of Bharatpur Metropolitancity in western Chitwan.

Keywords: Hematology, Blood-biochemistry, Pre-parturient, Buffalo

INTRODUCTION

Throughout the known history, buffaloes have been raised in Nepal and are the main source of meat and milk in the country. Buffaloes are also an important source of manure and draft power. Because of their multi uses, buffalo are more valuable than cattle in Nepal, atleast from an economic point of view. According to Rasali (2000), buffaloes are the key livestock species in Nepal contributing about 53% of the total livestock GDP, mainly through the production of milk and meat. Food and Agriculture Organization of the United Nations (2014), ranks Nepal on sixth in total buffalo milk production with the estimate of 1167773 MT annually and similar on animal population. The figures of buffalo products amounted to 71% of the total milk production and 60% of the total meat production in the country (DLS, 2014).

Chitwan district in the central Terai is a major dairy pocket of the country contributing 2.5% of total milk production of which 70% (29866 MT) is from buffalo. The similar is

with the total meat production in which the district alone produces 3.3% in which buffalo shares 57% (DLS, 2014). As per the government policy for buffalo improvement the activity of maintaining 62.5% of the Murrah breed blood level in Terai districts, through unrestricted artificial insemination (AI) with Indian Murrah semen has been running in the district since few decades (ABAI, 1997). Still intensive AI with frozen Murrah semen is being carried out since fiscal year 2068/069 via. AI Mission of Department of Livestock services (NLBC, 2016) the district at present has large number of Murrah cross buffaloes.

It has recently been proved that the metabolic profile (Complete hematological and biochemical) testing as a best tool for the assessment of dairy herd's nutritional status and is a pre-symptomatic diagnostic aid capable of giving early warning of certain types of metabolic derangement in dairy animals with simple blood test (Herd *et al.*, 2001). But these indices may vary depending on factors such origin, climate, management practice geographical distribution and in stages of animals. Cattle and buffaloes are more susceptible to metabolic derangements during peri-partum stage and prepartum period is more critical during which the cattle is most likely to render many metabolic disorders and also has sequel in postpartum like milk fever, hypomagnesaemia, retained placentas etc. (Bell, 1995). Moreover, the present study was aimed at assessment of different hematological and biochemical parameters of pregnant (pre-parturient) Murrah crossbreed buffaloes reared in the subtropical agro-climatic conditions of western Chitwan and to set the standard reference value.

MATERIALS AND METHODS

Site of study

The study was conducted in dairy pocket areas predominantly populated with dairy buffaloes inside Bharatpur Metropolitan city (i.e. Mangalpur, Bijayanagar, Shardanagar, and Shivanagar) of western Chitwan district.

Surveying and selection of Buffaloes

Surveying was done in all the four sites for the selection of pre-parturient Murrah cross buffaloes. Rectal palpation was done, and positive animals were selected. A total of 31 apparently healthy buffaloes ranging from 5-8 years old were included in the study.

Blood Collection, serum separation and transportation

Blood samples of all selected animals were collected from the jugular vein using vacutainer tubes. Blood for hematological analysis was stored in EDTA tubes (BD Vacutainer® with K2 EDTA 5.4mg) while blood for biochemical analysis was allowed to stand 1h at room temperature and transported in an ice pack thermocol to laboratory and centrifuged at 1500 rpm for 20 minutes to separate serum. The blood samples were examined for hematological indices within 48 hours of blood collection and serum sample was stored at -20 degree centigrade until analysis.

Analysis of blood and serum biochemicals

i) Hematology: It was including total RBC counts, total WBC counts, differential leucocytes count, hemoglobin percentage (Hb %), packed cell volume (PCV), Macro

corpuscular volume (MCV), Microhematocrit hemoconcentration (MCHC) and platelet counts. These all indices were determined using routine laboratory techniques at Kohinoor Patho-lab, Bharatpur.

ii) Serum biochemicals: Serum biochemical estimation will include liver function assay (ALT, AST), glucose, protein (albumin and total protein), kidney function assay (Blood urea nitrogen and creatinine), and macro minerals (Ca, P, and Mg). These were analyzed by using automated analyzer (Dimension RXL) in B.P.K. Memorial Cancer Hospital, Chitwan.

Bio-statistics and data analysis

Descriptive statistical analysis was done by using computer software Microsoft Excel 2010 and results are expressed as Mean, Standard Deviation and Standard Error.

RESULTS AND DISCUSSION

The haematological results reveals Hb (gm/dl) 11.03 ± 0.21 , PCV (%) 33.83 ± 0.51 , Platelet ($\times 1000/\text{mm}^3$) 566.33 ± 20.24 , MCV (fl) 53.37 ± 0.29 , MCH (pg) 15.33 ± 0.15 , MCHC (%) 30.90 ± 0.22 , RBC ($\times 10^6/\text{mm}^3$) 6.33 ± 0.10 , WBC (/mm³) 8743.33 ± 209.36 , Neutrophils (%) 31.2 ± 1.39 , Lymphocytes (%) 55.60 ± 1.64 , Eosinophils (%) 7.83 ± 0.82 , Monocytes (%) 4.4 ± 0.53 and Basophils (%) 0.47 ± 0.13 . Similarly, results for biochemical values showed Glucose (gm/dl) 36.35 ± 1.52 , BUN (mg/dl) 10.97 ± 0.49 , Creatinine (mg/dl) 0.79 ± 0.05 , Calcium (mg/dl) 6.76 ± 0.20 , Phosphorous (mg/dl) 4.03 ± 0.18 , Magnesium (mg/dl) 2.65 ± 0.10 , AST (U/L) 138.13 ± 6.47 , ALT (U/L) 70.68 ± 2.90 , Total serum Protein (gm/dl) 6.03 ± 0.22 and Albumin (gm/dl) 0.63 ± 0.03 .

Table 1: Hematological values for pre-parturent Murrah crossbred Buffaloes in western Chitwan of Nepal*

Parameters	Unit	Mean $\pm$ SE	Standard Deviation
Hb	gm/dl	11.03 ± 0.21	1.15
PCV	%	33.83 ± 0.51	2.81
Platelets	$\times 1000/\text{m}$	566.33 ± 20.24	110.85
MCV	fl	53.37 ± 0.29	1.61
MCH	pg	15.33 ± 0.15	0.84
MCHC	%	30.90 ± 0.22	1.18
RBC	$\times 10^6/\text{mm}$	$6.33 \pm 0.10^{**}$	0.45
WBC	/mm ³	8743.33 ± 209.36	1146.71
N	%	31.2 ± 1.3	7.6
L	%	55.60 ± 1.64	8.99
E	%	7.83 ± 0.82	4.48
M	%	4.4 ± 0.53	2.9
B	%	0.47 ± 0.13	0.73

*n=30 at 95% level of significance; **n=20

The result so obtained suggest for normal hematological and blood biochemical values within the range, but eosinophil and monocyte count were higher in the herd. Monocyte count quite similar with Canfield *et al.*, (1984), more than Patil *et al.*, (1992), Akhtaret *et al.*, (2007), Salong *et al.*, (1980) and Ciaramella *et al.*, (2005) which suggest subclinical chronic infectious disease in buffalo herd in the district. Eosinophil count were higher than Sharma *et al.*, (1985), Canfield *et al.*, (1984), Patil *et al.*, (1992), Akhtar *et al.*, (2007), Salong *et al.*, (1980) and Ciaramella *et al.*, (2005) and the higher value suggests gastrointestinal parasitic infestation and/or some allergic diseases. Variations in monocytes also may be due to difficulty in separation between large lymphocytes and monocytes. Selection of animals to be sampled was most daunting task because uniform age and physical condition of animals cannot be expected in different herds in field condition, so the sampling problem of animals did exist which may be responsible for minor variation.

Table 2: Serum biochemicals values for pre-parturient Murrah crossbred Buffaloes in western Chitwan of Nepal*

Parameters	Unit	Mean±SE	Standard Deviation
glucose	gm/dl	36.35±1.52	8.44
BUN	mg/dl	10.97±0.49	2.7
Creatinine	mg/dl	0.79±0.05	0.29
Ca	mg/dl	6.76±0.20	1.12
P	mg/dl	4.03±0.18	0.99
Mg	mg/dl	2.65±0.10	0.53
GOT/AST	U/L	138.13±6.47	36.03
GPT/ALT	U/L	70.68±2.90	16.13
TP	gm/dl	6.03±0.22	1.24
Albumin	gm/dl	0.63±0.03	0.18

* n=31 at 95% level of significance

CONCLUSION

A reference for hematology and blood biochemical values for pre-parturient (last trimester) Murrah crossbred buffaloes for Bharatpur Metropolitan city (western Chitwan) established. High eosinophil and monocyte count so obtained suggests parasitic infestations and/or allergic diseases along with some chronic infectious disease in the buffalo herd of Bharatpur Metropolitan city.

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EVALUATION OF OVSYNCH PROTOCOL ON REPRODUCTIVE PERFORMANCE OF ANESTROUS BUFFALOES DURING BREEDING SEASON IN CHITWAN, NEPAL

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ABSTRACT

This study evaluated the effectiveness of Ovsynch/FTAI protocol (day 0 fertirelin inj. 100µg I/M, day 7 cloprostenol inj. 500µg I/M, day 9 fertirelin inj. 100µg I/M, and day 10 fixed time artificial insemination 18 hours after second injection of fertirelin) in anestrous buffaloes of Chitwan, Nepal (n=14). Protocol was evaluated in terms of estrus characteristics, ovulation and pregnancy rate. Responses were analyzed by grouping the buffaloes based on type of anestrus, length of anestrus, body condition score (BCS), milking status, parity, age, management condition, follicle count on day of second GnRH treatment and site of semen deposition. Intensity of estrus characteristics on day of AI was mild. Major estrus signs observed were uterine tonicity, alertness, mucus discharge on rectal manipulation of internal genitalia, swelling of vulva and teat engorgement. Mucus discharge on rectal manipulation of internal genitalia and teat engorgement were clearly noticed in 50 % buffaloes. There was higher tendency ($P=0.08$) of estrus expression by buffaloes with anestrous period of 10 months or lower compared to those with anestrous period longer than 10 months. Overall ovulation rate was 85.7%. Pregnancy rate to FTAI was 28.6% (22.2% and 40% in true anestrous and silent estrus buffaloes). 64.3% (66.7% and 60% of true anestrous and silent estrus) buffaloes were pregnant when checked at 102 days of FTAI including subsequent breeding. Although Ovsynch/FTAI seems to be alternative breeding technique in silent estrus buffaloes, further study with increased sample size is recommended. This protocol can potentially be used for resumption of ovarian cyclicity in true anestrus buffaloes.

Keywords: cloprostenol, FTAI, ovisynch, anestrus

INTRODUCTION

Buffaloes contribute over 68% to the total milk production of Nepal, and over 58% to country's total meat production (Government of Nepal, 2014). Nepal ranked fourth in buffalo population and fifth in buffalo milk and meat production worldwide, worth 942 million USD as of 2013 Production, 2015. Infertility in buffalo was mostly due to anestrous condition (Devkota, Bohora and Yamagishi, 2012). True anestrous and silent estrous conditions were prevalent among Nepalese anestrous buffaloes (Sah & Nakao, 2010). After the work of Pursley *et al.*, in 1995, GnRH based protocol of ovulation synchronization become established as Ovsynch and widely used in dairy industry for timed AI of lactating dairy cow (Wiltbank & Pursley, 2014) which is schematically represented in (Fig 1).

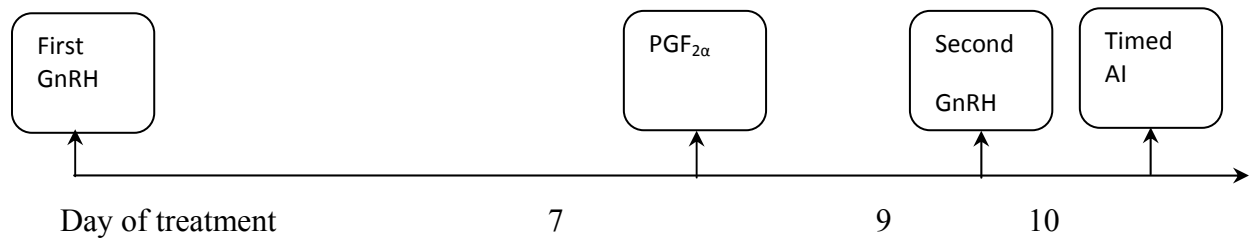


Fig 1. Schematic representation of Ovsynch protocol

Fixed time AI programs like Ovsynch without the need for estrus detection may be introduced in buffalo. There are few studies of Ovsynch protocol in different countries for fixed time insemination of buffaloes. There are scarce reports evaluating Ovsynch protocol in anestrus buffaloes during breeding season. In a study with 111 buffaloes, pregnancy rate of 36% was obtained when AI was performed 18 and 42 hours of second GnRH injection in Ovsynch protocol (Neglia *et al.*, 2003). Neglia *et al.*, (2003) rarely found estrus signs in Ovsynch treated buffaloes. Ovsynch protocol tested in 62 cyclic and 21 non cyclic buffaloes resulted in conception rate of 43.7% and 17% respectively (De Rensis *et al.*, 2005).

Paul and Prakash (2005) showed that 9 out of 10 non-lactating cyclic buffaloes ovulated. Ovsynch protocol is more effective in multiparous buffaloes than in primiparous ones (De Araujo Berber, Madureira and Baruselli, 2002). De Araujo Berber *et al.*, (2002) recorded the ovulation rate after second GnRH to be 93.3%. In a study designed to know the effect of parity to the efficacy of Ovsynch protocol in buffalo in Egypt, all 8 heifers ovulated to second GnRH whereas only 88.8% parous buffaloes ovulated out of 9 (Derar *et al.*, 2012). Conception rate of heifers was 62.5% out of 686 and that of parous buffaloes was 22.7% out of 273 buffalo cows (Derar *et al.*, 2012). Results obtained in study of Derar *et al.*, (2012) showed higher count of follicles in heifers compared to buffalo cows.

This protocol narrows down the window of ovulation and able to achieve the pregnancy rate of 66.7% in subestrus animals (Brar & Nanda, 2010) when pregnancy was checked 90 days including two subsequent breedings. All of anestrus buffalo heifers achieved ovulation but with poor conception rate of 18 percent (Ghuman *et al.*, 2009). Pregnancy rates of 20% at first AI and 60% upto third AI were obtained in applying Ovsynch protocol to 15 anestrus Murrah graded primiparous buffaloes (Malik *et al.*, 2011). Malik *et al.*, (2011) used 500 IU of eCG 3 days before first GnRH injection of the Ovsynch protocol and they named it Ovsynch Plus. All buffaloes were true anestrus animals in the study of recorded estrous signs like uterine tone and mucus discharge only in 40% of the animal Malik *et al.*, (2011).

In a study with 8 and 11 Nili Ravi buffaloes during peak and low breeding season ovulation rates of 87.5% and 36.36% respectively were reported as determined by LH peak and plasma progesterone rise using ELISA (Jabeen *et al.*, 2013). Jabeen *et al.*, (2013) included all parous buffaloes in their study. In a study with 10 cyclic lactating Nili Ravi

buffaloes, estrus expression rate of modified Ovsynch protocol was found to be 30% and conception rate was found to be 40% (Ullah, 2013). Ullah modified the protocol by presynchronization with PGF_{2α} 13 days before starting Ovsynch and AI was done at the day of second GnRH.

In Nepal, seven Murrah buffaloes of IAAS livestock farm were treated with the protocol in December 1999 (Sah, 2000). Sah used 50 µg fertirelin and 25 mg PGF_{2α} for treatment. Sah found only one pregnancy out of seven buffaloes. Devkota *et al.*, 2009 tested Ovsynch protocol in 5 lactating multiparous buffaloes of Institute of Agriculture and Animal Science livestock farm, Rampur, Chitwan during the breeding season. Devkota and Bohora confirmed ovulation by USG 24 hour after insemination. Devkota and Bohora reported ovulation in 40% of 5 animals and pregnancy in both ovulated buffaloes. There was absence of behavioral and physical estrus signs in Ovsynch treated buffaloes (Devkota *et al.*, 2009).

This study was designed to evaluate Ovsynch protocol in anestrus buffaloes of Chitwan for FTAI in silent estrus buffaloes and for resumption of ovarian cyclicity in true anestrus buffaloes. This study evaluates the protocol based on estrus characteristics, ovulation rate, and pregnancy rate. We did not know the quality of semen used for insemination. This study does not cover hormonal assay. Pregnancy loss or gain after 102 days of FTAI could not be incorporated in the study due to time limit.

MATERIALS AND METHODS

Study period and site

Ovsynch protocol was executed from 17 July 2015 to 26 July 2015 and study was continued till November 6, 2015 at AFU livestock farm of Rampur, Sharadanagar VDC and private farms of adjoining Shibanagar VDC of Chitwan district.

Maximum and minimum temperature during the study period recorded from 17 July, 2015 to 6 September, 2015, humidity percentage during July, and daily average hours of bright sunshine during July averaged 33.7±1.0⁰C, 26.5±1.0⁰C, 83.3±6.7, and 5.0±3.2 respectively in our study site Rampur (Department of hydrology and meteorology, 2015). The study sites in surrounding areas of Rampur are supposed to have similar agro climate. Treatment period is of decreasing day length, day length being slightly longer than the night. This agro-climate is representative of southern part of Nepal.

Animals

All six buffaloes in AFU livestock farm had passed at least eight months from the date of parturition without breeding. Buffaloes were kept in single row tie-stall, weaned calves being isolated. Buffaloes in Shivanagar area were either heifers or passed at least 9 months from parturition without any detectable heat signs. Farmers of Shivanagar area kept their buffalos in traditionally built sheds adjacent to their houses. In this study, the buffaloes were maintained in their routine management except FTAI after Ovsynch and followed them upto 102 days after AI.

MATERIALS

Ultrasonography Machine

Realtime B-mode diagnostic convex ultrasound scanner (HS-1500, Honda electronics, Japan) with multi frequency (5-7.5-10.0 MHz) rectal probe (HLV-375M) was used.

Hormones

Consultan injection 10 ml (a product of ASKA Japan): Each ml of Consultan injection contains 50 microgram fertirelin and Zenoadin C 20 ml (a product of ZENOAQ, Japan): Each ml of Zenoadin C contains 0.250 mg cloprostenol.

METHODS

Case classification

Fourteen anestrous buffaloes were categorized into 2 groups as shown in (Fig. 2). On rectal palpation and per rectal ultrasonographic examinations on first day of treatment, all animals had normal tubular genitalia. Buffaloes were classified into following two types based on the structures of ovary.



Fig 2: Embryo with amniotic and allantoic vesicles

- **True anestrus.** Buffaloes without detectable corpus luteum and follicles larger than 5 mm.
- **Subestrus (silent estrus).** Buffaloes with large follicles and detectable CL.

Ovsynch treatment

2 ml Consultan was injected I/M on July 17 of 2015 for the first time. Zenoadin C was injected 2ml I/M on July 24. Second shot of Consultan was given 2 ml I/M on July 26.

Recording of estrus characteristics at the time of FTAI

Estrus characteristics were recorded at the time of AI based on palpation of tubular genitalia rectally, external visual observation and history taking with animal attendants.

Fixed time artificial insemination at tenth day of Ovsynch protocol

Artificial insemination was done 18 hours after second injection of Consultan using frozen semen of Murrah breed from regular government supply.

Ovulation detection based on palpable CL on ovary on tenth day of AI

On tenth day of AI, ovaries were palpated per rectal for detection of any palpable CL (Amer & Mahdi, 2008) for confirmation of ovulation. Animals were then classified as ovulated and non-ovulated.

Breeding of animals in subsequent estrus

Natural breeding was allowed in subsequent natural estruses. Pregnancy diagnosis at 41 days of AI and at 102 days of AI including breeding of subsequent natural estruses. Pregnancy was diagnosed at 41 days of AI at 102 days of AI by rectal palpation and ultrasonographic scanning using 7.5 MHz rectal probe.

Statistical analyses

Independent-Samples T test assuming equal variances and Fisher's exact test in IBM SPSS Statistics version 21 were utilized to compare means of follicles in different group of animals and for testing independence of qualitative responses to factors affecting them respectively. Microsoft Office Excel was used for making bar diagrams.

RESULTS

Overall response of Ovsynch protocol

Response rates in true and silent estrus animals are summarized in (Table 1). Statistically significant difference was not found in responses of true and silent anestrous buffaloes.

Table 1: Overall response rate of Ovsynch protocol

Response indicator	Response rate (%)		P value	Overall Response rate (%)
	True anestrus	Silent anestrus		
Estrus Expression rate	55.6	40.0	1.00	50.0
Ovulation rate	77.8	100.0	0.50	85.7
Pregnancy rate at 41 days	22.2	40.0	0.58	28.6
Pregnancy rate at 102 days	66.7	60.0	1.00	64.3

Estrus characteristics

Since primary signs of estrus were absent and most of secondary signs were also not intense enough to describe, we may say that there was absence of characteristic estrus signs or estrus signs were subnormal in response to Ovsynch treatment. However, most frequent sign found were uterine tonicity and alertness of the animal. Other signs observed

include mucus discharge on internal genital manipulation during rectal palpation, swelling of vulva, teat engorgement, frequent urination, red color of vulva, and off-feed condition.

Although we did not find any statistically significant different response between groups of animals, the tendency of higher estrus response was observed in buffaloes having 10 months or shorter anestrus length (P value 0.08).

Ovulation rate

Ovulation rate was 85.7%. Ovulation response was not significantly different between groups of animals. Result is summarized in (Table 2).

Table 2: Ovulation rate as confirmed by detection of CL on tenth day of FTAI

Basis of categorization	Category	Number of animal	Ovulation rate (%)	P value
Type of anestrus condition	True	9	77.8	0.50
	Silent	5	100.0	
Length of anestrus period	Upto 10 months	5	100.0	1.00
	Above 10 months	6	83.3	
BCS	< 3	6	100.0	0.47
	≥3	8	75.0	
Milking status	Lactating	8	100.0	0.17
	Non-lactating	6	66.7	
Parity	Heifer	3	66.7	0.40
	Parous	11	90.9	
Age	Young	9	88.9	1.00
	Old	5	80.0	
Management condition	University mgmt.	6	83.3	1.00
	Farmers mgmt.	8	87.5	
Total		14	85.7	

Pregnancy rate

Pregnancy rate to FTAI checked at day 41 of FTAI was 28.6% without statistically significant difference between groups of animals. Pregnancy rate to FTAI and subsequent breeding diagnosed at day 102 of FTAI was found to be 64.3% without statistically significant difference between groups of animals. Among the ovulated buffaloes, 33.3 Û were pregnant to FTAI. Result is summarized in (Table 3).

DISCUSSION

Results of this study showed the effect of Ovsynch/FTAI treatment in anestrus buffaloes having at least 8 months of anestrus period. As Neglia *et al.*, (2003) and Devkota and Bohora (2009) mentioned, intense estrus signs were rare in the present study too. Estrus signs in 55.6% and 40% of true anestrus and silent estrus buffaloes in the present study are comparable with 40% result of Malik *et al.*, (2011) in true anestrus animals and 30% estrus expression rate found by Ullah (2013) in silent buffaloes.

Table 3: Pregnancy rate on 41 and 102 days of FTAI

Basis of categorization	Category	Number of Animal	Pregnancy rate			
			41 day		102 day	
			%	P value	%	P value
Type of anestrus condition	True	9	22.2	0.58	66.7	1.00
	Silent	5	40.0		60.0	
Length of anestrus period	Upto 10 months	5	20.0	1.00	80.0	0.55
	Above 10 months	6	33.3		50.0	
BCS	< 3	6	33.3	1.00	50.0	0.58
	≥3	8	25		75.0	
Milking status	Lactating	8	37.5	0.58	75	0.58
	Non-lactating	6	16.7		50	
Parity	Heifer	3	33.3	1.00	66.7	1.00
	Parous	11	27.3		63.6	
Age	Young	9	33.3	1.00	77.8	0.27
	Old	5	20.0		40.0	
Management condition	University mgmt.	6	16.7	0.58	50.0	0.58
	Farmers mgmt.	8	37.5		75.0	
Follicle count	<12	7	14.3	0.56	85.7	0.27
	≥12	7	42.9		42.9	
Site of semen deposition	Body of uterus	9	33.3	1.00	66.7	1.
	Cervix	5	20.0		60.0	
Total		14	28.6		64.3	

Ovulation rate was 85.7% as determined by detection of palpable CL on rectal palpation at least in any one ovary on tenth day of FTAI. Ninety percent ovulation rate in the study of Paul and Prakash (2005) with 10 non-lactating buffaloes is higher than 66.7% in non-lactating group of this study. This may be because of cyclicity in all buffaloes in their study. 93.3% ovulation rate in the study of De Araujo *et al.* in 2002 is closer to result of this study. In Heifers, Ghuman *et al.*, (2009) reported 100% ovulation rate which is higher than our finding of 66.7% in heifers. In the present study a heifer aged 2 years was unable to ovulate among 3 heifers included in the study. So, ovulation rate decreased to be 66.7% in the present result. 87.5% ovulation rate obtained by Jabeen *et al.*, (2013) is almost similar to 90.9% ovulation rate of parous buffaloes in this study.

Pregnancy rate of buffalo was found to be 28.6% at FTAI after Ovsynch while scanned by ultrasonography at day 41 of FTAI. Thirty six percent conception rate obtained by Neglia *et al.*, (2003) is closer to our study. 17% conception rate found in a study of De Rensis *et al.*, (2005) with 21 non cyclic buffaloes, and 20% in the study of Malik *et al.*, (2011) with 15 true anestrus buffaloes is closer to our result 22.2% of our true anestrus buffaloes.

Pregnancy rate of silent buffaloes under this study was 40% and was equal to that obtained by Ullah (2013) and was congruent with 43.7% of De Rensis *et al.*, (2005). Lower pregnancy rate of 14.28% obtained by Sah (2000) may be due to lower dose of fertirelin used. Forty percent pregnancy rate in lactating buffaloes obtained by Devkota *et al.*, (2009) is similar to 37.5% in lactating buffaloes of this study.

In the study including heifers and parous buffaloes to find out effect of parity on conception rates were higher (62.5%) in heifers as per the findings of present study (33.3%) conception rate of heifers was in the study by Derar *et al.*, (2012). Selection of cyclic heifers in their study might have increased the conception rates. Furthermore there might be some effect of sample size as this study had only 3 heifers included. Conception rate of 22.7% in parous buffaloes in the study of Derar *et al.*, (2012) which was similar to the findings of present study (27.3%).

Pregnancy rate was found to be 64.3% as examined at 102 days of FTAI which was the result of FTAI and subsequent natural breeding. Pregnancy rate at 102 days of FTAI including subsequent breeding in present study was found to be 60% in silent estrous buffaloes which is comparable with pregnancy rate of 66.7% obtained in the study of Brar and Nanda (2010). Sixty percent pregnancy rates of upto third AI in true anestrus animals reported by Malik *et al.*, (2011) is also closer to the result of 66.7% in true anestrus animals. Pregnancy rates of 20% at first AI and 60% up to third AI as obtained by Malik *et al.*, (2011) in anestrus buffaloes are like the results of this study.

CONCLUSION

Estrus signs expressed at the day of FTAI were mild. Major estrus signs were uterine tonicity, alertness, mucus discharge on rectal manipulation of internal genitalia, swelling of vulva and teat engorgement. Tendency of estrus expression was higher in buffaloes with 10 months or shorter anestrus period compared to those having longer anestrus period. Ovulation induction in 85.7% buffaloes indicates potential use of protocol for fixed time insemination. Conception rate of forty percent in silent estrus buffaloes are closer to national figures of conception rate to conventional breeding method. Ovsynch protocol with FTAI can be used in silent estrus buffaloes. Based on pregnancy rates of 66.7% in anestrus buffaloes within 102 days of FTAI, it may be concluded that the protocol causes good resumption of cyclicity in true anestrus buffaloes.

There was no statistically significant difference in the response rates of the protocol in heifers and parous buffaloes. This showed the prospects of using the protocol in heifers to shorten the age at first calving. So, it is recommended to further evaluate Ovsynch protocol in heifers with appropriate sample size as only 3 heifers were included in this study.

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COMMERCIAL FATTENING OF BUFFALO CALVES FOR ECONOMIC MEAT PRODUCTION

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ABSTRACT

An experiment was conducted on buffalo calves at Livestock farm of Regional Agriculture Research Station, Khajura, Nepal aiming to strengthen practice of commercial fattening. Experiment was done for a period of 7 months during the year 2015 with the main objective to develop low cost buffalo fattening technology emphasizing feeding management. Sixteen male buffalo calves aged 10-12 months were purchased from livestock haat bazaar at Rambhapur, Banke district and from farmers' field. The experiment was conducted in a Randomized Complete Block Design with 4 treatments each replicated four times. The treatment details were T1: Control- Farmer's practice, T2: Feed 1 + roughage adlib (straw and green at 3:1 ratio), T3: Feed 2 (Urea 2% and molasses 10 % of concentrate) + roughage ad lib (straw and green at 3:1 ratio), T4: Feed 3 (Oil seed cakes 20 %, soybean cake 10 %, urea 1%, Molasses 5 % of concentrate) + roughage ad lib (straw and green at 3:1 ratio). The dry matter requirement was fulfilled at the rate of 3 % of body weight. The concentrate was supplied at the rate of 25 % of total dry matter requirement and rest were fulfilled from roughages i.e straw and green forage at the ratio of 3:1. The buffalo calves were vaccinated against FMD and HS. Drenching was done at the beginning and during middle of the experiment. The results showed that the significantly higher average daily gain (g) was found for T2 (508.34 g) followed by T4 (503.57). Based on economic analysis the net benefit per animal was found highest on T3 i.e urea molasses supplemented group without soybean cake supplementation Rs 2853.

Keywords: fattening, dry matter, buffalo

INTRODUCTION

Buffalo is one of the important commodities in the Nepalese farming system. Buffalo meat contributes about 58.3 % of total meat production of Nepal (MOAC, 2015). It is the cheap source of animal protein for the people as compared to other meat like goat, sheep, pig and poultry. Buffalo meat demand is higher in Kathmandu, Pokhara and other larger cities of Nepal and supplied to these cities from various parts of Nepal like Banke, Nawalparasi, Siraha, and Bara districts of Nepal. Weekly livestock haatbazar are operated in these districts. Farmers sell their animals on haatbazar and traders purchase male buffaloes. Similarly, buffalo meat processing plants has been established in Bardiya and Bara districts of Nepal. These processed meats have been transported to China via Calcutta, India. There is a huge demand of male buffalo in the country for meat purpose. The demand of buffalo meat is not fulfilled yet (Devkota and Kolachapati, 2008). Buffalo

meat contains low amount of cholesterol so more people are attracted to buffalo meat. Sinclair *et al.*, 1982 reported that cholesterol content in buffalo meat is (46 %), sheep (68%) and goat (58%).

Most of the buffalo meat is produced from aged buffalo which have been used as draft (males) and milking (females). There is demand for the improvement in efficiency of buffalo meat production. The alternative use of young male calves may have advantage over old animals if they can be reared faster by feeding of nutritious diets. Kumagai *et al* 2012 indicated that 0.45 kg/day gain could be provided in crossbred male buffalo calves (Murrah x Nepal native buffalo) supplemented with limited amounts of concentrate at 0.75 % of their body weight (BW) from 128 to 250 Kg and higher daily gain, 0.72 kg, was attained by increasing the concentrate amount at 1.5% of Body weight. Previous studies indicated that average daily gain of buffaloes ranged between 0.433 and 0.780 kg (El-Feel *et al.*, 1993, Gigli *et al.*, 1993, Omar *et al.*, 1993, Sadek *et al.*, 1993 and Mehrez *et al.*, 1993).

Devkota and Kolachapati (2008) reported that 20 buffalo calves reared for a period 6 month and 10 month under two feeding system only forage based and forage plus concentrate 200 gms per head per day. The calf reared for 180 days under two feeding system showed Net profit per animal on forage based is Rs. 716.64 and forage plus concentrate supplementation had Rs 935.98 only. Similarly, on 300 days on two systems showed net profit Rs 539.41 and 547.57 respectively. Therefore, calf rearing for shorter period (180 days) was found profitable under two feeding system.

The Buffalo calf fattening could be one of the income generating activities. Accordingly, small calf with the age of 10-12 months can be purchased from local markets and reared for 6-7 months and could be sold for meat purpose. Despite fattening option, Nepalese farmers have not given any attention to raise buffalo calf. Majority of farmers culled male buffalo calf to increase the milk production. Devkota and Kolachapati (2008) reported buffalo calf fattening using only forage based and little concentrate supplementation on forage based so there is scope of research on growth performance of buffalo calf on increasing concentrate supplementation in their diet. Therefore, this study was conducted to fatten the buffalo calves for economic meat production with development of buffalo fattening technology using low cost feed stuff and identify the effect of concentrate supplementation on growth performance of buffalo calves and its economics.

MATERIALS AND METHODS

An experiment on commercial fattening of Buffalo calves was conducted on livestock Farm of Regional Agriculture Research Station, Khajura, Banke for a period of 7 months from 10 March 2015 to 6 October 2015. Sixteen Buffalo calves aged 10-12 months were purchased from weekly haat bazaar at Rambhapur, Banke and from Farmer's field drenched with Oxanide-L @ 10 mg /kg body wt on 13 March 2015. Prior drenching, the buffalo calves were vaccinated against FMD @ 3 ml subcutaneously on 11 March 2015. Similarly, H.S. and B.Q. combined vaccine was given @ 5 ml sub cutaneously on 17 March 2015. The experiment was conducted in a Randomized Complete Block Design

(RCBD) with 4 treatments and 4 replications. Dry matter requirement was fulfilled @ 3 % of the body weight. The treatment detail is as followed.

- T1: Control- Farmer's Practice (Straw and green forage with little concentrate i.e. rice bran)
- T2: Feed 1 (Maize-40 %, Rice polish- 15%, Soybean cake 20%, Rapeseed meal 18 %, Mineral mixture 1 %, Molasses 5 % and salt 1 %) + Roughage adlib (Straw and green at 3: 1 ratio)
- T3: Feed 2 (Maize-41 %, Rice polish- 20 %, Rapeseed meal 25 %, Mineral mixture 1 %, Molasses 10 %, urea 2 % and salt 1 %) (Urea and molasses) + Roughage adlib (Straw and green at 3:1 ratio)
- T4: Feed 3(Maize-47 %, Rice polish- 15%, Soybean cake 10%, Rape seed meal 20 %, Mineral mixture 1 %, Molasses 5 %, urea 1 % and salt 1 %) (Oil seed cakes) + Roughage adlib (Straw and green at 3:1 ratio).

Concentrate was supplied @ 25 % of total dry matter requirement. The feed compositions in different feeds were as followed. The concentrate mixture having 20% Crude protein level has been formulated.

Table: Different composition of feed

Feed 1	Feed 2	Feed 3
Maize: 40 parts	Maize: 41 parts	Maize: 47 parts
Rice Polish: 15 parts	Rice Polish: 20 parts	Rice Polish: 15 parts
Soybean cake: 20 parts	Rape seed meal: 25 parts	Soybean cake: 10 parts
Rape seed: 18 parts	Mineral mix: 1 parts	Rape seed meal: 20 parts
Mineral mix: 1 parts	Molasses: 10 parts	Mineral mix: 1 parts
Molasses: 5 parts	Urea: 2 parts	Molasses: 5 parts
Salt: 1 parts	Salt : 1 parts	Urea: 1 parts

Observations

Animals were adapted for a period of 7 days as a adaptation period before the initiation of experiment. All the animals were weighed at initial and fortnight intervals. The daily feed intake, feed conversion ratio was noted, and economic analysis was done at the end of experiment. All the collected data were analyzed using a statistical software MSTAT-C and Microsoft excel program.

RESULTS AND DISCUSSION

Chemical composition of Feedstuff

The samples of feed ingredients, prepared compound feed was sent to the Animal Nutrition Division, Khumaltar, Lalitpur for proximate analysis. Representative samples were analyzed for dry matter (DM), crude protein (CP), crude fibre (CF), and total ash contents (TA). The DM was determined by oven drying at 100°C for 10 hrs. Crude protein of the samples was determined using the Kjeldahl method. Ash content was determined by ashing at 550°C in a muffle furnace for 16 hrs (AOAC, 1980). Crude fibre of the samples

was determined using the Van Soest method (Goering, H.K. and Van Soest, 1970). The detail of chemical composition of feed stuff is presented in (Table1).

Table 1: Chemical Composition of feed stuff

S.N.	Name of ingredient	Dry matter (%)	Crude Protein (CP) %	Crude Fiber (CF) %	Total Ash %	Organic matter (OM) %
1	Maize	90.48	7.56	5.31	2.05	97.95
2	Calf feed 1	89.69	18.79	6.58	6.23	93.77
3	Calf feed 2	86.67	17.54	5.46	8.13	91.87
4	Calf feed 3	88.06	16.49	5.59	5.69	94.31
5	Soybean cake	90.27	44.39	8.67	7.45	92.55
6	Mustard cake	91.49	30.16	11.58	8.84	91.16

Dry Matter Intake

The dry matter intake of buffalo calves in different treatment groups is presented in (Table 2). The dry matter intake through green grass was found highest in treatment 2 and concentrate intake was highest in treatment 2 followed by treatment 4 and treatment 3. The highest dry matter intake was found in treatment 2 followed by T4 and T3 respectively.

Table 2: Dry matter intake by different treatment groups

Treatments	Total DM intake (Mean $\pm$ SD)			
	Green grass (Kg)	Straw (Kg)	Concentrate (Kg)	Total
T1				
T2	179.96 $\pm$ 29.53	411.85 $\pm$ 86.67	228.92 $\pm$ 45.9	820.23 $\pm$ 160.43
T3	162.03 $\pm$ 12.79	358.75 $\pm$ 45.7	125.04 $\pm$ 26.0	645.83 $\pm$ 62.78
T4	168.07 $\pm$ 26.64	411.85 $\pm$ 76.68	197.20 $\pm$ 35.86	777.12 $\pm$ 137.31

The average daily dry matter intake in treatment T2, T3 and T4 were 3.9, 3.07 and 3.7 % of body weight.

Growth rate

There was no significant difference ($P > 0.05$) on initial body weight of animals on different treatments. The significantly difference body weight was found on final body weight ($P < 0.05$). The highest body weight was found for T2 i.e. 221.4 Kg followed by T4 216.43 Kg, where as body weight of T3 and T1 respectively were similar and significantly difference ($P < 0.01$) on average daily weight gain was found highest on T2 (508.34 g /day) followed by T4 (503.57 g/day), T3 (372.15 g/day) , and T1(285.71 g/day) respectively (Table 3).

Table 3: Body weight of animals in different treatment groups

Treatments	Initial weight (Kg)	Body Final weight (Kg)	Body Average Weight (ADG)	daily gain
T1	112.45	172.45 ^b	285.71 ^b	
T2	114.65	221.4 ^a	508.34 ^a	
T3	113.63	191.78 ^{ab}	372.15 ^b	
T4	110.68	216.43 ^a	503.57 ^a	
Significance	NS	(P<0.05)	(P<0.01)	
CV	5.34	9.27	14.65	
Grand mean	112.85	200.51	417.44	

Feed Conversion Ratio

The feed conversion ratio of male calves during fattening period in different feeding regimes has been presented in (Table 4). The feed conversion ratio in treatment 2, 3, and 4 were 7.68, 8.26 and 7.34 respectively. Better feed conversion ratio was found for treatment (Table 4).

Table 4:Feed conversion ratio in fattening male buffalo calves

Treatments	Total DM intake (Kg)	Total Live weight gain (Kg)	Feed ratio (Kg DM/Kg LWG)
T1			
T2	820.23	106.75	7.68
T3	645.83	78.15	8.26
T4	777.12	105.75	7.34

Economic Analysis

The cost of fattening calves in different feeding regimes and output has been presented in (Table5). The net benefit (Rs) per animal was found highest in treatment 3 i.e. Rs 2853.53 per animal followed by T4, T1 and T2 i.e 2574.66, 1246 and 598.24 respectively.

Table 5: Economic Analysis of fattening male buffalo calves (Mean $\pm$ S.D.)

S.N.	Treatment groups	Total cost (NRs)	Total output (NRs)	Net benefit (NRs)
1	T1	15998.33 $\pm$ 2355.31	17245 $\pm$ 2396.67	1246 $\pm$ 867.65
2	T2	21541.76 $\pm$ 3726.57	22140 $\pm$ 3261.87	598.24 $\pm$ 772.63
3	T3	16323.98 $\pm$ 1049.61	19178 $\pm$ 1158.23	2853.53 $\pm$ 747.71
4	T4	19506.99 $\pm$ 2879.16	21643 $\pm$ 2832.51	2574.66 $\pm$ 1023.30

Note: concentrate @ Rs 38.3/kg for T2, 30.33/kg for T3, T1 and 34.43/kg for T4, Rice straw @ 1/kg and green grass @ 5/kg on DM basis. The green grass cost also includes

labor cost for calf management. The buffalo calf has been estimated @ Rs 100/kg live weight.

DISCUSSION

El-Ashry *et al.*, (1972) showed that Feed conversion values for buffalo calves were 11.5, 5.6 and 5.7 Kg for roughages solely supplemented, 50 % concentrate (1: 1 roughages and concentrate supplemented) and 80 % concentrate ration and 20% roughages supplemented group. The FCR Value in 50 %, 80% concentrate supplemented group is less and roughages only has high comparable to present study.

Feeding 20 % less CP than NRC (2001) recommendation for small cattle breeds are adequate for 400 g growth rate of Bhadawari calves and had no adverse effect on feed intake and dietary fiber utilization (Singhet *al.*, 2009). The experiment on fattening of buffalo calves was conducted in three categories of ration i.e (T1) mixed grass and straw adlibitum, (T2) mixed grass and rice straw and concentrate ration (roughage and concentrate ration (70: 30 in DM basis) and (T3) mixed grass and rice straw and concentrate ration (50 : 50 in DM basis). The body weight gain for T1, T2, and T3 were 202, 455 and 618 gm/day. The more economical combination was mixed grass and rice straw, concentrate supplementation (70: 30 in DM basis) (Banstola *et al.*, 2004). The feeding combination of roughage and concentrate (70: 30 in DM basis) growth rate is at par with the present findings.

Devkota and Kolachapati (2008) reported that buffalo calves were reared on on-farm condition using 15 buffalo calves in three treatment groups with three animals in each treatment replicated five times. The treatment combination was urea treated rice straw with 1.5 kg Berseem and little molasses (5 % of dry matter requirement), urea untreated rice straw with little concentrate (10 % of total dry matter requirement) and berseem forage (straw and berseem in the ratio of 1:1), untreated rice straw and berseem (1:1 ratio out of which 80 % dry matter from straw and 20 % from berseem) for a period of 150 days found per kg meat production cost was 67.77 on untreated rice straw and berseem supplemented group . The daily weight gains were 360 g, 323 and 305 g respectively. Net profit per kg meat production was found highest Rs 39.61 on urea non-treated and berseem feeding group. The growth rate found better on urea treated cum berseem feeding and molasses supplemented group. On molasses unavailability, condtion straw and berseem could be used for feeding. The growth rates in present findings were greater than reported by Devkota and Kolachayapati (2008).

Response of different level of concentrate feeding on local male buffalo calves was conducted at RARS Lumle for a period of 210 days, the ratio of concentrate to roughages assigned in the three treatments were 40:60, 50:50 and 60: 40. The average daily gain were 645 g, 728 g, 747 g, respectively and the net return per animal were Rs 4528, 4330 and 4630 in three treatment groups respectively (Jha *et al.*, 2004). The growth rate on present study is 508 g/day in concentrate to roughages ratio (25: 75 %) which is less than (40: 60 concentrate and forage ration)supplementation reported by Jha *et al.*, 2006.

Meat production potential and carcass characteristics of eight local male buffalo calves were studied. A group of 4 calves (treatment group) were kept under concentrate feeding, whereas, another group having similar number of calves (control group) were kept under general village feeding practices. The calves on treatment group received 70 % of total requirement was consisted from concentrate and 30 % from roughage. The animals of treatment group received significantly ($P < 0.05$) higher daily weight gain (719 g) compared to control group (469 gm) (Rana *et al.*, 2000). Increasing the concentrate level increased the weight gain, the weight gain in this treatment group is higher where concentrate had contribution of 70 % dry matter requirement. The present study has 508 gm average daily gain on 25 % dry matter requirement fulfillment from concentrate and rest is from roughages.

The economics of buffalo meat production was compared at veal stage (200 kg body weight) and at beef stage (300 kg weight) under four feeding planes. The calves under 6 hr grazing and concentrate supplementation @ 1 % of body weight and adlib bhusa feeding found to more economical i.e net return per animal was found to be Rs. 1898. Concentrate mixture consisting of barley grains (50%), deoiled rice bran (10%), deoiled sunflower cake (20%), deoiled mustard cake (17%), mineral mixture 2% and common salt 1 %. The fodder to concentrate ratio was maintained at 60: 40 in all these treatments (Kamboj *et al.*, 2007). The present research net return per animal is similar with reported by Kamboj *et al.*, (2007) in urea molasses supplemented group.

Eighteen male calves from native, crossbred (Holstein *Native) and buffalo (six animals for each group) with an average body weight (BW) of 160 Kg were fatten till 300 kg. Total mixed ration feed (5 % over animal appetite) offered to each animal 3 times daily. The average daily gain (gm/day) found to be 777, 641 and 912 for buffalo, native and crossbred (HN). Similarly, the significant dry matter intakes (Kg/day) were 5.58, 5.31, and 6.3 and feed conversion ratio was 7.2, 8.3 and 6.9 which was found non-significant (Azary *et al.*, 2007). The dry matter intake found higher than present study, but feed conversion efficiency was found similar.

Growth rate of buffalo calves reported by Thapa *et al.*, (1997) was 389 g per day fed on concentrate. Similarly, the growth rate varied from 200 to 550 g per day (Langer *et al.*, 1985; Punia and Sharma 1988; Kakker *et al.*, 1991). However, Pathak *et al.* (1987) observed a daily weight gain of 576 g on high protein and high energy diet. These growth rate are comparable with present findings.

CONCLUSION

The highest average daily gain was found in soybean cake supplemented group, but the economical analysis showed that urea and molasses supplemented group had the highest net benefit, so fattening of buffalo calves could be done using urea, molasses feed mixture without soya cake supplementation meeting 25% dry matter requirement from concentrate and 75% from roughages (straw and green forages in 3:1 ratio). There is still scope of research on soybean cake supplementation in concentrate ration for further verification.

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BENEFICIAL EFFECTS OF STINGING NETTLE SUPPLEMENTATION ON MILK PRODUCTION

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ABSTRACT

After having demonstrated the beneficial effects of stinging nettle supplementation in poultry and pigs, a month long on-station pilot trial in 10 lactating cattle of second to third stage of parity at CattleResearch Programme at Nepal Agricultural Research Council, Lalitpur was carried out. Daily supplementation of 20 gm nettle powder to dairy cattle resulted in enhanced milk production by 0.9 litres on an average compared to milk yield before nettle supplementation (3.2 litres/day versus 4.1 litres/day). After the on-station pilot trial; an on-farm trial in Tanahun, 150 km west of Kathmandu was carried out in 12 lactating Jersey cross cows by daily supplementation of 20 gm (T1) and 40 gm (T2) of nettle powder in two groups (n=4 cows/group) of cattle and productive performance was compared with control. Milk yield in terms of quantity and quality was recorded one week prior and during nettle supplementation. Qualitative analysis of milk samples was done every 14 days to measure levels of milk fat, solid non-fat and milk protein using Lactoscan. Available data on on-farm trial showed that there was increment in milk production in both treatment (T1 and T2) groups by 8.08% and 10.19%, respectively against control. Similarly, fat percentage was also increased compared to fat level prior to nettle supplementation in both T1 (3.52% vs. 4.15%) and T2 (3.46% vs. 4.46%) groups while in control group there was very negligible change (3.24% vs. 3.33%). Likewise, there were also remarkable increment in solid non-fat (SNF) and milk protein in nettle fed groups against control. Furthermore, overall body condition score in nettle supplemented group was better than that of control towards the end of supplementation. Our findings exhibited the beneficial effects of stinging nettle supplementation in enhancing the quantity and quality milk yield besides improving body condition score of the dairy cattle.

Keywords: stinging nettle, milk yield, milk quality, body condition score

INTRODUCTION

In the recent years, various efforts, ranging from the use of milk formula to bovine somatotrophin (BST) hormone or bovine growth hormone (BGH) have been used by the researchers and farmers to enhance milk yield in dairy animals. Use of BST is debated across the globe; it is legal in USA while it is not in Europe (Global Healing Center,

2014). The commercial milk formula consisting especially of Calcium, Phosphorus and Vitamins A, D and E in oral liquid form is quite popular in the peri-urban households of Nepal. However, such commercial formula involves cost and thereby reduction in the net profit from the sale of milk. Some subsistence farmers in the hills and mountains of Nepal feed nettle (*Urtica dioica*) gruels to their lactating animals with the perception of getting more milk yield with higher level of ghee (clarified butter) without any scientific backing. The current study was aimed at scientific validation of beneficial effect of nettle supplementation and creating awareness among producers and consumers about organic way of milk production using locally available natural resource that goes wasted in rural areas.

Few years earlier, supplementations of stinging nettle powder in poultry and cooked preparation in pigs demonstrated beneficial effects in terms of productive performances (Khanal, 2008; 2009; 2015). Egg laying performance was enhanced up to 35% in poultry in addition to improved growth and carcass quality in nettle-supplemented group. Furthermore, antibody titre against New Castle disease was maintained at a protective level in nettle-supplemented group when compared to control. The egg yolk appeared very intact and appealing even after storage at room temperature for 4 weeks in nettle supplemented group. Similarly, the crude protein percent was higher in broiler carcass supplemented with nettle.

Similar study conducted at Agricultural Research Station, Dhankuta, Nepal in piglets revealed the growth performance of nettle feeding at 30% inclusion level as compared to control, receiving only commercial ration. Encouraged by the results in pig and poultry, we wanted to investigate the effect of nettle supplementation in milk yield and its quality, both at on station and on farm. This paper discusses about the results of a pilot test at the National Bovine Research Programme, Khumaltar, NARC and that of on-farm trial in farmers' field at Tanahun.

MATERIALS AND METHODS

Preparation of nettle powder

Young, immature leaves of nettle were collected from Dhading and Tanahun districts and dried in shade for 3-4 days. Nettle powder was prepared using a blender. About 18 kg of nettle powder was prepared for supplementation in cattle and stored appropriately in a cool and dry place.

On-station trial (Experiment I)

A total of 10 cattle of similar age and parity of Bovine Research Programme, Khumaltar were divided into two groups: treatment (n=5) and control (n=5). All cattle were dewormed with 3-4 boli of Oxytoclozanide hydrochloride depending on the body weight one day prior to nettle supplementation. Lactating cows of treatment group received daily supplementation of 20 gm nettle powder in the commercial concentrate feed during the evening while control received only the commercial concentrate feed for 21 days. Maintenance and production ration were given to all cattle as per their milk yield. The daily milk was recorded one week prior to supplementation until the end of experiment. Experiment lasted 3 weeks. Milk was assessed qualitatively for fat%, Solid non-fat (SNF)

%, milk protein % and conductivity using Lacto-scanner before and after nettle supplementation.

On-farm trial (Experiment II)

Inspired by the positive result of the on-station pilot testing, further investigation with on-farm testing was carried out at Pokhari Bhanjhyang Village Development Committee (VDC), of Tanahun district in Year 2012 with two levels of nettle supplementation (T1: 20 gm and T2: 40 gm daily for 60 days). A total of 12 crossbred Jersey cattle was selected with similar age, lactation and parity (2-4) and divided into three groups (by purposive sampling) consisting of two treatments: T1 (n=4), T2 (n=4) and one control (n=4). All cattle were kept in similar management. Lactating cows of treatment groups-T1 and T2 received daily supplementation of 20 gm and 40 gm nettle powder, respectively for 60 days in the concentrate feed in the morning while control (T0) received only the concentrate feed. Their daily milk yield was recorded from one week prior to supplementation until the end of experiment. Body condition score (BCS) was recorded prior to nettle supplementation and at the end of 60 days trial. Milk was assessed from all three groups qualitatively for fat%, solid non-fat (SNF) and milk protein using Lactoscanner every two weeks and compared.

Statistical calculations

The statistical calculations were limited to the determination of the mean value of the parameters of milk quantity (Milk Yield) and quality (Fat%, SNF%, Protein% and Conductivity) in both on-station and on-farm trials. The standard error of the mean was calculated for each of the parameters and means were compared Tukey MSD (IBM SPSS Statistics Version 23).

RESULTS AND DISCUSSIONS

Experiment I (On-station trial)

On an average there was significant increment ($p < 0.042$) in milk yield by 400 ml after nettle supplementation (4100 ml/day) compared to milk yield before (3700 ml/day) nettle supplementation. In control group, the milk yield remained unchanged (slightly decreased) when compared to nettle supplemented group (Fig. 1).

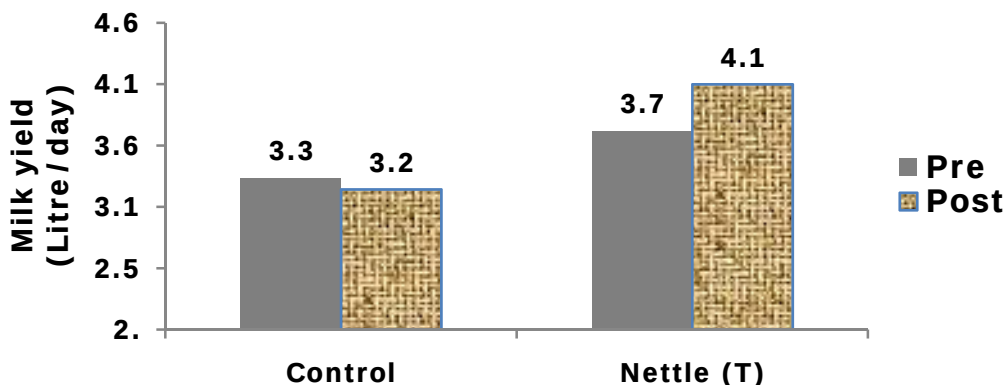


Fig. 1: Showing productive performance of nettle supplemented cattle against control receiving only commercial ration

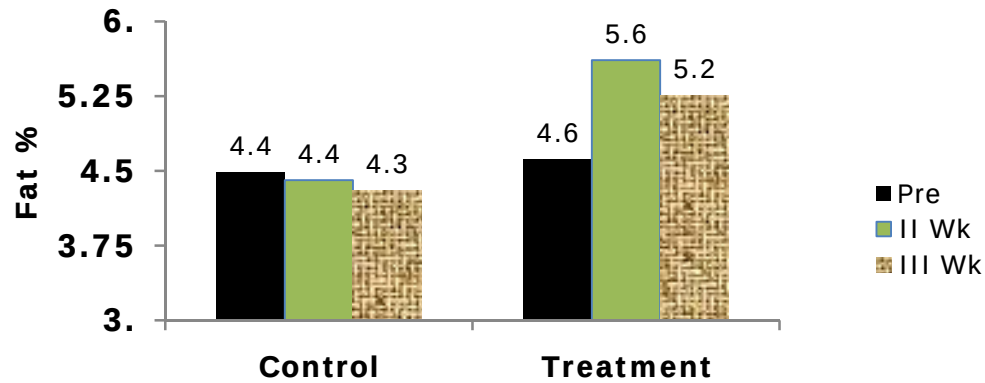


Fig.2: Showing the effect of nettle supplementation (Treatment) on milk fat% in two groups of cattle

Similarly, there was increment in fat percentage in milk of nettle supplemented group (Fig. 2) when compared to that of control. Milk fat rose from 4.61% to 5.61% level two weeks after nettle supplementation in treatment group whereas it did not change in control. Contrary to the general expectation, there were no remarkable differences in SNF%, protein% and conductivity in milk samples in both groups before and during nettle supplementation (Figs. 3-5).

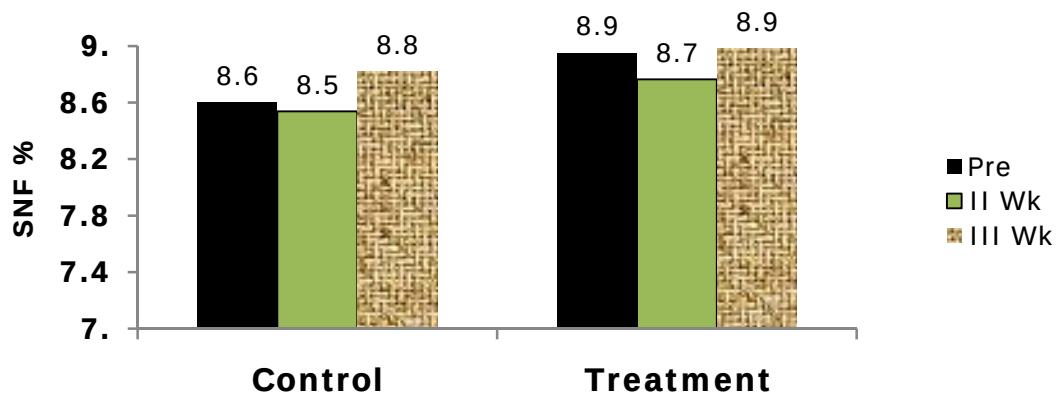


Fig. 3: Showing the level of Solid Non-Fat (SNF) % in milk of cattle supplemented with nettle (Treatment) and without nettle (Control).

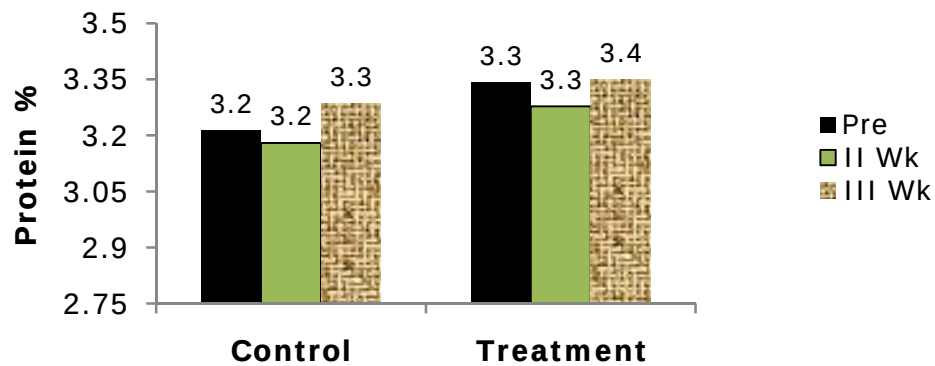


Fig. 4: Showing the level of protein% in milk of cattle supplemented with nettle (Treatment) and without nettle (Control).

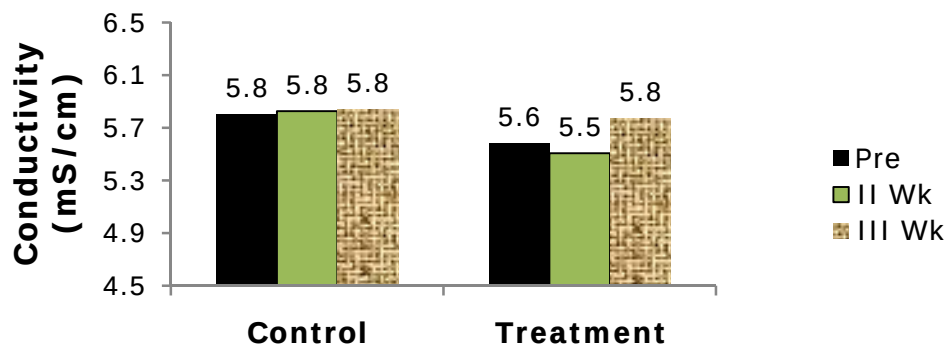


Fig. 5: Showing the conductivity in milk of cattle supplemented with nettle (Treatment) and without nettle (Control)

Experiment 2 (On-farm trial)

Results of nettle supplementation at two levels were encouraging for all parameters: adjusted average daily milk yield increment were 940 ml in T1 and 1900 ml in T2 compared to control (C). Similarly, adjusted increments in milk fat% compared to control were 0.54% and 0.91%; milk protein were 0.07% and 0.09% and SNF were 0.16% and 0.25%, respectively in T1 and T2 groups. BCS also improved by 7% and 10%, respectively in T1 and T2 groups (Figs. 6-10).

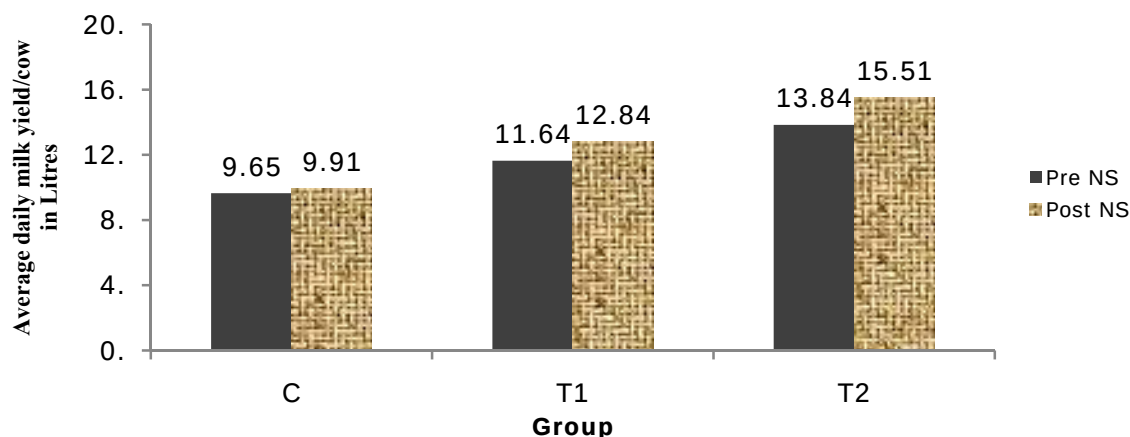


Fig. 6: Showing comparison of milk yield in litres (L) before (7 days) and after nettle supplementation (NS) in control (C) and nettle supplemented groups (T1: 20 gm/day and T2: 40 gm/day for 60 days)

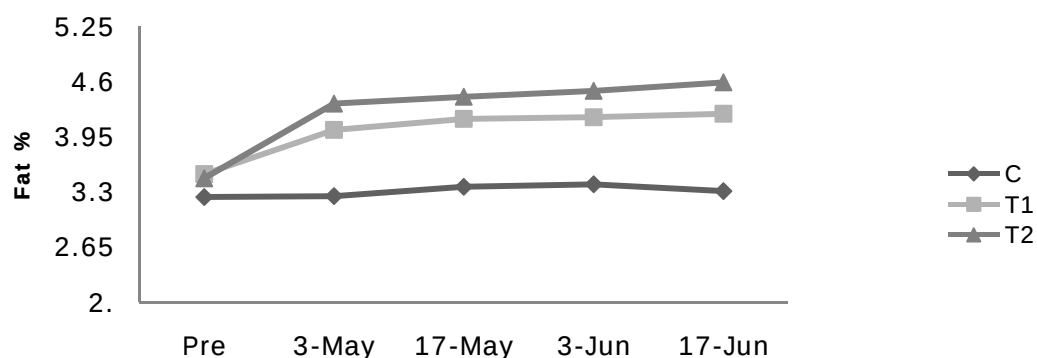


Fig. 7a: Showing temporal comparison of fat% in treatment (T1= 20 gm/day and T2= 40 gm/day) and control (C) cattle before and after nettle supplementation (NS).

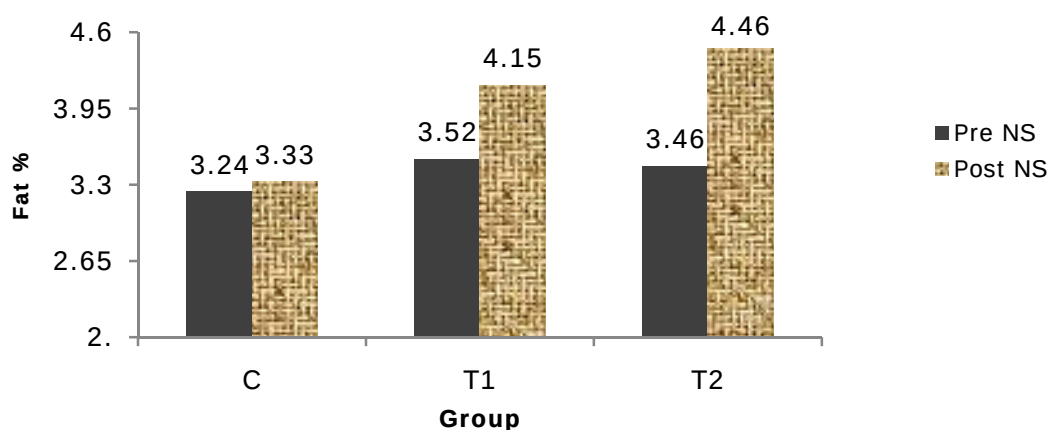


Fig. 7b: Showing comparison of average fat% in treatment (T1= 20 gm/day and T2= 40 gm/day) and control (C) cattle before and after nettle supplementation (NS) for 60 days.

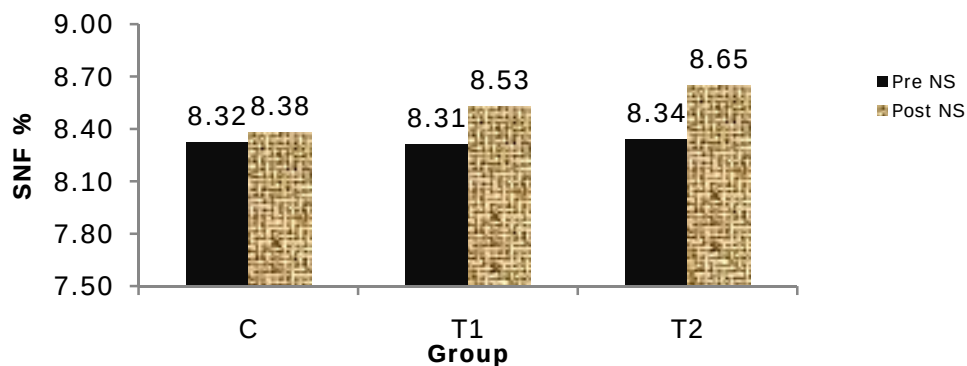


Fig. 8: Showing comparison of solid non-fat (SNF) % in control (C) and treatment (T1=20 gm/day and T2= 40 gm/day) groups before and after nettle supplementation (NS)

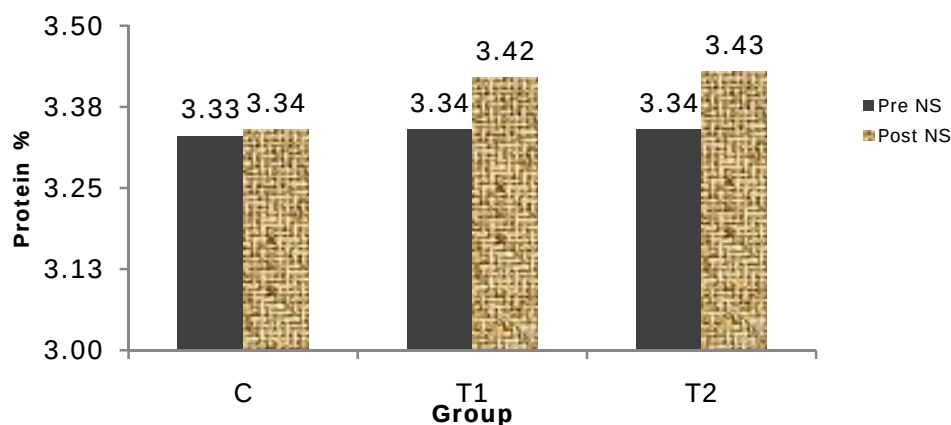


Fig. 9: Showing comparison of milk protein% in control (C) and treatment (T1=20 gm/day and T2= 40 gm/day) groups before and after nettle supplementation (NS)

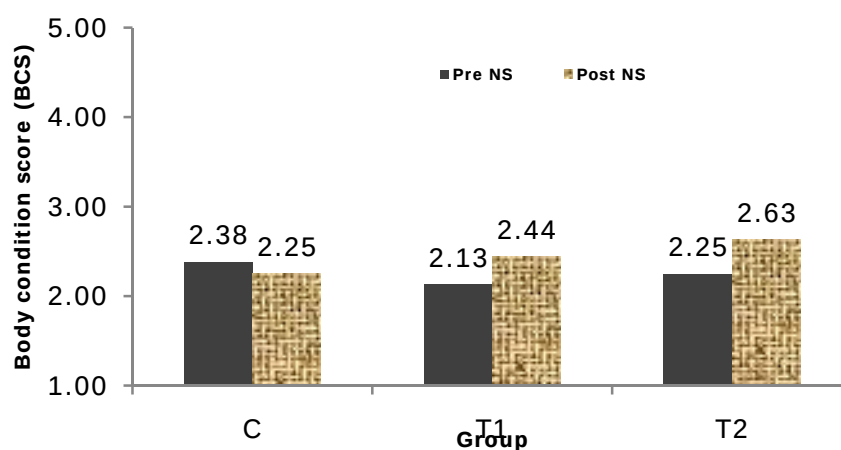


Fig. 10: Showing comparison of body condition score (BCS in 1-5 Scale) in control (C) and treatment (T1=20 gm/day and T2=40 gm/day) groups before and after nettle supplementation (NS)

After nettle supplementation in both T1 and T2 groups, rise in average fat % (Figs. 7a and 7b) was obvious. As expected, there has been enhancement of milk quality in terms of SNF% and protein% in both nettle supplemented groups (Figures 8 and 9). Similarly, the body condition score of cow improved in nettle supplemented groups. The on-farm results have clearly indicated that nettle supplementation has the potential to enhance productive performance in dairy cattle not only by increasing the milk quantity but also by improving the quality besides exerting positive effect on body condition score. Furthermore, economic analysis revealed that extra profit Rs. 15,410 (US \$ 200) was generated from increased milk yield, fat% and SNF% in top of regular sale of milk to the cooperative. In line with earlier studies in poultry (Khanal *et al.*, 2008; Maharjan, 2008; Regmi, 2010; Poudel and Khanal, 2011; Dahal and Khanal, 2012; Khanal *et al.*, 2015) this study has proved that nettle supplementation is beneficial for imparting positive effects towards organic production of milk among dairy farmers especially in rural areas where stinging nettle is abundantly available with virtually low labour cost towards in collecting and processing.

Considering the cost and availability of nettle powder, the optimum dose would be 20-40 gm day although toxic level not has been established. Since nettle powder is rich in non-specific nutrients such as Vitamins E and C, Calcium, Phosphorus, Iron and lectins, its supplementation will directly enhance the milk production besides boosting up the immunity in animals. Although *in vivo* anti-parasitic effect in pig was not seen, but it needs to be studied in cattle.

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PROGRESSIVE PATHOLOGICAL CHANGES IN SUBCLINICAL EXPERIMENTAL PARATUBERCULOSIS IN GOATS

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ABSTRACT

*An experiment was carried out to study the development of sequential histological lesions and efficacy of certain diagnostic tests in experimentally induced subclinical paratuberculosis infection in goats. Twelve goats of 8-12 weeks age were infected with 4.23×10^9 *Mycobacterium avium* subsp. *Paratuberculosis* on 8 occasions. Eight goats were kept as in-contact controls and 4 as uninfected controls. Diagnostic tests, gross and histopathological studies were carried out at 3, 6, 9 and 12 months post-infection. Two goats were positive in the AGID test at 12MPI. Three goats, one at 9 MPI and two at 12 MPI were positive in faecal culture. Tissue PCR detected one goat positive each at 9 and 12 MPI. Six (50%) of 12 infected goats had gross and histological lesions. Marked enlargements of the mesenteric lymph nodes were observed in the early stages (3 and 6 months post infection) of infection. Mucosal thickening and corrugation of the jejunal and ileal mucosae, enlargement and oedema of the mesenteric lymph nodes, dilatation of lymphatics and gelatinisation of mesenteric fat were observed in goats euthanised at 9 and 12 MPI. Histological lesions consisting of flat and broad villi with mild infiltration of lymphocytes and macrophages in the intestinal villi and crypts were common at 3 MPI and 6 MPI. Focal infiltration of macrophages in Peyer's patches and giant cells were found in the mesenteric lymph nodes at 6 MPI. Broadened villi and increased infiltration of lymphocytes and macrophages with Langhan's giant cells were observed in the villi and crypts at 9 MPI and 12 MPI goats. Acid-fast bacilli were detected in 3 goats, one each at 6, 9 and 12 MPI.*

Keywords: paratuberculosis, goat, sub-clinical, pathology

INTRODUCTION

Johne's disease is an economically important chronic bacterial disease of all domestic and wild ruminants. The disease, caused by *Mycobacterium avium* subsp. *paratuberculosis* is characterized by chronic granulomatous enterocolitis, lymphangitis and lymphadenitis. The clinical manifestations of the disease include incurable diarrhoea, progressive weight loss and decline in the productivity of animals (Williams *et al.*, 1979; Chiodini *et al.*, 1984; Benedictus *et al.*, 1987; Buergelt and Ginn, 2000; Godfroid *et al.*, 2000; Kramsky *et al.*, 2000). The bacterium of Johne's disease also has been implicated with an inflammatory bowel disease in humans known as Crohn's disease (Stabel, 1998; Selby, 2000).

In India, paratuberculosis in goat was first reported by Pande in 1942 from Assam. The disease now been reported from many parts of the country with prevalence varying from 2.5 to 18.9 % (Srivastava and More, 1987; Kumar *et al.*, 1988; Singh *et al.*, 1998; Tripathi and Parihar, 1999, Tripathi *et al.*, 2002). Various authors have described lesions in paratuberculosis, which occurs due to host's inflammatory immune response to the invading mycobacteria (Paliwal and Rajya, 1982; Rubin and Habekar, 1999; Hirsh and Zee, 1999).

The pathological changes in natural cases of paratuberculosis of goats were studied in detail by several workers (Nakamatsu *et al.*, 1968; Majeed, 1972; Paliwal and Rajya, 1982; Corpa *et al.*, 2000). Corpa *et al.*, (2000) described lesions in the natural cases of caprine paratuberculosis as focal, diffuse multibacillary, diffuse lymphocytic and diffuse mixed based on the severity and pattern of cellular infiltration. Sigurdardottir *et al.*, (1999) studied the pathological and immunological response in early subclinical stages of experimental paratuberculosis in goat kids. Similarly, Storset *et al.*, (2001) carried out immunological and microbiological study in subclinical paratuberculosis in goats following experimental infection. Valheim *et al.*, (2002) described lesions in subclinical paratuberculosis in goats, which were associated with persistent gut associated lymphoid tissues (jejunal and ileo-caecal valve Peyer's patches). The studies on sequential development of lesions and their relationships with immunological, serological and bacteriological findings in early stages of paratuberculosis are scarce in goats. Therefore, this study was aimed for comparative assessment of commonly used diagnostic tests during subclinical infection in experimental infection in goats.

MATERIALS AND METHODS

Experimental design

Twenty-four goats were randomly divided into three groups, namely, the infected (n=12), the in-contact control (n=8) and the uninfected control (n=4). The animals in the infected group were orally administered with 5 ml of bacterial suspension (4.23×10^9 /ml) on eight occasions at 3 days interval within a month. Similarly, the uninfected control animals received 5 ml sterile PBS. The animals of the in-contact group were administered similarly with PBS and kept along with the animals of the infected group. Five animals (3 infected, one in-contact and one uninfected control goats) were euthanised by intravenous injection of Thiopentone injection (Anesthal, Jagsonpal Pharmaceutical, Faridabad, India) at 3, 6, 9 and 12 months post-infection (MPI).

Preparation of inoculum

Small intestine from mid jejunum to the terminal ileum of an advanced case of paratuberculosis goat (multibacillary goat, case no.12A/2003) was collected at necropsy and the bacterial suspension was prepared as described previously (Kurade *et al.*, 2004). The material was then filtered through the sterile gauze to remove tissue debris and to recover the bacterial suspension. The approximate number of acid-fast organisms in the suspension was found to be 4.23×10^9 AFB/ml (Talwar, 1983). The inoculum was stored in screwcapped plastic containers in 50 ml aliquots at -20°C.

The suspension was subjected to culture on the Herrold's egg yolk medium (HEYM) and mycobactin – J dependent *M. a. paratuberculosis* was isolated. The identity of the strain was confirmed by the demonstration of the IS900 sequence by polymerase chain reaction and confirmed by restriction enzyme analysis (Sivakumar *et al.*, 2005).

Clinical signs

All the goats of the infected, in-contact and uninfected control groups were examined regularly for development of the clinical signs. The body weights of all the animals were recorded at monthly intervals.

Johnin testing

The test was carried out by single intradermal inoculation of 0.1 ml of Johnin purified protein derivative (1 mg/ml, BP Division, IVRI, Izatnagar) at a shaven site on one side of the neck. The thickness of skin was measured before inoculation and at 72 hours after injection with the help of Vernier callipers. Animals showing an increase of 2 mm in thickness of skin were considered positive (OIE Manual, 2000; Perez *et al.*, 1996).

Faecal and tissue smear examination

Faecal samples (2.0 g) collected directly from the rectum of each goat, was mixed well in 15 ml of distilled water and left overnight to settle. 5 ml of the supernatant was centrifuged at 1500 g for 20 min and smears were prepared from the sediment. Similarly scraping smears were prepared from the jejunum, ileum, ileocaecalcalve and mesenteric lymph nodes. After heat fixation, smears were stained by Ziehl Neelsen's (ZN) method and examined under microscope using oil-immersion objective. The presence of 2-3, strongly acid-fast bacilli in clumps were considered as positive (OIE Manual, 2000, Anonymous, 2004).

Bacterial culture of faeces and tissue samples

Faeces (2.0 g) and pooled tissue samples (2.5 g) from small intestine and mesenteric lymph node were homogenized in sterile distilled water and decontaminated with 0.9% hexadecylpyridinium chloride (final concentration 0.75%) as described previously (Whipple *et al.*, 1991; Rajukumar, 1998; Tripathi *et al.*, 2000). The samples were cultured on Herrold's egg yolk medium (HEYM, pH 7.1-7.4), with and without Mycobactin - J (Allied Monitor Inc., Missouri, USA) at 37°C for 16 weeks. The culture was checked every 4 weeks. The growth of acid-fast colonies on Mycobactin J supplemented HEYM tubes were identified as positive, which were also subjected to the IS900 PCR confirmation.

Polymerase Chain Reaction (PCR)

IS900PCR was carried out in faecal and tissue samples at 3, 6, 9 and 12 months post infection. The PCR mixture included 10x buffer(5µl), Mgcl₂(5µl), dNTP(10µl), primers BN₁ and BN₂(2.5µl each), Taq. Polymerase(0.5µl), DNA(0.5µl) and water(24µl). Oligonucleotide primersBN₁: 5' GTT ATT AAC GAC GCC CAG C-3' and BN₂: ACG ATG CTG TGT TGG GCG TTA G-3' flanking a region of 626 bp fragments, designed from published sequence of IS 900 of *M.a. paratuberculosis*(Sivakumar *et al.*, 2005 and Green *et al.*, 1989) was used. Initial denaturation (1 cycle) was carried out at 94°C for 4

min, denaturation (30 cycles) at 94° C for 1 min., annealing (30 cycle) at 60°C, synthesis (30 cycles) at 72°C for 4 min, final elongation (1 cycle) at 74°C for 4 min and holding at 4°C for 30 min.

Serological tests

a) Agar gel immunodiffusion test (AGID): AGID test was carried out in 1% agarose prepared in PBS (pH 7.4) as per the procedure already developed in the laboratory (Goat Lab Division of Pathology IVRI).

b) Absorbed ELISA: The capture antigen from MAP strain 316 and the adsorbing antigen from *Mycobacterium phlei* were prepared as described previously (Rajukumar *et al.*, 2001). The flat bottom 96-well plates (Maxisorp, Nunc, Denmark) were used. The ELISA procedures including optimum concentration of capture and adsorbing antigens, sera and conjugate (antigoat IgG-HRP, Sigma, St. Louis, MO, USA) were carried out at Goat Disease Lab, Division of Pathology, IVRI. The colour was developed by addition of hydrogen peroxide and O-phenylenediamine (Sigma). The optical density was measured at 492 nm in and ELISA reader (Multiscan Ex; Becton Dickinson, Franklin Lakes, NJ, USA). The serum samples were incubated with optimum concentration of adsorbing antigen (*M. phlei* antigen).

The positive ELISA results were determined by S/P value (sample to positive ratio) using the following formula:

$$S/P = \frac{\text{Sample mean} - \text{Negative control mean}}{\text{Positive control mean} - \text{Negative control mean}}$$

The samples showing S/P value of 0.35 (cut off value) or more were considered positive. The cut off value was determined based on sera collected from 100 goats from an organized farm that had no history of occurrence of paratuberculosis and for last 3 consecutive years, had been testing negative in faecal culture, PCR and AGID.

Pathological studies

The experimental animals were euthanised at 3, 6, 9 and 12 months post infection. Detailed necropsy examination was conducted, and the gross pathological changes were recorded. The tissue collection for the microscopic examination included 1 section of duodenum, 4 sections of jejunum, 3 sections of ileum, 1 section each of the mesenteric lymph node, ileocaecal lymph node, caecum, colon and rectum. One section each from other organs such as liver, kidneys, spleen, adrenals, pancreas, heart, lungs and aorta were also collected. All tissue samples were fixed in 10% neutral buffered formalin and cut into pieces of 2-3 mm thickness before conventional processing. The embedded tissues were sectioned at 4µm thickness and stained with haematoxyline and eosin (Culling, 1968). Adjacent sections from all tissues were also stained by Ziehl Neelsen's (ZN) method.

RESULTS

Clinical signs

The body weight did not show significance difference between the infected, in-contact and the uninfected control animals. Three goats, one at 9 MPI and two at 12 MPI. showed unthriftiness and mild muscle wasting. Oedema of the intermandibular space in two goats (One at 9 MPI and one at 12 MPI) and intermittent soft faeces (two at 9 MPI and one at 12 MPI) which were observed during 7 months of infection and persisted for 3 weeks. The remaining animals of the infected and control groups had normal appetite and pelleted faeces during the observation period.

Johnin testing

Three goats (Goat No 3, 4 and 28) tested positive at 6 MPI. Five goats (Goat No. 1, 3, 4, 5, and 6) tested positive at 9 MPI. Two goats (Goat No 3 and 4) tested positive both at 6 and 9 MPI. Two of 3 goats that were positives at 9 MPI became negative at 12 MPI (Table 1).

Table 1: Result of different diagnostic tests employed in 12 experimental infected animals

Goat No.	Sacrifice MPI	DTH	AGID	ELISA	Faecal smear	Faecal culture	Faecal PCR	Tissue smear	Tissue culture	Tissue PCR	H/p lesion	ZN stain
1	12	+	+	+	+	+	-	+	+	+	+	+
2	12	-	-	-	-	-	-	-	-	-	-	=
3	12	+	+	+	-	+	-	-	-	-	-	=
4	9	+	-	-	+	+	-	+	-	+	+	+
5	9	+	-	-	-	-	-	-	-	-	-	=
6	9	+	-	-	-	-	-	-	-	-	-	=
7	3	-	-	-	-	-	-	-	-	-	-	=
23	6	-	-	+	-	-	-	-	-	-	-	=
26	3	-	-	-	-	-	-	-	-	-	-	=
27	6	-	-	+	-	-	-	-	-	-	+	+
28	6	+	-	+	-	-	-	-	-	-	-	=
31	3	-	-	-	-	-	-	-	-	-	-	=

Serological examination

Agar gelimmunodiffusion test (AGID)

In the infected group, 2 goats (Goat No.1 and 3) were positive at 12 MPI in the AGID test. All goats of the in contact and uninfected control groups were tested negative in the AGID test.

Absorbed ELISA

There were increased S/P values in both infected and in-contact groups as compared with the control group. However, the mean S/P values were negative at all time points. A total of 5 experimentally infected goats (Goat N. 1, 3, 23, 27 and 28) were found positive at various month post infection. Only one goat showed positive after 1 MPI, two were found positive after 5 and 6 MPI and other two goats were tested positive after 8 and 10 MPI.

However monthly mean S/P values of entire infected, in-contact and control group were found negative for ELISA for paratuberculosis infection (Table 1).

Bacterial culture

M.a. paratuberculosis was isolated from faeces of 3 animals, one goat (Goat no.4) at 9 MPI and two goats (Goat No.1 and 3) at 12 MPI. Remaining animals of infected group and all the animals of the in contact and uninfected control groups were negative for bacterial culture at all time points. Tissues from one infected goat (Goat No 1) was positive for bacterial culture (Table 1).

Polymerase Chain Reaction (PCR)

All the experimental animals were negative in the faecal PCR at all time points. IS900 PCR carried out on tissue samples revealed positive amplification in two goats, one each at 9 MPI (G4) and 12 MPI (G1) (Table 1).

Gross Pathology

The important gross lesions consisted of thickening and corrugations of the intestinal mucosa and enlargement and oedema of the mesenteric lymph nodes. Enlargement of the mesenteric lymph nodes was marked in goats necropsied on 3 and 6 MPI. Pale discolouration of mucosa, thickening and corrugation of jejunum and ileum were more prominent in goats necropsied at 9 and 12 MPI. Gelatinisation of mesenteric fat was observed in two goats (G1 and G3) each at 9 and 12 MPI (Fig 1 and 2).



Fig.1: Intestinal tract (6 MPI): enlarged MLN (blue arrow) and thickened intestinal wall (white arrow).



Fig.2: Jejunum and MLN (12 MPI): Mucosal corrugation in the jejunum and note enlarged and oedematous jejunal MLN with distinct grayish white area (blue arrow) in the cortex.

Histopathology

In goats at 3 MPI, slightly flat and broad villi with increased infiltration of lymphocytes and macrophages were noticed. Focal infiltration of macrophages was observed in the ileal Peyer's patches of two goats (G26 and G31) and ileo-caecal valve Peyer's patches and mesenteric lymph node of one goat. Acid-fast bacilli were not detected in any of 3 infected goats. In the infected goats at 6 MPI, there were more infiltration of lymphocytes and macrophages in the ileum and jejunum. The Peyer's patches of jejunum and ileum showed focal infiltration with macrophages. Multinucleated giant cells of Langhans type were observed in the paracortical region of the mesenteric lymph node. Acid-fast bacilli were detected in ZN stained sections of the jejunum and ileocaecal valve of one goat (G27) (Fig 3 and 4).

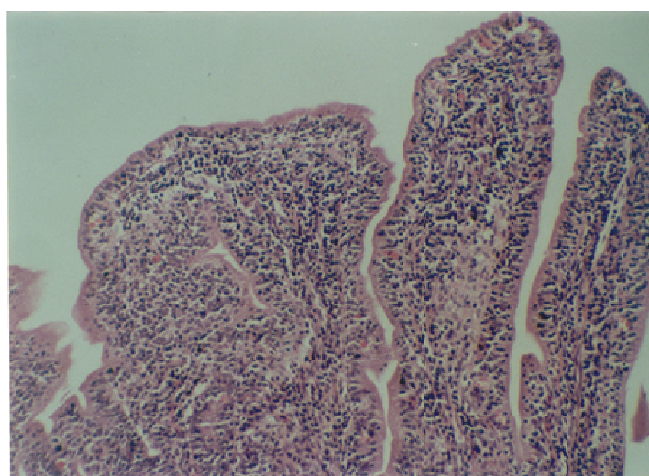


Fig.3: Ileum (3 MPI): Broad villi showing infiltration of lymphocytes and macrophages. H&E X 160

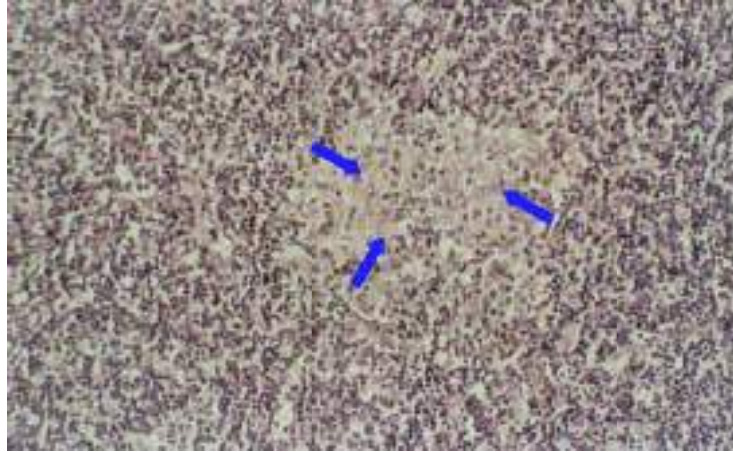


Fig.4: Mesenteric lymph node (3 MPI): Focal granuloma (blue arrow) consisting of macrophages in the paracortical area. H&E X160.

Microscopic lesions in infected goats at 9 months post infection were more severe as compared with 3 and 6 MPI groups. The intestinal villi were broad, and at places fused and showed diffuse infiltration with lymphocytes, macrophages and occasionally multinucleated giant cells in the mucosa of the jejunum and ileum. Focal granulomas were frequently detected in the jejunal and ileal villi in one goat (G4). In the same goat, ganglion cells of Meissner's plexus were surrounded by the lymphocytes and macrophages. Acid-fast bacilli were detected in the ZN stained sections of jejunum, ileum and ileocaecal valve of one goat (G4). In the mesenteric lymph nodes, microgranulomas along with multinucleated giant cells were observed in the cortical and paracortical regions without demonstrable acid-fast bacteria.

In infected goats at 12 MPI villi were broad, short and had shown diffuse infiltration with lymphocytes and macrophages. There were infiltrations of macrophages in the Peyer's patches of jejunum. Giant cells were present in the crypt region of ileum. Acid-fast bacilli were detected in ZN stained tissue section of ileo-caecal valve of one goat (G1). Interfollicular areas of cortex, and the paracortex showed infiltration with macrophages and formation of granuloma at the cortico-medullary junction. The in-contact control goat at 12 MPI revealed mild thickening but mild infiltrations of lymphocytes and macrophages in small intestine were common in most of the in-contact control goats (Fig 5 and 6).

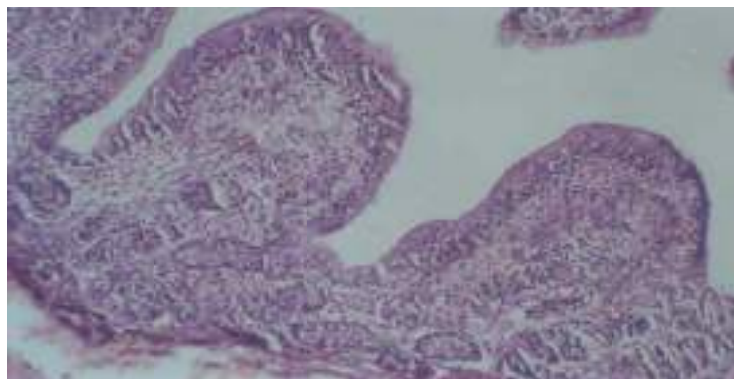


Fig. 5: Ileum (12 MPI): Shortening and broadening of villi with infiltration of macrophages. H&E X160.

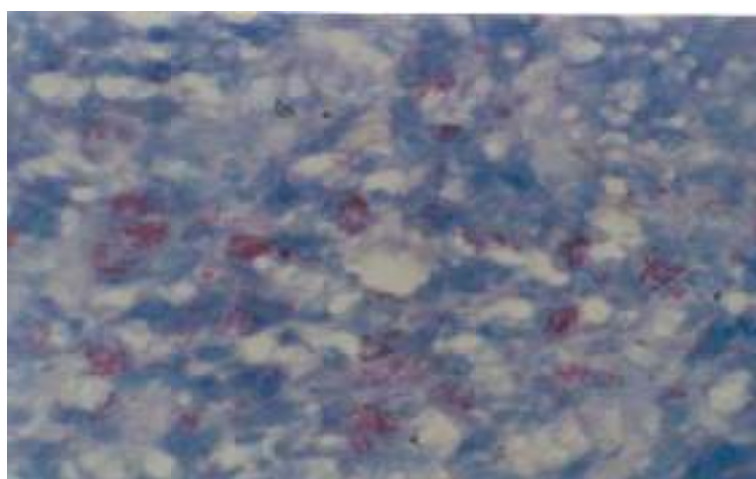


Fig. 6: Ileocaecal valve(12 MPI) with clumps of acid fast Bacilli in crypts

DISCUSSION

In this study few infected animals showed unthriftiness, mild muscle wasting, intermittent soft faeces and oedema of the inter mandibular space, which was in contrast to previous long term studies in which there were no symptoms of paratuberculosis (Sigurdardottir *et al.*, 1999; Munjal, 2004).

Six goats from the infected and none from the in-contact groups yielded positive delayed type hypersensitivity (DTH) reaction using Johnin PPD at different MPI. Two animals, which were positive at 9 MPI in DTH failed to respond positively at 12 MPI. Fluctuation in CMI response has been reported frequently in paratuberculosis in goats (Munjal, 2004; Storset *et al.*, 2001) and other small ruminants (Kurade *et al.*, 2004). Out of 6 DTH positive goats, acid-fast bacilli were detected in 3 goats, which also had lesions compatible with paratuberculosis. In other 3 goats, although there were specific tissue reactions, acid-fast organisms could not be demonstrated. The negative DTH results in all the in-contact goats suggested that the excretion of bacilli from the infected animals was not sufficient to sensitise the in-contact goats.

In the present study, only 2 of 3 goats at 12 MPI were positive in the AGID test, suggesting poor sensitivity of the test in the subclinical paratuberculosis (Shulaw *et al.*, 1993; Clarke *et al.*, 1996). Out of 12 infected goats, 5 goats were found to be positive in the ELISA, 4 of these were positive at 5 MPI. In the experiment of Sigurdardottir *et al.*, (1999), only one goat of 8 infected goats was positive in the ELISA after 8 months of infection. Storset *et al.*, (2001) detected antibodies against *M. a. paratuberculosis* from 15-20 weeks in 4 of 7 animals and one additional animal became positive by 35th week. Munjal (2004) detected 4 goats positive in ELISA at 180 days post infection. Our results alongwith the previous studies suggested the utility of the ELISA in diagnosing subclinical infection in goats.

In the present study faecal or tissue culture and tissue PCR detected 2-3 goats positive after 9 months of infection (write culture results of other workers (Sigurdartottir *et al.*, 1999, Storset *et al.*, 2001, Vathiem *et al.*, 2002, Munjal, 2004).

Sensitivity of bacterial culture and PCR have been reported to be comparable (Sigurdardottir *et al.*, 1999; Collins *et al.*, 1993; Anonymous, 2004) and poor in the experimentally infected small ruminants because of variable incubation period (Colgrove, 1989; Collins *et al.*, 1993; OIE Manual 2000; Carrigan and Seaman, 1990; Sigurdardottir *et al.*, 1999).

The sensitivity of the bacterial culture and PCR was reported to be variable in the clinical sample (Collins, 1996) and depends on various factors such as number of bacteria, DNA extraction procedure, concentration of eukaryotic DNA and presence of PCR inhibitors (Van der Geison. *et al.*, 1993; Collins *et al.*, 1993; Collins, 1996). In the current study, low numbers of bacteria in subclinical infection appeared to be the major factor for poor sensitivity of the bacterial culture and PCR.

Initially (3 and 6 MPI) gross lesions were more prominent in the merenteric lymph node than in the small intestine where thickening and corrugations were more obvious after 9 months of infection. While these findings generally agree with those reported previously (Fodstad and Gunnarson, 1979; Paliwal and Rajya, 1982; Collins *et al.*, 1984; Rajukumar, 1998; Sigurdardottir *et al.*, 1999; Corpa *et al.*, 2000, Munjal, 2004), mesenteric lymph node lesions should be examined carefully in early cases. In the small intestine, comparatively severe lesions observed in the jejunum than ileum corroborated earlier observation correlating lesions with the Peyer's patches, which persist in adult life (Corpa *et al.*, 2000; Valheim *et al.*, 2002; Munjal, 2004).

At 3 MPI, except for the presence of increased proliferation of lymphocytes and macrophages in the mucosa and infiltration of macrophages in the peyer's patches specific granulomas with acid-fast bacteria were not observed. While these lesions cannot be considered specific for paratuberculosis, there was no means to absolutely rule out that such initial lesion would not be caused by *Mycobacterium avium paratuberculosis*. Sigurdardottir *et al.* (1999), failed to detect lesions in experimental goats at 11 weeks post infection (WPI) but at 17 WPI, inflammatory cell infiltrate and giant cells were detected in

the jejunum and ileum. Munjal (2004) reported granulomatous lesion in the ileo-caecal valve in one goat at 2 MPI. Experimental infection of goats with *M. a. paratuberculosis* (human isolate) produced granulomatous lesions consisting of clusters of epithelioid cells and giant cells in the interfollicular and basal regions of the ileal and jejunal Peyer's patches at 3 MPI (Van Kruiningen *et al.*, 1986; Clarke 1997). The mild lesions observed in the present study could be due to the dose and strain variation and age of the animals, which were comparatively younger in previous studies (Van Kruiningen *et al.*, 1986; Clarke 1997).

At 6 MPI histological lesions were more developed along with demonstration of AFB than those seen at 3 MPI and were similar to those reported by Sigurdardottir *et al.*, (1999) at 24-week post-infection, and Munjal (2004) at 30 weeks post-infection in the experimental goats. In the experimental goats sacrificed at 9 MPI had increased infiltration with lymphocytes and macrophages, and multinucleated Langhan's giant cells in the lamina propria of the jejunum and ileum of one goat. Proportions of macrophages were more in comparison to lymphocytes. Acid-fast organisms were detected in the jejunum, ileum and ileo-caecal valve of one goat. Sigurdardottir *et al.*, (1999) detected microscopic lesions of inflammatory infiltrates in the jejunum and ileum at 37 weeks post infection. Munjal, (2004) detected lesions in intestine consisting of few multinucleated giant cells and accumulation of AFB in the intestinal villi at 270 DPI.

In the present study, experimental goats sacrificed at 12 MPI also revealed broad and flattened villi with more infiltration of macrophages. Multinucleated Langhan's type of giant cells was also seen in the crypt region of ileum. Peyer's patches were infiltrated with macrophages. Granulomas were observed in the cortical and paracortical areas of the mesenteric lymph nodes. Acid-fast organisms were detected in the ileo-cecal valve of one goat. In comparison to other studies that were carried out for more than one year (Sigurdardottir *et al.*, 1999; Storset, 2001), typical histological lesions of paratuberculosis were observed in goats in the present study.

In spite of observation of corrugations of intestinal mucosa in 9 infected goats, granulomas and giant cells were seen in the small intestines of one infected goat each at 9 and 12 MPI. The presence of giant cells in the paracortical region of lymph node was observed in one goat at 6 MPI. These findings suggest that thickening and corrugation of intestinal mucosa in goats can be due to infiltration of lymphocytes and macrophages in subclinical paratuberculosis without presence of classical lesions. This is important from diagnosis point of view in naturally infected cases, wherein such lesions even without demonstration of acid-fast bacteria should be suspected for paratuberculosis.

In the present study, acid-fast organisms were mainly detected in the Ileo-caecal valve and associated Peyer's patches indicating the major involvement of Ileocaecal valve in the progression of subclinical infection in goats. A previous study on experimental paratuberculosis using young goats showed that ileal Peyer's patches can take up the bacteria and hence it could be a site of early histological lesions (Valheim *et al.*, 2002). In the present study, most of the infected goats had infiltration with macrophages in the Peyer's patches. Only one goat at 12 MPI revealed the presence of acid-fast bacilli in the

Peyer's patches of ileo-caecal valve confirming that these were the sites of bacterial invasion multiplication and further dissemination. Munjal (2004) demonstrated acid-fast bacilli in 2 experimentally infected goats, one at 60, and other at 210 days post infection with similar lesions as observed in the present study.

Van Kruiningen *et al.*, (1986) reported that *M. a. paratuberculosis* infection began in the ileo-caecal or jejunal Peyer's patches, the only intestinal lymphoid tissues persisting in the adult animals (Landverk *et al.*, 1991; Perez *et al.*, 1996). As seen in this study, macrophage infiltration in the jejunal Peyer's patches were also noticed by Corpa *et al.*, (2000) in natural cases of paratuberculosis suggesting that initial focus of histological changes starts around Peyer's patches.

In the present study, mononuclear cell infiltration around ganglion cells of Meissner's plexus in small intestine in one goat at 9 MPI was like those observed in cattle and sheep with histological evidence of paratuberculosis (Gwozdz *et al.*, 2001). Buergelt *et al.*, (1978) described the intestinal neural lesions, which were characterised by infiltration of myenteric ganglia with globular leucocytes. Chiodini *et al.*, (1984) highlighted the possibility of axonal degeneration of sciatic and brachial plexuses. Binford, (1985) noticed distinct aggregation of mononuclear cells around nerves in the ileal submucosa and muscular layer of 8 naturally and 5 experimentally infected sheep. It has been suggested that the neural lesions may represent an autoimmune reaction triggered by antigens expressed in nerves or nerve associated cells in affected parts of the intestine since nerve runs parallel to lymphatics (Gwozdz *et al.*, 2001). Though, in the present study, goat had only soft faeces, neuritis causing involuntary muscle relaxation in the small intestine during infection, may potentiate diarrhoea that has been correlated with type 1 or III hypersensitivity (Tizard, 1996).

Mild thickening of intestines in the in-contact control goats at 12 MPI and infiltrations mainly of lymphocytes in the small intestine and ileo-caecal valve in most of the in-contact goats could be the result of the sensitization of animals by exposure to *M. a. paratuberculosis* organisms but it might not have been enough to cause the detectable infection. None of the in-contact animals could be detected positive by any diagnostic tests employed. Moreover, it should be kept in the mind none of infected animals were heavy shedders as most of them were in subclinical stage of disease in the present study.

The results of the present and earlier studies (Sigurdardottir *et al.*, 1999; Valheim *et al.*, 2002; Storset *et al.*, 2001; Munjal, 2004), initially there were no distinct granulomas and clinical disease could not be induced even with higher dosage up to 2 years of age (Storset *et al.*, 2001). On the contrary, in sheep with similar experimental design (Kurade *et al.*, 2004) like the present study and in others (Begara-McGorum *et al.*, 1998) well delineated granulomas were reported as early as 4 weeks after infection and production of clinical disease within a year of infection. Though comparison of infection process in goat from sheep was not the aim of this study, evidences suggest that early pathogenetic mechanism differ between these two species. While goat could be a better experimental model for persistence of infection without developing immune granuloma, sheep for the clinical disease model that early pathogenetic mechanism differs between sheep and goat and

latter does not appear to be a better experimental model for clinical paratuberculosis. This conclusion may be true only for experimental paratuberculosis, but it raises other questions, as clinical disease is not produced in goats, if goat is resistant, it should develop immune granulomas to resist and clear the infection, which was not obvious in any experimental studies. This means that the mechanism of resistance to *Mycobacterium avium paratuberculosis* infection in goat differs from sheep, inferring that the mechanism to intracellular resistance could be characteristics of the species and hence may vary. While goats may not be a good experimental model for paratuberculosis, it could be a good subject to study the resistance of Map infection in goats for quite long time without showing any distinct granuloma.

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EFFECT OF BREED AND GENDER ON HEMATOLOGICAL PARAMETERS AND SOME SERUM BIOCHEMICAL PROFILES OF APPARENTLY HEALTHY INDIGENOUS SHEEP OF NEPAL

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ABSTRACT

This research was conducted to investigate the effect of some factors such as breed and sex on biochemical and serum parameters as well as to establish normal physiological reference values for indigenous breed of sheep of Nepal. Blood sample were collected from 25 apparently healthy sheep (11 males and 14 females) from on station flock at Khumaltar, Nepal Agriculture Research Council (NARC) were used. In case of serum biochemical parameters, overall LS mean for total Protein (TP) was less than physiological range while Albumin (Alb) was recorded higher than normal physiological range. The breed differences were observed in TP where Lampuchhre sheep (lowland sheep) showed the lower value than hill sheep breeds (Baruwal and Kage sheep) taken in the study. The same breed exhibited gender variation in the same parameter with values in males were significantly higher ($P < 0.05$) than females. Comparing LS Mean for Alb and Phosphorous (P) for Nepalese sheep, value recorded for females were found to be significantly higher ($P < 0.05$) than males in case of serum Alb while serum P was significantly lower ($P < 0.05$) in females. For RBC indices, Nepalese sheep exhibited higher Mean Corpuscular Haemoglobin Concentration (MCHC) was higher than normal physiological range. LYM and NE value was significantly higher in Kage breed in comparison to Lampuchhre and Baruwal. The highly significant gender variation for Mean Corpuscular Volume (MCV) ($P < 0.01$) and Mean Corpuscular Hemoglobin MCH ($P < 0.05$) where female showed the higher value than males. The result of the preliminary study revealed the breed and sex variation in case of hematological and biochemical indices Further research with more number of samples is warranted for concrete information which could be used to monitor animal health status to improve the management and conservation of these breeds.

Keywords: sheep, Nepalese breed, hematological parameters, serum biochemical indices

INTRODUCTION

Hematological tests are important tools for evaluation of physiological and health status of farm animals (Oduye, 1976). Hematological analyses in farm animals have been extensively discussed as an essential part of clinical examination often pointing to a specific differential diagnosis or suggesting a prognosis (Braun *et al.*, 2010; Polizopoulou, 2010).

Sheep are an important livestock commodity in the country, particularly for the hills and mountains. There are total 0.79 million sheep in the country (Central Bureau of Statistics, 2016); out of which five to ten percent is exotic (pure or crossbred) and remaining is indigenous suggesting more

contributions come from indigenous breeds (Neopane and Pokhrel, 2005). The raising of sheep in Nepal is primarily destined to the production of wool, meat, manure and draught; particularly in the hills and mountains. Considering the high economic values of sheep among the livestock farming system, it is important to perform clinical and para-clinical examination to guarantee sanitary strategies control, prevention or treatment of diseases and to assure good management practices. It is well-recognized that hematological parameters in sheep show several variations in relation to disease, nutrition, management system, physiologic status (Šimpragaa *et al.*, 2013); stress, location and season (Oramari *et al.*, 2014); sex, breed, age (Addass *et al.*, 2010, 2012). Moreover, the productivity and reproductive efficiency of animals are co-related with the blood parameters and values are affected by internal and external environment (Oramari *et al.*, 2014). These differences have under scored the need to establish appropriate physiological baseline values for livestock, which could be used in the realistic evaluation of the management practice, nutrition and diagnosis of health condition as well as in determining the physiological status of animals (Šimpragaa *et al.*, 2013).



Fig. 1:Four breeds of Nepalese sheep: A. Bhyanglung, B. Baruwal, C. Lampuchhre, D. Kage

In this study, we have focused on three out of four breeds of Nepalese sheep (Fig1.) according to their economic contribution and/ or population, namely Baruwal (63%), Kage (21%) and Lampuchhre (12%). Baruwal sheep are found from mid hill to high hill; Kage are distributed from low land to mid hills; and Lampuchhre are predominant in low land. These animals particularly

adapted to their existing environment and exhibit disease resilience against the economic diseases. Although considerable information is available on the normal hematological parameters of exotic sheep breeds under their home tract environment and management conditions (Tibbo *et al.*, 2004), these values could not serve as baseline information for comparison in conditions of nutrient deficiency, physiological and health status of indigenous sheep breed in Nepalese condition. Hence, the aim of this study was to evaluate the variation on age and breed on some hematological and biochemical parameters of indigenous sheep breeds of Nepal and moreover to bring forth normal hematological and biochemical reference values for these breeds.

MATERIALS AND METHODS

Experimental location and climatic condition

In this study, three Nepalese sheep breeds, namely Baruwai, Kage and Lampuchhre, was used which were maintained at farm of Animal Breeding Division, Nepal Agriculture Research Council (NARC), Khumaltar. All the animals considered for study were around one year of age. The average minimum and maximum temperature during the sample collection season was 9.7°C and 18.5°C respectively. Relative humidity was 48%, pressure 1.0037 atm, wind speed 1.1m/s and wind degree 231.501°.

Sample Collection

Blood samples were collected from the jugular vein of 25 apparently healthy sheep (11 males and 14 females) of different breeds (Baruwai, Kage, and Lampuchhre). Average live weights of adult males and females were 48 kg and 30 kg respectively. The sheep were bled through jugular vein and 10 ml blood collected. 3ml of the blood samples were collected into in anticoagulant free serum vial and allowed to clot at room temperature within 3 hrs of collection. Serum samples was obtained through centrifugation at 8000 rpm for 10 minutes and stored at -20°C for biochemical studies. The remaining 7ml of blood samples were deposited in vacutainer containing EDTA for hematological studies.

Table1:List of individual number for different breeds of indigenous sheep

Breed		Total
Baruwai		9
Kage		11
Lampuchhre		5
	Male	11
	Female	14
		25

Haematological analysis

Hematological parameters such as RBC indices i.e. Total Red Blood Cell count (RBC), Haemoglobin (Hb), Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC) and WBC indices i.e. Total White Blood Cell Count (WBC) and differential count were estimated by fully automated Abacus Junior Vet 5 Haematology Analyser at Central Veterinary Laboratory Tripureswore.

Biochemical analysis

The biochemical metabolites were analyzed using Randox kits on manually operated colorimeter. The serum biochemical metabolites measured were Total Protein (mg/dl) by Biuret, albumin (mg/dl) by Bromocresol Green dye binding method, glucose (mg/dl) by GOD-PAP method and Calcium (mg/dl) by ARSENAZO III method and phosphorus by UV method.

Statistical analysis

Least Square Mean values and standard errors were calculated using Harvey (1990) computer software package based on fixed effect model stated by Henderson (1953). One-way ANOVA was used to assess the statistical difference between male and female and among breed variation.

$$Y_{ijk} = \mu + a_i + b_j + e_{ijk}$$

Where,

Y_{ijk} = Observational value of the k^{th} animal

μ = Overall mean

a_i = i th effect of breed (Kage, Lampuchhre and Baruwai)

b_j = j th effect of sex (Male and Female)

e_{ijk} = error term

RESULT

Hematological parameters

Red Blood Cell indices

Red Blood Cell indices i.e. total Red Blood Cell count (RBC), Haemoglobin (Hb), Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC) were found to be $12.74 \pm 1.90 \times 10^{12}/l$, $14.97 \pm 1.90 \text{ g/l}$, $36.74 \pm 4.76\%$, $28.92 \pm 2.41 \text{ fl}$, $11.81 \pm 0.82 \text{ pg}$, $40.52 \pm 2.98 \text{ g/dl}$ respectively. Nepalese sheep exhibited higher MCHC than normal physiological range (31- 34g/dl). Highly significant gender variation was observed for MCV ($P < 0.01$) and MCH ($P < 0.05$) where females showed comparatively higher value than males.

Table2: Nepalese Sheep Red Blood Cell indices (Least Square $\pm$ Standard Error)

Factors	No.	RBC($10^{12}/l$)	Hb(mg/dl)	PCV (%)	MCV(fl)	MCH(pg)	MCHC(g/dl)
Overall Mean	25	12.61 ± 0.36	14.7 ± 0.38	36.29 ± 1.01	28.82 ± 0.42	11.79 ± 0.17	40.62 ± 0.67
Breed		NS	NS	NS	NS	NS	NS
Baw	9	12.11 ± 0.57	14.3 ± 0.60	35.92 ± 1.59	29.61 ± 0.67	11.92 ± 0.26	40.16 ± 1.05
Kag	11	13.85 ± 0.54	16 ± 0.57	38.57 ± 1.51	27.94 ± 0.63	11.59 ± 0.25	40.65 ± 1
Lam	5	11.87 ± 0.77	14 ± 0.8	34.39 ± 2.14	28.90 ± 0.90	11.86 ± 0.34	41.05 ± 1.41
Sex		*	NS	NS	**	*	NS
Male	11	13.37 ± 0.52	15.2 ± 0.55	36.64 ± 1.46	27.31 ± 0.61	11.41 ± 0.24	40.89 ± 0.96
Female	14	11.84 ± 0.50	14.3 ± 0.50	35.94 ± 1.39	30.33 ± 0.59	12.16 ± 0.23	40.34 ± 0.91
CV		13.57	12.04	13.01	6.95	6.68	7.77

Mg/dl=milligram per deciliters, fl= femtolitre, pg= pictogram, g/dl=gram per deciliters,
NS=Not Significant, CV= Coefficient of Variation

White Blood Cell indices

White Blood Cell indices i.e. White Blood Cell Count (WBC), Lymphocytes (LYM), Monocytes (MO), Neutrophils (NE) were found to be $10.56 \pm 3.87 \times 10^9/l$, $46.81 \pm 18.59\%$, $0.50 \pm 0.00\%$, and $52.69 \pm 18.59\%$ respectively while Eosinophils and Basophils were undetectable for Nepalese sheep. LYM and NE value was significantly higher in Kage breed in comparison to Lampuchhre and Baruwal. The highly significant gender variation for MCV ($P < 0.01$) and MCH ($P < 0.05$) where female showed the higher value than males.

Table 3: Nepalese sheep White Blood Cell Indices (Least Square $\pm$ Standard Error)

Factors	No. of individual	WBC ($10^9/l$)	LYM (%)	NE (%)
Overall Mean	25	10.31 ± 0.84	49.02 ± 3.23	50.48 ± 3.23
Breed		NS	*	*
Baw	9	10.77 ± 1.33	56.94 ± 5.10	42.56 ± 5.10
Kag	11	11.35 ± 1.26	36.89 ± 4.38	62.60 ± 4.82
Lam	5	8.81 ± 1.79	53.23 ± 6.85	46.26 ± 6.86
Sex		NS	NS	NS
Male	11	10.96 ± 1.21	53.74 ± 4.67	45.76 ± 4.67
Female	14	9.66 ± 1.16	44.30 ± 4.45	55.19 ± 4.45
CV		37.75	32.62	28.98

NS=Not Significant CV= Coefficient of Variation

Biochemical indices

The overall Least Square (LS) mean for Total protein (TP), Albumin (Alb), Glucose (Glu), Calcium (Cal) and Phosphorous (P) were $5.94 \pm 0.53 \text{ mg/dl}$, $3.15 \pm 0.06 \text{ mg/dl}$, $55.66 \pm 12.89 \text{ mg/dl}$, $11.39 \pm 0.77 \text{ mg/dl}$ and $6.60 \pm 1.60 \text{ mg/dl}$ respectively. The result of present study revealed that the overall LS mean for TP for Nepalese breed was lower while that for Alb was higher than physiological range. The breed differences were observed in TP where Lampuchhre sheep (lowland sheep) showed the lower value than hill sheep breeds (Baruwal and Kage sheep) taken in the study. The same breed exhibited gender variation in the same parameter with values in males were significantly higher ($P < 0.05$) than females. Comparing LS Mean for Alb and P for Nepalese sheep, value recorded for females were found to be significantly higher ($P < 0.05$) than males in case of serum Alb while serum P was females.

Table 4: Nepalese sheep biochemical indices (Least Square $\pm$ Standard Error)

Factors	No.	TP (mg/dl)	Alb (mg/dl)	Glu (mg/dl)	Cal (mg/dl)	Phos (mg/dl)
Overall Mean	25	5.88 ± 0.10	3.10 ± 0.06	56.55 ± 2.94	11.41 ± 0.18	6.60 ± 0.31
Breed		NS	NS	NS	NS	NS
Baw	9	5.73 ± 0.16	3.06 ± 0.08	54.37 ± 4.48	11.45 ± 0.28	6.27 ± 0.48
Kag	11	6.32 ± 0.17	3.16 ± 0.08	57.29 ± 4.52	11.45 ± 0.28	7.36 ± 0.48
Lam	5	5.60 ± 0.22	3.07 ± 0.11	57.98 ± 6.09	11.33 ± 0.38	6.17 ± 0.65
Sex		*	*	NS	NS	*
Male	11	5.91 ± 0.15	2.97 ± 0.08	52.68 ± 4.14	11.31 ± 0.26	7.59 ± 0.44
Female	14	5.86 ± 0.15	3.23 ± 0.08	60.41 ± 4.16	11.51 ± 0.26	5.61 ± 0.44
CV		8.27	8.01	24	7.37	21.68

mg/dl=milligram per deciliters, NS=Not Significant, CV= Coefficient of Variation

Table 5:Range of hematological and serum biochemical indices of Nepalese sheep

Haematology (RBC Indices)			
Parameters	Unit	Nepalese sheep range	Normal range of sheep*
RBC(Total Count)	($10^{12}/l$)	9.56-16.96	9-15.8
Hb	(mg/dl)	11.9-19.9	9 - 15
PCV	%	26.22-47.47	27 - 45
MCV	fl (femto litre)	26-34	28 - 40
MCH	pg (picogram)	10.4-13.6	8 - 12
MCHC	g/dl	35.71-48.4	31 - 34
Haematology (WBC Indices)			
WBC (Total Count)	$10^9/l$	5.26-19.8	4 - 12
Lymphocyte	%	0-71	40 - 70
Neutrophile	%	28.5-65	10 - 63
Monocyte	%	0.5	0 - 6
Eosinophile	%	0	1 - 8
Basophile	%	0	0 - 3
Biochemistry			
TP	(mg/dl)	4.93-7.04	6-7.9
Alb	(mg/dl)	2.68-3.54	2.4-3
Glu	(mg/dl)	33.33-75	42-76
Cal	(mg/dl)	10.03-12.61	11.5-12
P	(mg/dl)	5.05-8.42	5-7.3

*Adapted from: Research Animal Resources [RAR] (2009)

DISCUSSION

Hemoglobin is the iron-containing oxygen-transporting protein in the red blood cells of vertebrates. The deficiency of hemoglobin in the red blood cells decreases blood oxygen-carrying capacity leading to symptoms of anemia while higher Hb value indicates higher oxygen carrying capacity of the blood and greater ability to resist disease infection. Nepalese Breed exhibited higher concentration of Hb (14.7 ± 0.38 mg/dl) than Northern Nigeria sheep breed i.e 12.90 ± 0.22 mg/dl (Njidda *et al.*, 2014) and Iranian sheep breed i.e. 8.93 ± 1.07 mg/dl (Oramari *et al.*, 2014) which suggests Nepalese Sheep breeds have higher oxygen carrying capacity and therefore more resistance to infectious disease. Among three breeds, Kage recorded the highest Hb concentration than others though it is not statistically significant. It could be because Kage can adapt in quite a range of altitude. Hb concentration for Baruwal and Lampuchhre breeds are comparable. The result of RBC shows that males had significant higher value than females, which is the reflection of health status of the breed studied. Breedwise variation in RBC value was also observed. Kage and

Baruwal breed, which have been well adapted in higher altitude compared to lowland breed-Lampuchhre, have recorded higher value for RBC number, Hb and PCV, which could provide evidence of the adaptation of these breeds to low atmospheric oxygen. The effect of altitude on erythrocyte values has been studied by many investigators and it is now a well-established fact that reduced oxygen tension in mountainous regions leads to an increased production and release of erythropoietin, thereby, stimulating erythropoiesis as a coping or adaptive mechanism to low oxygen level in such an environment (Tibbo *et al.*, 2004).

PCV or Haematocrit value refers to percentage of whole blood comprises of RBC and therefore is crude marker of red cell number. The present study revealed that breed had no significant effect on PCV and Hb values. In contrast, the breed differences for these parameters was reported by (Forhead *et al.*, 2002 and Jawasreh *et al.*, 2009). Similarly, gender exhibited no significant effect on same parameters and this result concords with other workers (Tibbo *et al.*, 2004; Shumaila *et al.*, 2012). While Egbe-Nwiyi *et al.*, (2000) reported the sex differences in HB and PCV of Nigeria sheep. PCV value in Nepalese breed (36.29 ± 1.01) was within physiological range and considerably higher than Iranian breeds (27.35 ± 3.16) which might be a sign of superior health status of Nepalese sheep.

The values of MCV, MCHC and MCH are very important in the diagnosis of anemia and serve a useful index of the capacity of the bone marrow to produce red blood cells (Awodi *et al.*, 2005). The MCV, MCHC and MCH values are greatly influenced by age and sex (Egbe-Nwiyi, 2000). In this study, MCHC value in Nepalese breed was higher than physiological range. The highly significant gender variation for MCV ($P < 0.01$) and MCH ($P < 0.05$) where female showed the higher value than males.

The major functions of the white blood cell and its differentials are to fight infections, defend the body by phagocytosis against invasion by foreign organisms and to produce or at least transport and distribute antibodies in immune response. Thus, animals with low white blood cells are exposed to high risk of disease infection, while those with high counts are capable of generating antibodies in the process of phagocytosis and have high degree of resistance to diseases (Soetan *et al.*, 2013) and enhance adaptability to local environmental and disease prevalent conditions (Kabir *et al.*, 2011; Okunlola *et al.*, 2012; Iwuji and Herbert, 2012; Isaac *et al.*, 2013). The mean total WBC count obtained in this study fell within the normal physiological range, with majority of WBC made up of lymphocytes and neutrophils. Leucocyte cell distribution is affected by breed, temperature, environmental as well as body's demand and health status (Mbassa and Poulsen, 2003). Lymphocytic count was significantly lower in Kage breed compared to others while same breed recorded significant high value for neutrophil count. Males showed higher value for lymphocytes than females while females showed higher value for neutrophils and this finding agrees with values (Egbe-Nwiyi *et al.*, 2000) for sheep and goats in arid zone of Nigeria but contradicts with the record reported by Njidda *et al.*, 2014. Monocytes were consistent for both breed and gender while Eosinophils and Basophils were undetectable in this study.

Serum biochemical indices is used to determine the level of heart attack, liver damage and to evaluate protein quality and amino acid requirements in animals (Harper *et al.*, 1977). All biochemical parameters in this study were not significant for breeds while TP, Alb and P were observed statistically significant for gender. This result is consistent with earlier findings which

illustrated the effect of breed on total protein, albumin was not significant (Gundogan, 2005). The value for TP in was less than normal physiological range, which might be attributed to poor nutrition. The biochemical parameters are usually influenced by nutrition level and closely associated with metabolic activity of individual animals (Shumaila *et al.*, 2012). The breed difference for TP was observed where Kage breed recorded the highest value and Lampuchhre breed the lowest. Males exhibited significant higher value for TP than females. The Overall LS mean for Alb was observed higher than normal range and same parameter is significantly higher in females.

The result obtained for glucose value fell within normal physiological range for ovine species and females showed higher values than males. This value was found consistence among breeds. The value of glucose depends upon metabolic demand of body, exercise and diet. The value for calcium was consistent for both breed and sex while value for phosphorous was significantly higher in males. About 80% of the body phosphorous is present in the bones and teeth. The 20% in the soft tissues make up phosphor-protein, nucleoprotein, phospholipids, hexose phosphate and other compounds, which are essential in organ structure, nutrient transport and energy utilization.

CONCLUSION

The result of the preliminary study revealed that breed has no significant effect on all biochemical parameters under study and most of the haematological parameters except WBC indices. While most of the biochemical and haematological parameters were influenced by gender. The hematological and biochemical parameter values obtained from this study can be used as normal reference to assess health status of Nepalese breed of sheep. Further research with more number of samples is warranted for concrete information, which could be used to monitor animal health status to improve the management and conservation of these breeds.

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STATUS OF BRUCELLOSIS IN DAIRY CATTLE OF KAPILVASTU AND BHAKTAPUR DISTRICTS OF NEPAL

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ABSTRACT

Brucellosis is a zoonotic disease that causes abortion in dairy cattle. To find out its status, serological tests were conducted, during June-July 2013, in 48 sera samples from dairy cattle (23 from Kapilvastu and 25 from Bhaktapur districts) having the recent history of abortion. Out of 48 samples 6 (12.5%) were positive on Rose Bengal Plate Test. Among 6 positive samples, 2 (8.69%) were from Kapilvastu and 4 (16%) from Bhaktapur. Considering the positive cases of brucellosis in the dairy pocket areas and its threat of transmission to other animals and human, a suitable preventive and control measures including the regular test and segregation of sero-positive animals, effective quarantine, legislative measures and awareness programs for farmers, veterinarian, technicians and stakeholders are recommended.

Keywords: abortion, brucella, cattle, rose bengal plate test, zoonosis

INTRODUCTION

Brucellosis is one of the most widespread bacterial zoonotic diseases of cattle, buffalo, swine, goats, sheep, dogs and human, resulting into tremendous economic losses in endemic regions. In human, it causes Malta or Mediterranean fever (Godfroid *et al.*, 2005). It is caused by gram-negative coccobacilli of the genus *Brucella* that contains a group of very closely related bacteria. The first member of the group, *Brucella melitensis*, affects primarily sheep and goats. The second member of the group, *Brucella abortus*, affects primarily cattle, the third member of the group, *Brucella suis*, affects primarily pigs, the fourth member of the group, *Brucella ovis*, affects primarily rams and ewes, the fifth member of the group, *Brucella neotomae*, affects primarily the desert wood rats, and the sixth member of the group, *Brucella canis*, affects primarily male dogs and bitches (Corbel and Macmillan, 1998). Cattle infected with *B. melitensis* poses a public health problem. *B. abortus* is communicable from cattle to sheep or sheep to sheep or sheep to cattle (Chakrabarti, 2007).

Brucellosis has both public health and economic significance in most developing countries. The disease is transferred from meat, milk and other contaminated materials. Epidemiological information could be important tool in control and eradication. Establishing the epidemiology of the disease could be an important source of information for farmers, students, future researchers, control strategy and other persons as well. There have been little studied on abortive diseases in dairy cattle in Nepal. This study was therefore, aimed at assessing the status of brucellosis in dairy cattle of selected pocket areas of Kapilvastu and Bhaktapur districts.

MATERIALS AND METHODS

Study areas

Kapilvastu and Bhaktapur districts were selected as the study area where the large numbers of abortion occurred in dairy cattle. The selected areas have high potential for milk production in recent year. Nowadays, the numbers of cross breeds are increasingly used for large amount of milk production in this area. Abortion causes huge economic losses in that area and small dairy farmers are suffering from this problem. The blood samples were collected from aborted cattle at Motipur, Kopawa, Bhalwad, Banganga, Hathausa, Budhi, and Gajehada of Kapilvastu district and Balkot, Dadhikot, Jhaukhel, Sirutar and Bageswori of Bhaktapur district.

Blood sampling

A total of 48 blood samples (23 from different VDCs of Kapilvastu district and 25 from different VDCs of Bhaktapur) were collected from cattle with recent history of abortion. About 5 ml of blood was collected aseptically from the jugular vein and serum samples were extracted in serum vials and stored in refrigerator at 4°C until tests.

Rose Bengal plate test (RBPT)

Laboratory examinations of samples were conducted at Animal Health Research Division, NARC, Khumaltar, Lalitpur. The RBPT test was performed according to information provided by OIE guidelines (2009). Briefly, the serum samples and antigen were placed to room temperature ($22 \pm 4^\circ\text{C}$); only sufficient antigen for the day's tests was removed from the refrigerator. 30 µl of each serum sample was placed on a clean grease free glass slide. The antigen bottle was well shaken, gently, and placed equal volume of antigen near each serum spot. Immediately after the last drop of antigen has been added to the plate, the serum and antigen were mixed thoroughly (using a clean glass or plastic rod for each test) to produce a circular or oval zone approximately 2 cm in diameter. The mixture was agitated gently for 4 minutes at ambient temperature manually. It was then read for agglutination immediately after the 4-minute period was completed. Any visible agglutination was considered to be positive and none agglutinated mixture was considered to be negative.

Questionnaire survey

The questionnaires were designed to retrieve information from each farmer on the usual main risk factors, such as the introduction of infected animals into the herd, the level of management system in the farm and the exposure of the herd to an infectious disease. It is also concerned with the general management of the farm, recording system and health problems.

Data Analysis

The data were analyzed to determine the percentage of positive cases for brucellosis.

RESULTS

Management status and animal details

Questionnaire survey based management status of animals in the study area is shown in Table 1. Survey revealed that (85%) of the farmers of both Kapilvastu and Bhaktapur districts adopted semi intensive system while remaining (15%) practiced intensive farming system. Furthermore, it was understood that (80%) of farmers (households) used artificial insemination for breeding purpose

whereas only (20%) used bull for breeding their dairy cattle. Regarding the cases of abortion and repeat breeding in dairy cattle, 25% of household reported the cases of abortion in first trimester, 15% in second trimester, 8% in third trimester and 52% of household had no history of abortion in their dairy cattle. Similarly, 47% of household had experienced the cases of repeat breeding in their dairy cattle.

Table 1: Status of animal management in Kapilvastu and Bhaktapur districts

Activities	Households	
	Number	Percentage (%)
Animal details		
1. Breeding of animal		
a. Artificial Insemination	80	80%
b. Natural service	20	20%
2. History of abortion		
a. First trimester	25	25%
b. Second trimester	15	15%
c. Third trimester	8	8%
d. No history of abortion	52	52%
3. History of repeat breeding	47	47%
4. Farming system		
a. Intensive	15	15%
b. Semi intensive	85	85%

Rose Bengal Plate Test (RBPT) result

Out of 48 samples screened by the RBPT six (12.5%) were found positive of which 4 were from Bhaktapur and 2 were from Kapilvastu district (Fig. 1).

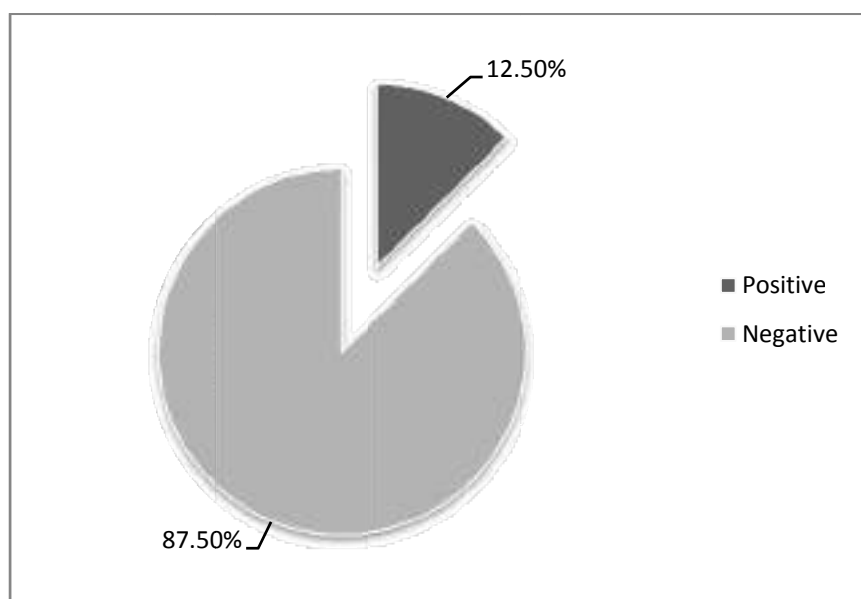


Fig.1: Showing result of Rose Bengal test for brucellosis

District wise status of brucellosis

Out of six positive samples four were from Bhaktapur and two were from Kapilvastu district (Fig. 2).

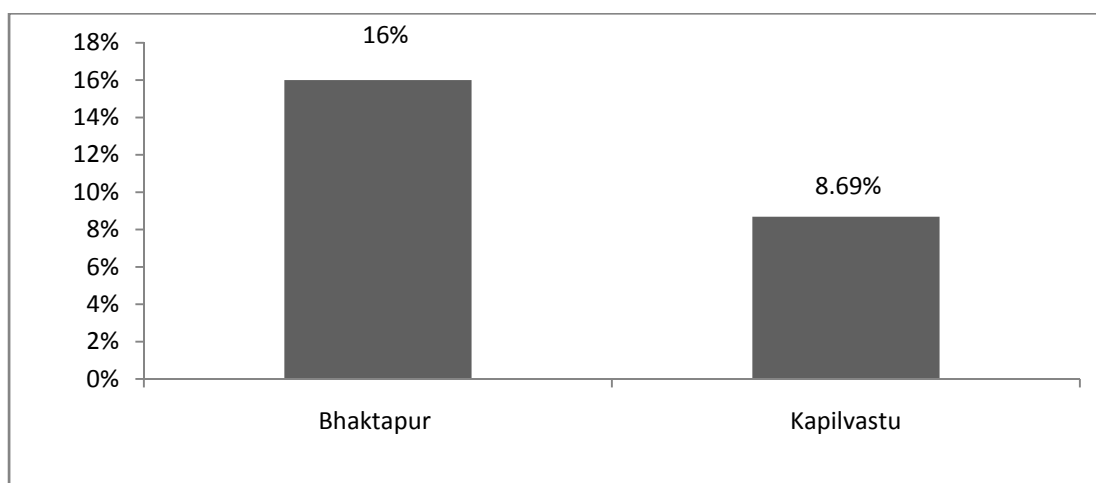


Fig. 2: District wise prevalence of brucellosis

VDC wise status of brucellosis in Bhaktapur district

Out of four positive samples of Bhaktapur district two samples Jhaukhel were positive for brucellosis and one sample each was found positive in Balkot and Sirutar VDC no positive samples were found in Dadhikot and Bageswori (Fig. 3).

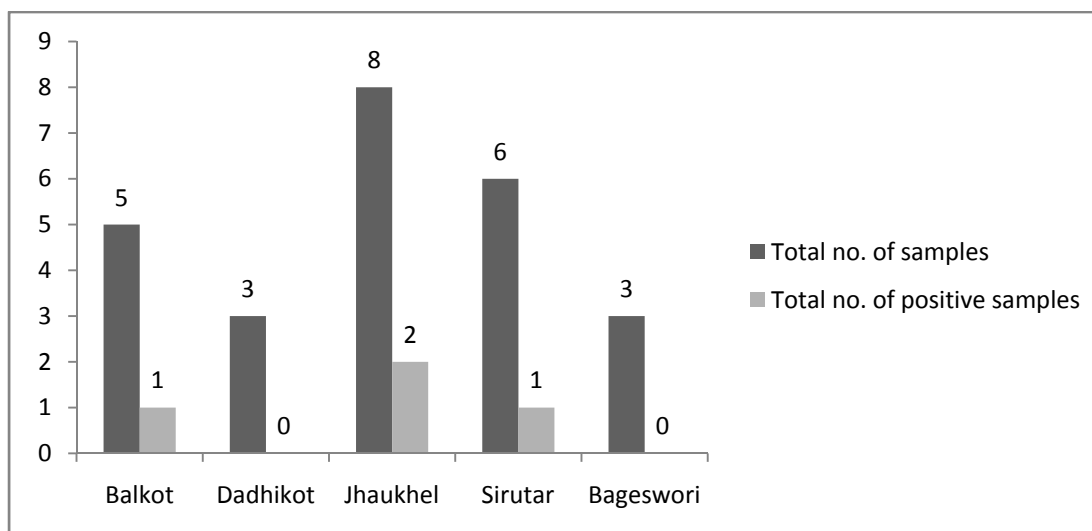


Fig. 3: VDC wise prevalence of brucellosis in Bhaktapur district

VDC wise status of brucellosis in Kapilvastu district

Out of two positive samples of Kapilvastu district each positive case was from Motipur and Banganga VDC (Fig. 4).

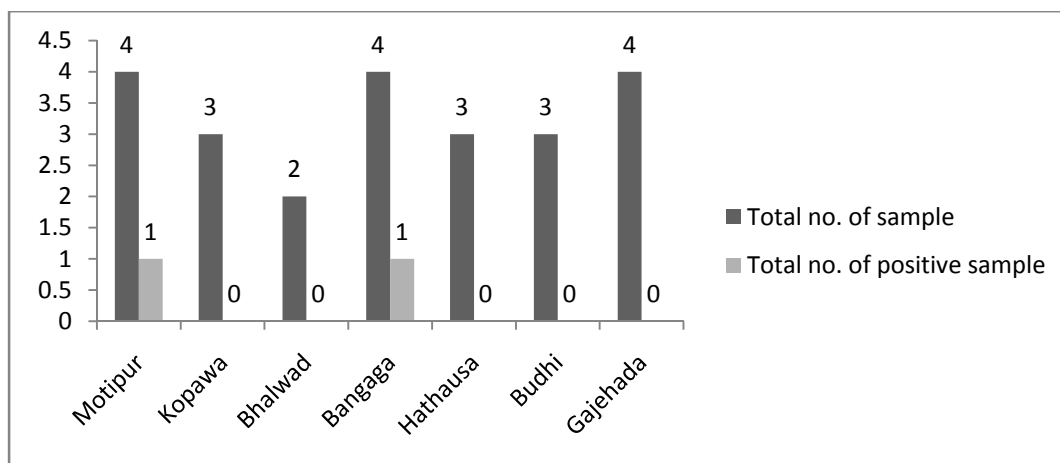


Fig. 4: VDC wise prevalence of brucellosis in Kapilvastu district

Age wise status of brucellosis in dairy cattle

Out of six positive samples two were from 0-4 year's age group and rest were from above the four years age group (Fig. 5).

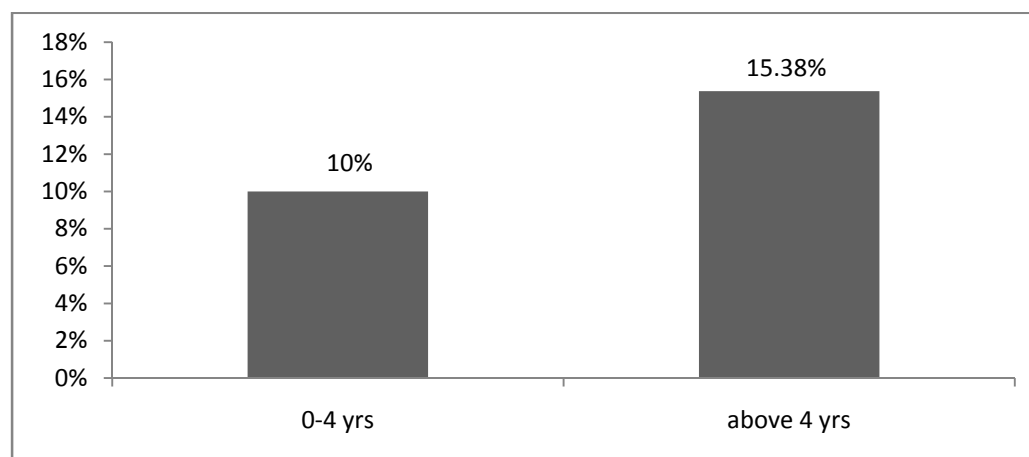


Fig. 5: Age wise prevalence of brucellosis

DISCUSSION

The husbandry system adopted in Bhaktapur and Kapilvastu are mostly traditional sedentary without scientific housing system. There is close contact between human and animal during milking, feeding, grazing, care during parturition, which may result in the transmission of zoonotic diseases. The present finding is like the result obtained by Pandeya (2013) in different animal species of Kailali district. Lack of quality semen for AI, use of the unscreened bull for natural service, improper handling of aborted materials, improper import of bulls and lack of awareness on brucellosis among the farmers and the public are causes for the transmission.

Earlier Pyakurel and Mishra (1977) found 22.64% positive cases of brucellosis in buffalo, which is quite higher than the present finding. Pyakurel and Mishra (1977) tested for the prevalence of

brucellosis in cattle of Kathmandu and found (17.47%) were positive. Present finding is quite like the finding of Pyakurel and Mishra (1977).

The analysis carried out by DHS (2003) indicated that in Nepal around (2-3%) of the cattle and buffalo are sero-positive, which is quite lesser than this finding. This contrast result might be due to less number of animals and the aborted dairy cattle whose main cause might be brucellosis for abortion. Dhakal *et al.*, (2005) carried out the serological study in 35 slaughtered buffaloes in Chitwan and (2.86%) of the buffaloes were found to be positive for brucellosis. Mishra (2008) studied the sero-prevalence of brucellosis in buffaloes of Bhaktapur district and found (15%) to be positive, which is quite like this finding. This might be due to the reason of rearing of cattle and buffalo in the same farm of those areas where there is chance of transmission of the disease from infected to the healthy ones.

CONCLUSION

Serological screening of dairy cattle with the history of abortion in Kapilvastu and Bhaktapur districts has indicated 12.5% of cattle (6 out of 48) positive for brucellosis, the latter showing more positive cattle (16%) than Kapilvastu (8.69%). Since dairy cattle are raised near with human population, direct contact with aborted materials and placenta in absence of protective gears shall increase the likelihood of contracting brucellosis in humans in affected districts. Thus, the status of brucellosis in human is another facet to be studied.

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ACCURACY OF PREGNANCY DIAGNOSIS WITH COMMERCIALY AVAILABLE PROGESTERONE KIT IN DAIRY COWS

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ABSTRACT

Present study was carried out for the accuracy of commercially available progesterone ELISA kit at NCRP farm in the fiscal year 2015/16. Twenty crossbred Jersey and Holstein dairy cows were selected at different time periods of post insemination. Blood serum was collected in those animals and progesterone was quantified with the commercially available progesterone ELISA kit. Pregnancy diagnosis was performed by rectal palpation and Ultrasonography (USG) as a Gold standard and compared for the accuracy to ELISA kit. Results of the ELISA kit revealed the accuracy of the kit to be only 80 % with high sensitivity 92 % and very low specificity 57 % at 95 % confidence interval. Out of twenty artificially inseminated cows, thirteen were pregnant and seven were non-pregnant by the Gold standard test with their significantly different mean progesterone (at $P < 0.05$) 8.93 ± 1.10 ng/ml and 4.36 ± 1.21 ng/ml respectively. Hence, it can be used in the early pregnancy diagnosis at only after 24 days of the insemination, however, progesterone quantification by ELISA is not the confirmatory tests for the pregnancy diagnosis as this results accuracy of only 80 %.

Keywords: sensitivity, specificity, pregnant, true positive, false positive

INTRODUCTION

Progesterone is the most biologically active progestogen in cattle and is primarily produced and secreted by the corpus luteum (CL) during estrous cycle and by both placenta and CL during pregnancy. Quantification of progesterone in blood or milk can be achieved in a laboratory using Radioimmunoassay (RIA) or Enzyme-Linked Immuno-Sorbent Assay (ELISA) methods. The biology of early pregnancy and maintenance of the CL results in distinct progesterone profiles for pregnant compared with non-pregnant cows. Hence, progesterone quantification could be helpful in pregnancy examination that could be diagnosed as early as 21-day post insemination (Fricke *et al.*, 2016).

Pregnancy diagnosis is an important part of reproductive management to maintain the reproductive efficiency of the herds. In general, Nepalese dairy farms must wait more than a month to know the pregnancy status by rectal palpation and since, only the expert and trained skilled person can detect the pregnancy within that period by rectal examination; it may not be always accurate. Ultrasonography (USG) which gives almost 100 % accuracy in the pregnancy confirmation is also not used in Nepal because it is expensive and needs well trained and skilled person for the examination (NCRP, Annual Report, 2016).

Progesterone estimation by the commercially available kits is an important tool for early detection of pregnant animals. Cows can be diagnosed as early as 24 days after insemination. Progesterone analysis may be performed on the farm or in a veterinary clinic, but users must become familiar with the procedures, proper interpretation of results, and limitations of this tool (Pen State University, 2016).

Hence, there is clearly a potential in many dairy production systems of Nepal to apply this method as a diagnostic service to farmers. Therefore, the objective of this study was to verify the accuracy of these kits by progesterone estimation for the pregnancy diagnosis test in context to dairy cows of Nepal. Although the kits have several advantages like to carry out the test by milk samples, furnish the results by color changes in a micro-plate well and semi quantitative test, this study is confined in the analysis of the quantitative results only.

MATERIALS AND METHODS

Total 20 dairy cows with no uterine and ovarian pathology at the National Cattle Research Program (NCRP), Rampur, Chitwan were taken under the study. Four blood serum samples of within 37 to 40 days and rest samples beyond 45-day post insemination were taken and stored at -20°C before carrying out the progesterone estimation test at the Animal Health Research Division, Khumaltar, Lalitpur. Commercially available progesterone ELISA kit from the Abraxis LCC USA Company was used. Standard curve and average progesterone level (ng/ml) of high control were quantified from the ELISA optical density (OD) readings and its spreadsheet Excel calculation was identified as a cut-off point to differentiate positive and negative pregnancy. Mean $\pm$ SEM of progesterone concentration (ng/ml) of the pregnant and non-pregnant cows were obtained and compared with t-test by using SPSS version 16. True positive, true negative, false positive and false negative count were used for the calculation of the sensitivity and specificity and finally, accuracy of the test at 95 % Confidence Interval (CI) was done from the calculator or programme written by Hutchon (2016). Transrectal palpation after two-month post AI as the Gold Standard test for the pregnancy diagnosis was also included and confirmation by B-mode Ultrasound Machine of 7.5 MHz was followed which was assumed to be 100 percent accurate.

RESULTS AND DISCUSSION

Standard Curve for the kit readings OD Values against progesterone quantification

Four-parametric logistic fitting standard curve for the progesterone ELISA (Duplicates) obtained from those sample tests is given below in the Fig. 1. Average progesterone level of high control for micro plate well was quantified as 4.156 ng/ml and the concentration above this value was considered as pregnancy positive for every sample and the below as negative.

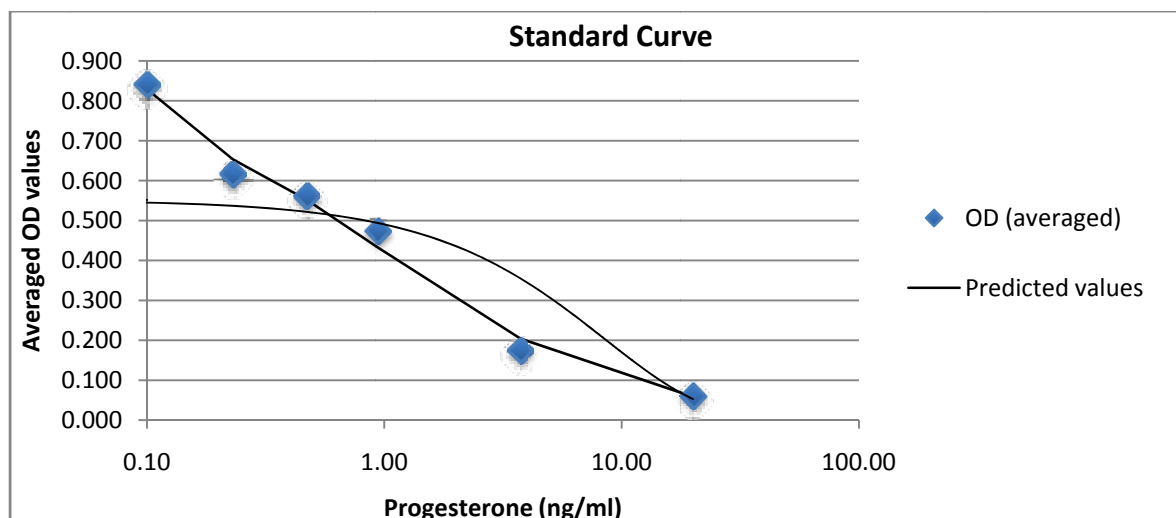


Fig. 1: Four-parametric logistic fitting standard curve for the progesterone ELISA

Progesterone concentration of the pregnant cows

Mean progesterone concentration in the Gold standard pregnant positive cows was found to be significantly different at $P < 0.05$ with 8.93 ± 1.10 ng/ml in positive cows in comparison to negative cows (Table 1) which is consistence as reported by Gumen *et al*, 2003. However, the mean of the progesterone in negative cows was found to be more than individual set cut off point as calculated from kits for the average high control. This may be due to false positive results generated from the kit or luteal phase effect.

Table 1: Progesterone concentration in pregnant and non-pregnant cows

Pregnancy Status	Individual (n)	Mean $\pm$ SEM (ng/ml)
Positive	13	8.93 ± 1.10
Negative	7	4.36 ± 1.21

Mean $\pm$ SEM (ng/ml) significantly different at $P < 0.05$

Sensitivity, specificity and accuracy of the progesterone test kit

Out of 15 tests positive as detected by the kit, 12 were detected as true positive against the 13 positive Gold Standard Test with 3 false positive having the sensitivity of 92 % at 95% CI. Similarly, out of 5-test negative detected against 7 negative Gold Standard test, 4 were true negative and 1 false negative having the specificity 57 % at 95% CI (fig. 2). And the accuracy of the kit results was only 80 %.

Sensitivity and Specificity of the progesterone ELISA kits for the pregnancy diagnosis

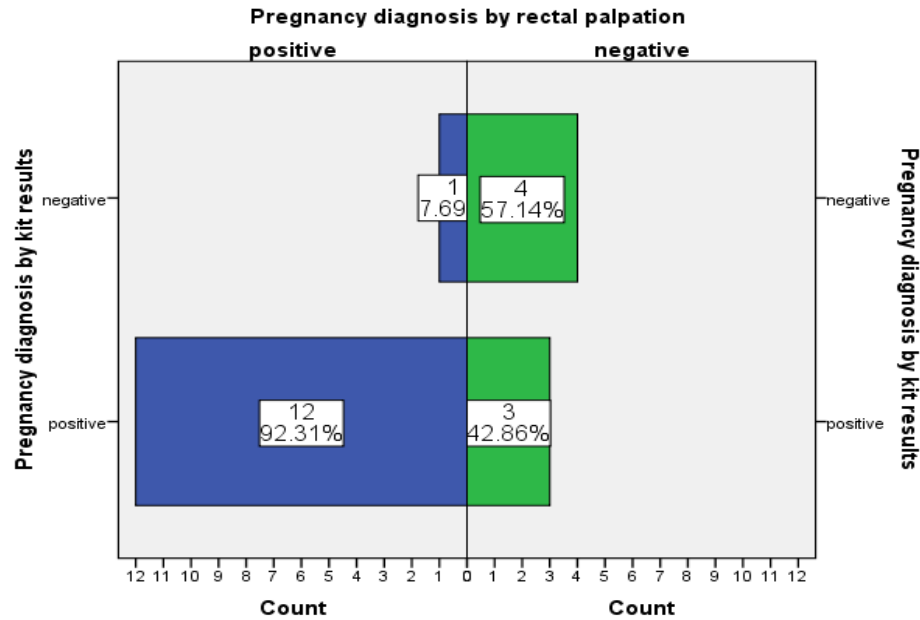


Fig. 2: Graph representing the ELISA kits test results against the Gold Standard Test Rectal palpation

Accuracy of pregnancy diagnosis was found to be 84% at an early stage 37-41 day (Alam and Ghosh, 1994) which contrasts with this present study (Table 2) and 100 % as reported by (Muhummad *et al.*, 2000) which is similar to this study.

Table 2: ELISA Kits Pregnancy diagnosis results of the cows against Rectal Palpation Test at different stages of pregnancy

Days Post Artificial Insemination		Pregnancy Diagnosis by ELISA Kit test		
		Positive Count % of Total	Negative Count % of Total	Total Count %
37- 45 days pregnant Pregnancy Diagnosis by Rectal Palpation	Positive Count	3		3
	% of Total	75.0%	0	75.0%
	Negative Count	1	0	1
	% of Total	25.0%		25.0%
Total Count %		4	0	4
		100.0%		100.0%
46- 60 days Pregnancy Diagnosis by Rectal Palpation	Positive Count	2	1	3
	% of Total	66.7%	33.3%	100.0%
	Negative Count	0	0	0%
	% of Total			
Total Count %		2	1	3

		66.7%	33.3%	100.0%
61- 90 days Pregnancy Diagnosis by Rectal Palpation	Positive Count	1	0	1
	% of Total	33.3%		33.3%
	Negative Count	2	0	2
	% of Total	66.7%		66.7%
	Total Count %	3	0	3
		100.0%		100.0%
91- 120 days Pregnancy Diagnosis by Rectal Palpation	Positive Count	0	0	0
	% of Total			
	Negative Count	0	2	2
	% of Total		100.0%	100.0%
	Total Count	0	2	2
			100.0%	100.0%
beyond 120 days Pregnancy Diagnosis by Rectal Palpation	Positive Count	6	0	6
	% of Total	75.0%	0.0%	75.0%
	Negative Count	0	2	2
	% of Total	0.0%	25.0%	25.0%
	Total Count	6	2	8
		75.0%	25.0%	100.0%

Similarly, there was non-significant results on the accuracy of diagnosis upon parity basis as reported by (Muhummad *et al*, 2000) which is as similar to this study results (Table 3).

Table 3: Pregnancy diagnosis by the ELISA kit results against Rectal Palpation test in cows at different parity

Parous		Pregnancy Diagnosis by ELISA Kit Test			
		Positive Count of Total	Negative Count % Total	Total of %	Count
Nulliparous Pregnancy Diagnosis by Rectal Palpation	Positive Count	5	0	5	
	% of Total	83.3%	0.0%	83.3%	
	Negative Count	0	1	1	
	% of Total	0.0%	16.7%	16.7%	
Primiparous Pregnancy Diagnosis by Rectal Palpation	Total Count	5	1	6	
	%	83.3%	16.7%	100.0%	
	Positive Count	2	0	2	
	% of Total	33.3%	0.0%	33.3%	
	Negative Count	2	2	4	
	% of Total	33.3%	33.3%	66.7%	
	Total Count	4	2	6	
	%	66.7%	33.3%	100.0%	

Pluriparous Pregnancy Diagnosis by Rectal Palpation	Positive Count	5	1	6
	% of Total	62.5%	12.5%	75.0%
	Negative Count	1	1	2
	% of Total	12.5%	12.5%	25.0%
	Total Count	6	2	8
	%	75.0%	25.0%	100.0%

Several studies in the U.S. and Europe have evaluated milk progesterone analysis as a method of pregnancy diagnosis. Generally, it has been shown that high progesterone in 20 to 24 days post-breeding is only 75 percent accurate in confirming pregnancy. There are various conditions such as severe uterine infection, some cystic ovarian conditions and early embryonic death other than pregnancy can cause high progesterone concentrations 20 to 23 days' post breeding (Penn State University, 2016).

CONCLUSION

It can be said that progesterone quantification by EIA is not the confirmatory tests for the pregnancy diagnosis as this also gives the false positive and false negative results with the accuracy of only 80 % however, it can be used in the early pregnancy diagnosis as a screening purpose after 21 to 24 days of insemination as this can almost predict the positive pregnant animals as a positive with high sensitivity.

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PREVALENCE OF BLOOD PARASITES IN DOGS OF KATHMANDU VALLEY

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ABSTRACT

Study in blood parasitic diseases in dog was carried out from August 2014 to November 2014 to find out the prevalence of haemoparasites in clinical cases of hyperthermia in dogs. Blood sample from 50 cases of hyperthermia were collected and examined for any blood parasites by the smear method. The haematological parameter (RBC, WBC, PCV, Hb, & DLC) of each sample was also assessed and analyzed. Data was analyzed to determine prevalence of various species of blood parasites to establish the correlation of the infections with age, sex and breed. Out of 50 samples examined, blood parasites were determined in 12 percent samples of which 2(4%) were positive for Babesia canis and 4 (8%) were positive for Ehrlichia spp. The percentage of infection was greater in female 4(16.67%) than male 2(7.14%). The prevalence of blood parasite was higher in German shepherd. The prevalence of blood parasite was higher in 2-4 years of dogs. To determine significant difference between the hematological parameter of infected and non-infected cases, t-test was need (R, version 3.0.3). The confidence level for the analysis was set at 95percent with significance level assessed at $p < 0.05$. The study between the infected and non-infected host revealed statistically significant difference in PCV, Neutrophil and Eosinophil. Whereas other parameters did not have any significant difference. The mean PCV was significantly low ($P < 0.05$) in infected dog than in non-infected dog. Mean Neutrophil was significantly decreased whereas Eosinophil was increased in infected dogs than in non-infected dogs.

Keywords: breed, dog, haemoparasites, haematological parameter, hyperthermic, sex

INTRODUCTION

Endoparasitic infestations have always been a major health problem in animal species. These blood parasites affect the blood vascular system, which may be the intraerythrocytic parasite, the intraleukocytic parasite or those living freely (Urquhart, 1987). The common diseases caused by blood parasite in dog are babesiosis, trypanosomiasis and ehrlichiosis (Urquhart, 1987). The principal vector for *E. canis* is *Rhipicephalus sanguineus* (brown dog tick). *E. canis* is passed only transtadially in the tick, so unexposed ticks must feed on a rickettsemic dog to become infected and perpetuate the disease. *E. canis* is maintained in the environment by passage from ticks to dogs. These blood parasites are mostly diagnosed and identified by blood smear examination. Companion animals such as dogs are associated with different haemoparasites and can pose serious health concern worldwide (Manandhar, 2008) and has significant economic impact from veterinary standpoint.

Study conducted by (Maharjan *et al.*, 2014), the prevalence of haemoparasites in feral dogs in Lalitpur district was found to be of 10%. In the study, 10(9.3%) samples were detected positive for the *Babesia spp.* and 2(1.8%) samples were positive for *Ehrlichia spp.* Dogs negative for haemoparasites had Packed Cell Volume (PCV) within normal range (35-51%) whereas PCV was decreased in infected dogs.

The organism parasitizes the leucocytes mainly monocytes and lymphocytes. Some strains, which parasitize neutrophils have been reported, which are like *E. equi* (Kirk & Bistner, 1985). (Bhattacharjee and sarmah, 2013), showed the prevalence of 57.3 percent in the hospital population comprising pet (58.03%) and working (54.54%) dogs and 63.64% in stray dog population. (Tuna and Ulutas, 2009) Investigated the prevalence of *Ehrlichia canis* infection in thrombocytopenic dogs. Blood samples were collected from 224 dogs of different ages and of both sexes. Eighty-one (36.2%) of 224 dogs were positive to *E. canis* infection. *E. canis* infection occurs in the average of 5.22 years in the dogs and the range has been found within 2 months to 14 years (Tilley *et al.*, 1997).

Diagnosis of a disease as per blood examination is not a common procedure in Nepalese context. Fortunately, some cases are diagnosed in time and treated whereas most of the cases are treated symptomatically and supportively with the help of antibiotics and some cases are luckily recovered whereas some remain ill because of disease which may be caused by haemoprotozoan. Though the occurrence is low it is sometimes confused with other similar disease and the veterinarian overlooked the hemoprotozoan disease. So this research was mainly conducted to know the prevalence of blood parasites namely *Babesia spp.*, *Trypanosomes spp.*, and *Ehrlichia spp.* in dog based on breed wise, age wise & to compare the hematological parameters in blood parasites positive and negative cases of dogs.

MATERIAL AND METHODS

Materials such as sterile syringe, EDTA vial, glass slides, cotton, spirit, Giemsa's stain (Himedia), methanol, RBC and WBC diluting fluids, wintrobe's tube were used for this study.

Selection of dogs

Blood samples was taken from dogs with hyperthermia (temperature >103-degree F). These samples were randomly selected (irrespective of age, sex and breed) from hospital and clinics of different district

Collection of blood

About 2 ml of blood was withdrawn in sterile blood collecting vial containing EDTA. The blood was immediately transferred to a sterile vacutainer. The tube was kept under refrigeration at -4 °C until further laboratory works were performed. Collection of blood, preparation of slide and staining, RBC count, DLC, PCV and hemoglobin determination will be performed as per Benjamin (2010).

Preparation of slide and staining

A drop of blood was taken in a clean glass slide and by using another slide tilted at an angle of 45 degree, a thin smear was prepared and air dried immediately. Then the smear was fixed in methyl

alcohol for 2- 3 minutes. This fixed slide was then stained in working solution of Giemsa stain (Himedia) in coplin jar for 20-30 minutes. The slide was washed and dried for further examination.

Examination of slide for blood parasite

The slide was examined under oil immersion by using binocular compound microscope (Olympus). The smear was examined for the presence of blood parasite. Mainly the RBC's and WBCs was carefully looked. In the RBCs protozoans like *Babesia spp.*, and WBCs, especially monocytes for the presence of *Ehrlichia spp.*, and *Trypanosoma spp.* were looked between the blood cells. Mainly the periphery of the smear was examined.

Babesia species appeared as blue stained piroplasms having V or comma shape. They are singly or multiple present in the RBCs. *Ehrlichia spp.* was morulae in monocytes, lymphocytes and sometimes neutrophils.

RBC count and WBC count

RBC and WBC diluting pipette were taken separately, and blood and dilution fluid were drawn as per required. After mixing it well, a drop of the mixture was charged on a hemocytometer (Hawksley) and it was fixed in 100X microscope (Olympus). RBC and WBC were then counted.

Differential leukocyte count (DLC)

The same Giemsa stained smear was used for differential leukocyte count.

Packed cell volume (PCV) determination

PCV was determined by microhematocrit tube to separate the cells from plasma.

Hemoglobin

Hemoglobin was estimated by acid hematin method using Sahli's hemoglobinometer (Singhla Company).

Estimation of prevalence

The prevalence of infection was assorted in terms of breed, age and sex. The basic formula for calculating the prevalence in percentage is given below:

$$\text{Prevalence} = \frac{\text{Number of individual having a disease at a particular point of time}}{\text{Total number of individual in the population at risk sampled at that point of time}} \times 100$$

Data analysis

The collected data were entered spreadsheets and analysed with statistical software; a Statistical Package for Social Sciences (SPSS, version 16). Data were summarised as descriptive statistics (mean and percentages) and displayed as tables. Data regarding the hematology was analyzed by studentst test using R, version 3.0.3 (R Core Team, 2014). The confidence level for the analysis was set at 95 percent and $p < 0.05$ was considered significant.

RESULT

Overall prevalence of blood parasites

In the study done in 50 pyrexia dogs, 6 (12%) were found to be caused by blood parasites.

Blood parasitic species isolated in positive blood samples of dogs

Two different blood parasites were observed in 6 positive blood samples. *E. canis* was found highest 4(8%) followed by *Babesia canis* 2 (4%).

Sex-wise prevalence of blood parasites

The study comprised of 28 males and 22 females. Among 28 males, 2(7.14%) and among 22 females, 4 (16.67%) in infected cases.

Breed wise prevalence of blood parasites

Out of the 6 total positive cases 4 cases (20%) were encountered in German Shepherd breed of dog followed by 2 cases (15.38%) were encountered in Mongrel.

Age wise prevalence of blood parasites

Out of 50 blood sample, 29 samples were from age 1-5 years and 21 sample were from dog greater than 5 years. The age group of 1 –5 years i.e. 12 percent of canine population were infected.

Temperature

The mean temperature of the infected cases was found to be 103-105°F, whereas for the non-infected cases was 103.73 °F.

Mean RBC count, HB and PCV estimation

The mean RBC, Hb and PCV in infected dogs was $4.7 \times 10^6/\mu\text{l}$, 9.34 g% and 23.17% and in non-infected dogs was $5.61 \times 10^6/\mu\text{l}$, 10.8 gm% and 33.31% respectively.

DISCUSSION

The prevalence of blood parasites under study closely resembles the findings of (Maharjan *et al.*, 2014; Subedi, 2009; Manandhar and Rajawar, 2008) who found 10%, 14% and 17.14% respectively in dogs of Kathmandu valley. This may be due to the selection of hyperthermic dogs for the study or may be due to the seasonal variation (summer) or selection of dogs with tick infestation (Maharjan *et al.*, 2014; Subedi, 2012; Manandhar and Rajawar, 2006).

On the other hand, our study reported high prevalence of *Ehrlichia canis* followed by *Babesia canis*. Occurrence pattern is almost like the findings of Manandhar and Rajawar, (2006) (*Ehrlichia spp.* 11.43% and *Babesia Spp.* 5.71%) but vary from the (Maharjan, 2014) (*Babesia spp.* 9.09% and *Ehrlichia spp.* 1.08%). Under sex wise prevalence of blood parasites in the study higher prevalence in female species than in male species. This finding was similar with findings reported by (Shitta, 2009; Gadahiet *et al.*, 2008) who observed high prevalence of infection on female dogs than male. But findings of our study were contrast to that of Subedi (2009) who found that male dogs have a significantly higher prevalence than female dogs. This could be attributed to the greater propensity for male dogs as compared to females (Papa, 2016).

In this study, the prevalence of blood parasites was higher in German shepherd followed by Mongrel. This may be due to inadequate immunogenic response and the owner's preference to German shepherd (Subedi, 2009; Manandar *et al.*, 2008).

The prevalence of blood parasites was higher in age group 1-5 years of canine population. This may be due to greater attention paid by the pet owners to the dogs before 1 years to control vectors than to the dogs greater than 1 years (Jalali *et al.*, 2013; Subedi, 2009)

The above results of the blood tests showed that the temperature in positive cases ranges from 103 - 105°F. This result due to biological response to a blood parasitic disease (Richardson *et al.*, 1964).

The hematological parameter under study revealed statistically significant low PCV in infected cases than in non-infected cases whereas there was no significant difference in the RBC count and Hb estimation between infected and non-infected group. This finding agrees with the findings of (Maharjan *et al.*, 2014; Shitta *et al.*, 2012) who also have observed a lower mean PCV in the infected dogs than the non-infected dogs. This may be due to immune mediated damage to the bone marrow stem cells caused by blood parasites. And is attributed due to immunomediated phenomena by autoantibodies directed against component of membrane of infected and uninfected erythrocytes, production of toxic hemolytic factors of the parasite, mechanical damage by trophozoite intra-erythrocytic binary fission, erythrophagocytosis and through release of vasoactive molecules such as kallikrein. (Schetters *et al.*, 2009 & Brockus and Andreasen, 2003)

It has been reported statistically no significant difference in the TLC between infected and non-infected dogs. This study resembles with the findings in which leucopenia is not specific in the positive cases. In differential leucocyte count (DLC) there was statistically significant difference in Neutrophil and Eosinophil counts between infected and non-infected cases, whereas lymphocyte and monocyte count did not vary significantly. Eosinophilia in infected cases may be due to parasitic infection. Neutropenia was observed in infected cases, which satisfies the findings of (Weiser *et al.*, 1991) which has stated neutropenia in a hemoprotozoan infection. (Manandhar and Rajawar, 2008).

CONCLUSION

In conclusion, our study identified the prevalence of Babesia spp. and Ehrlichia spp. in clinical cases of dogs with hyperthermia. The study showed higher prevalence of disease in female with respect to male. High occurrence was encountered in German shepherd breed of dog and rest in Mongrel. Eventhough, the prevalence of blood parasite was higher in 2-5 years of dogs. In comparative study of hematological parameter between the infected and non-infected host revealed statistically significant difference ($P < 0.05$) in PCV, Neutrophil and Eosinophil whereas other parameter doesnot have any significant difference. The mean PCV was significantly low in infected dogs than in non-infected dogs. Neutropenia and Eosniophiliawas observed in infected dogs. A holistic approach is required for the prevention and control of haemoparasites in Nepal and this will require the active involvement and cooperation of veterinary and allied professionals and government regulatory agencies at all levels.

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STUDY OF TUBERCULOSIS IN RHESUS MACAQUES OF NEPAL

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ABSTRACT

Tuberculosis is one of the prioritized zoonotic diseases in Nepal producing severe health hazard in humans and animals including both wildlife species and livestock species. It is also one of the most important bacterial diseases of nonhuman primates, especially macaques which is caused by Mycobacterium tuberculosis and M. bovis. A study was conducted from 2010 to 2012 in macaques of Nepal to find out the status of tuberculosis. Altogether 326 macaques were tested for tuberculosis by Intradermal Skin test on the eyelid and M. tuberculosis/M. bovis antibody testkit. Out of 326 macaques tested, 62 were found positive for the tuberculosis. So, the overall prevalence rate of tuberculosis in rhesus macaques was found to be 19.02%. From the study it is concluded that not only humans, but the monkeys are also equally vulnerable to this disease condition and produce more severe health hazards, resulting even in death. These macaques should also be given priority concerning this as they may pose risk to other wild life species and humans. More researches should be directed towards exploring disease condition and status in these animals to protect other wildlife species and human contacting this disease.

Keywords: tuberculosis, zoonotic, macaques

INTRODUCTION

Tuberculosis (TB) is one of the prioritized zoonotic diseases in Nepal. It has been reported that it creates serious health hazard in nonhuman primates as well as to other animals and human as it causes economic and life losses in both (Min *et al.*, 2013). It is one of the most important bacterial diseases of nonhuman primates, especially macaques which is caused by *Mycobacterium tuberculosis* & *M. bovis*. Also, it has been reported that natural infection is not very common in primates i.e. they acquire illness primarily from humans and then spread rapidly within another monkey (Bennett *et al.*, 1998). The main route of infection is via the respiratory tract by inhalation of fine particles containing the bacilli. Transmission may also occur by ingestion, through fight wounds, tattoo machines, needles, rectal thermometers, contact with fomites including contaminated thermo-fomites nets and gloves (Bennett *et al.*, 1998 & Lecu *et al.*, 2013). So, other humans and animals with active disease represent the main reported sources of TB infection in captive primates. Retransmission of the infection to humans has been reported as well (Lecu *et al.*, 2013).

Based on the incidence, the disease is common in Asian monkey species, especially those from India, less frequent in African species and rare in New World monkeys and prosimians (Lecu *et al.*, 2013). More specifically, rhesus monkeys are more susceptible to *M. tuberculosis* than other experimental monkeys and apes (Min *et al.*, 2013). Rhesus macaques develop an acute, progressive,

and often fatal form of tuberculosis (Langermans *et al.*, 2001). In nonhuman primates, it is well documented that the primary infection might not result in active TB and the latent infection might occur without any overt disease (Capuano *et al.*, 2003; Gormus *et al.*, 2004). Although animals with latent TB are not infectious, there is a significant risk of reactivation and the development of active tuberculosis (Lerche *et al.*, 2008). So, macaques are considered to be disease reservoirs (Alfonso *et al.*, 2004).

In macaques, TB classically involves the respiratory system; clinical signs may include coughing labored breathing and exercise intolerance (Bennett *et al.*, 1998). However, the most common sign is cough. In some cases, it may remain subclinical for upto 6 months. The disease progresses slowly, taking up to one year to cause death in a rhesus. As TB progresses, monkeys may become fatigued, anorectic and experience chronic and marked emaciation. In general, intermittent coughing and chronic weight loss should always be regarded as possible indicators for the disease (Lecu *et al.*, 2013). Hepatomegaly and splenomegaly in some cases may be detected by palpation. Hence, this study was conducted as there was an outbreak of T.B in the macaques breeding centre at Nepal Biotechnology Research Centre (NBRC), Lele, Lalitpur. The main objective of this study was to identify the T.B positive macaques and to isolate them from the breeding colony.

MATERIALS AND METHODS

A total of 326 macaques were collected from 2010-2012 A.D from various places of Nepal; Hetauda Bhutandevi temple (Hetauda), Melamchi (Sindhupalchok), Dhadingbeshi (Dhading), Parsa and Nepalgunj. These monkeys were collected from the places surrounding the human dwellings in case of Hetauda, Sindhupalchowk and Dhading. While from Parsa and Nepalgunj, they were collected from the woods. Thus, captivated monkeys were brought to Nepal Biomedical Research Center (NBRC), Lele-8, Lalitpur. For the diagnosis of tuberculosis in these macaques, 2 types of test procedures were used; Intradermal skin test on the eyelid (Delayed hypersensitivity test) and M. tuberculosis/M. bovis rapid antibody test kit

Intradermal skin test

Macaques weighing above 1.5 kg were sedated with ketamine while baby macaques were properly restrained. Then, 0.1ml (irrespective of the body weight) of a solution containing 1250 IU mammalian purified protein derivative (PPD), USA was intradermally injected into upper eyelid of eye (midway between the medial and lateral canthi) as shown in (Fig. 1 and 2). Subsequently, observations were made at 8, 24, 48 and 72 hours post inoculation. Positive and negative cases were identified based on the following changes in eyelid. (Table 1).

Table 1: Classification of tuberculin test

Test reaction	Changes in eyelid	Inference
0	No reaction	Negative
1	Bruising, no swelling	Negative
2	Pronounced erythema of eyelid spread beyond the injection site, no swelling	Negative
3	Slight swelling and drooping of eyelid. No redness noted	Indeterminate
4	Marked drooping of eyelid due to swelling. Redness may be present or absent	Positive
5	Eyelid is swollen and closed. Redness may be present or absent	Positive

***M. tuberculosis/M. bovis* rapid antibody test**

The antigen rapid bovine TB test kit is a chromatographic immunoassay for the qualitative detection of *Mycobacterium bovis* antibody in bovine whole blood, plasma or serum. The Antigen Rapid Bovine TB test kit shows two letters which are test (T) line and control © line on the surface of device. The control line is a reference line which provides the test is performing properly. The control line has to appear at all times after the test has performed. If the antibodies against *Mycobacterium bovis* exist in sample, a purple test line would appear in the result window.

The highly selective recombinant *Mycobacterium bovis* antigens are used as a capture and detector in the assay. These antigens are capable of detecting *Mycobacterium bovis* antibodies in sample with high accuracy. For this, venous blood was collected from saphenous/ femoral vein, of which 30 µl was added onto the center of the test well. After which 3 drops of diluents were added dropwise and reading was taken 20 minutes thereafter. Positive reading taken within after 20 minutes was shown as in (Fig. 3). Whereas, results read after 30 minutes were considered invalid.



Fig. 1: Intradermal skin test on the eyelid



Fig.2: Positive reactor on intradermal test after 24 hour



Fig. 3: Kit detecting positive TB specimen

RESULTS

Out of the 326 macaques tested 62 were found positive both in PPD and antibody rapid test for the tuberculosis. All the 62 PPD positive reactors were subjected for rapid antibody test and all the positive reactors for PPD was also positive for rapid test. So, the overall prevalence of tuberculosis in rhesus macaques was found to be 19.02%. At first the macaques were screened using the tuberculin test and the cases were classified as mentioned in (Table 1) above.

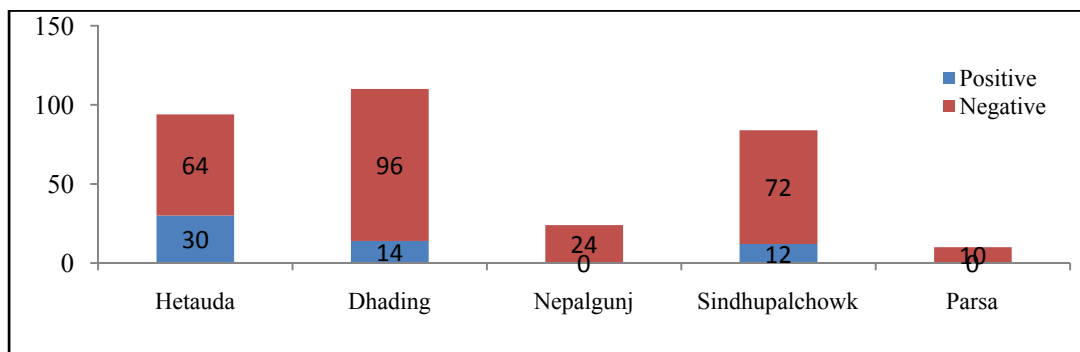


Fig.4: Proportion of positive cases in different locations

The cases positive to tuberculin test were further confirmed by the antibody test kit. The confirmed cases were euthanized, and post-mortem examination was done. In the postmortem examination, several multifocal pinpoint, miliary to nodular lesions were seen in various organs especially lungs, spleen, intestine, liver, trachea-bronchial and mesenteric lymph nodes, kidney as shown in (Fig. 4 to 7) below. The lymph nodes especially trachea-bronchial were enlarged with gray, soft, cheesy, somewhat gritty centers. Caseous tubercles were prominent in the lungs. Consolidation, airway exudation and gross bronchopneumonia were also seen in the lungs.



Fig.5: Viscera of Macaque died from TB



Fig.6: TB infected liver of macaque



Fig.7: Spleen of a TB infected macaque



Fig. 8: Lungs of a TB infected macaque

DISCUSSION

Macaques are the lab animals used for the research based on mycobacterial tuberculosis mainly used for investigating tuberculosis pathogenesis and preclinical testing of drugs and vaccines owing to their anatomical and physiological similarities with human (Pena *et al.*, 2015). However, natural infection is not seen in these nonhuman primates. It has been reported that the outbreak may have been through contact with an infected human, birds, contaminated water (Garcia *et al.*, 2004). Similarly, the monkeys captured from Colombia where the human incidence of TB was high, were found to be infected with TB, suggesting that these animals could have acquired the infection through contact with tuberculous people (Alfonso *et al.*, 2004). Our result also suggests the same finding as the macaques collected from woods from Parsa and Nepalgunj showed no tuberculosis at all. But the monkeys collected from Bhutandevi temple, Hetauda where monkeys roam around the temple, showed high infection rate. It suggests that these monkeys had high contact with humans (might be infected) visiting the temple. While the monkeys collected from Dhading and Sindhupalchowk were not always in close contact with humans as they returned to inhabitant after visiting the human premises for short interval. Also, during our research we noticed that there was rapid transmission of the pathogens among the healthy macaques from the sick ones. So, infection in captive macaques spread like wild fire.

Similarly, intradermal method and kit test were found to be equally efficient for the diagnosis of the disease condition. All the cases found positive by intradermal tests were found infective following kit test and post-mortem examination as well.

CONCLUSION

This study concludes that not only humans, but the monkeys are also equally vulnerable to this disease condition and produce more severe health hazards, resulting even in death. These macaques should also be given priority concerning Tuberculosis as they may pose risk to other wild life species and humans. Also, the control of human TB is impossible without control of TB in macaques- near human dwellings. Hence, more researches and initiatives should be directed towards exploring disease condition and status in these animals to protect other wildlife species and human contacting this disease.

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FIRST RECORD OF ARGULUS JAPONICUS (CRUSTACEA: BRANCHIURA) ON CYPRINUS CARPIO IN NEPAL, WITH ADDITIONAL NOTES ON MORPHOLOGY AND PREVALENCE OF A. JAPONICUS AND ITS TREATMENT

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ABSTRACT

The present study recorded the parasite Argulus japonicus (Crustacea: Branchiura) as the first discovery in Nepal. The description was based on using 11 males (4.12 mm average TL) and 5 females (4.18 mm average TL) of Argulus japonicus which were collected from Godawari fish farm, Lalitpur. Biological behavior of one summer old common carp (Cyprinus carpio) infected with Argulus japonicus were sluggish movement, jumping and poor growth grown in earthen pond in winter at water temperature 15.5 °C and pH 7.4. Heavily infected fishes showed brown-grey to reddish points throughout their body surface, especially on the caudal fins and on the body portion. Prevalence rate of this parasite was 80% in infected common carp fish with an average weight 261.80±85.12 g and length 22.9±4.9 cm. Argulus mean intensity count was averaged 10.2 per infected fish with relatively high infestation in body surface (2.8±3.9) followed by caudal fin (1.8±4.5) and dorsal fin (1.1±1.5). Prevalence rate of this parasite in treated common carp reduced by 95% and Argulids assembled in several regions of the fish was in average 0.1/treated fish by feeding Duroclean treated feed. Duroclean, an aquaculture drug was found effective to control this parasite. The effective dose of the drug was 0.3 g per kg fish feed and fed for 3 days consecutively with one day off and then again for 2 days.

Keywords: *Argulus japonicus*, ectoparasite, prevalence rate, duroclean

INTRODUCTION

The family Argulidae contains a valid Genus *Argulus* Muller, 1785, more commonly known as fish louse cause argulosis disease. Species of the *Argulus* are found distributed throughout the world (Fryer, 1968; Post, 1987; Rushton-Mellor, 1992) and 143 species in the Genus have been described (Anon, 2001), although many of these species are synonymous (Taylor *et al.*, 2005). Further Poly (2008) has enlisted about 129 valid species, occurs around all continents, except Antarctica, in marine and estuarine (n= 44) and freshwater habits (n=85), in total, the diversity of freshwater Branchiura stands at 113 species. Among Crustacean parasite *Argulus japonicus*, *Argulus foliaceus* and *Argulus coregoni* are widespread and the most documented species (Post, 1987; Taylor, *et al.*, 2005).

In due course Argulosis disease is one of the major economic concerns in all phase of the aquaculture industry from production to marketing (Menezes *et al.*, 1990; Taylor *et al.*, 2006). Heavy infestations of *Argulus* parasite can induce significant morbidity and mortality in cultured fish population (Yamaguti 1963; Benz *et al.*, 2001; Northcott *et al.*, 1997; Wilson, 1902), both in

carp (Rahman, 1996) and trout fish (Menezes *et al.*, 1990). In Nepal ectoparasite *Argulus* are currently considered to be one of the most commercially pathogenic parasite and known to infect a wide variety of fish. *Argulus* was reported as threat to production of Rohu (*Labeo rohita*) and Naini (*Cirrhinus mrigala*) and breeding performance of cultured carp fish (Mandal and Prasad, 2011). Pantha (1998) has reported annual losses about 15-20% loss in total fish production and 30-40% loss in fish seed production due to various disease problems every year which accounts an economic loss of about Rs 1513.3 million per year in Nepal. Since the *Argulus japonicus* is the first record for Nepal, therefore current study has initiated to assess the infestation rate, undertaken the morphological study of the adult stage of *Argulus japonicus* including determine the promising drug against this parasite.

MATERIALS AND METHODS

A total of 20 Common carp (*Cyprinus carpio*) of average weight 261 ± 80 g and length 22.9 ± 4.9 were collected from the earthen pond of Godawary fish farm, Lalitpur, at central Nepal for detection of *Argulus* infection in December 2015. Localities of fish farm at altitude N $27^{\circ}35.989'$ and latitude E $085^{\circ}23.246'$ and 1576 masl elevation. *Argulus* were removed from head, body, fins and caudal fin of common carp using brush and forceps and *Argulus* number/fish was noted and fixed in 70% ethanol. Clinical examination of collected fish was adopted using the methods as described by Lucky (1977) for the determination of any lesions or abnormalities on the external body surface. Identification and morphological study of sampled parasite *Argulus* was according Bykhovskaya Pavlovskaya *et al.*, (1962) and Rushton-Mellor (1994). Description of identified *Argulus japonicus* was based on studies of their 16 adult specimens (males $n=11$, females $n=5$) (Table 1). The measurement of total length (TL), total width (TW), carapace length (CL), carapace length as percent of total length, abdomen length (AL), abdomen length as percent of total length and abdomen width (AW) of *Argulus japonicus* was taken in millimeters under light olympus microscope. Photos of male and female *Argulus japonicus*, were also taken under olympus microscope (4x) using digital camera. Sex determination was accomplished as mentioned by Wilson (1902). Prevalence, mean intensity and mean abundance were calculated as following the formula suggested by Margolis *et al.*, (1982):

$$\begin{aligned} \text{Prevalence (\%)} &= \frac{\text{Number of individual infected fish}}{\text{Total number of fish examined}} \times 100 \\ \text{Mean intensity} &= \frac{\text{Number of collected parasite}}{\text{Number of infected fish}} \\ \text{Abundance} &= \frac{\text{Number of parasite}}{\text{Number of fish examined}} \end{aligned}$$

Efficacy of Duroclean (herbal extracts of satavari, copper salicylic acid, vitamin E and K) an aquaculture drug was tested on common carp as feed additive against *Argulus* parasite at research station: Godawary fish farm, Lalitpur district, Kathmandu. Further, drug verification trial was carried at farmer pond condition in Karaiya, Khairani Nagarpalika-1, Chitwan central Nepal.

RESULTS

The present study recorded the *Argulus japonicus* as the first representative of the genus isolated from the common carp fish. *Argulus japonicus* is recognized by the abdomen lobes which have nearly rounded terminal end (slightly pointed than *Argulus foliaceus*); covered marginally with small spines and not covered by carapace. Furthermore, the posterior incisures of abdomen reaches middle of the body and pair of respiratory areas occurs on the ventral surface of lateral lobe of the carapace, the anterior respiratory area was smaller and roughly ovular in shape and posterior was much larger kidney bean shaped.

Body length of male *Argulus japonicus* 4.12 ± 0.46 (3.35-4.68) mm, body width 2.39 ± 0.29 (1.98-2.71) mm and abdomen length 1.03 ± 0.10 (0.90-1.15) mm, comprising 25.02 ± 1.17 (23.16-27.61) % of total length; its posterior lobes tapering to relatively rounded points. Carapace length is 3.08 ± 0.43 (2.45-3.80) mm, comprising 74.62 ± 3.40 (69.80-81.28) % of total length (Table 1). Cephalothoracic carapace extended beyond the beginning of abdomen and cover all four pairs of swimming legs (Fig. 1a, b). The males *Argulus japonicus* are more abundant than females (sex ratio=68.8%).

Table 1: Measurements of *Argulus japonicus* isolated from common carp

SN	Measurements (mm)	Male n=11	Female n=5
1	Total length (TL)	4.12 (3.35-4.68 $\pm$ 0.46)	4.18 (3.58-4.75 $\pm$ 0.46)
2	Total width (TW)	2.39 (1.98-2.71 $\pm$ 0.29)	2.35 (2.11-2.50 $\pm$ 0.16)
3	Abdomen length (AL)	1.03 (0.90-1.15 $\pm$ 0.10)	0.93 (0.78-1.05 $\pm$ 0.11)
4	AL as % of TL	25.02 (23.16-27.61 $\pm$ 1.17)	22.35 (21.28-23.33 $\pm$ 0.86)
5	Abdomen width (AW)	0.60 (0.41-0.80 $\pm$ 0.11)	0.56 (0.45-0.66 $\pm$ 0.09)
6	Carapace length (CL)	3.08 (2.45-3.80 $\pm$ 0.43)	3.26 (2.75-3.80 $\pm$ 0.40)
7	CL as % of TL	74.62 (69.80-81.28 $\pm$ 3.40)	78.56 (76.67-80.85 $\pm$ 1.86)



Fig. 1: Male of *Argulus japonicus* Thiele 1900 viewed under a light microscope (4x)
(1a dorsal view, 1b ventral view; 1c abdomen with a pair of testis (3.75 mm TL, 0.90 mm AL))

Body of female *Argulus japonicus* is slightly elongate, with length 4.18 ± 0.46 (3.58-4.75) mm and body width 2.35 ± 0.16 (2.11-2.50) mm. Abdomen length 0.93 ± 0.11 (0.78-1.05) mm, comprising 22.35 ± 0.86 (21.28-23.33) % of total length; its posterior lobes tapering to relatively rounded points. Carapace length is 3.26 ± 0.40 (2.75-3.80) mm, comprising 78.56 ± 1.86 (76.67-80.85) % of total length (Table 1). Cephalothoracic carapace extended beyond the beginning of abdomen and cover all four pairs of swimming legs (Fig. 2a, b).

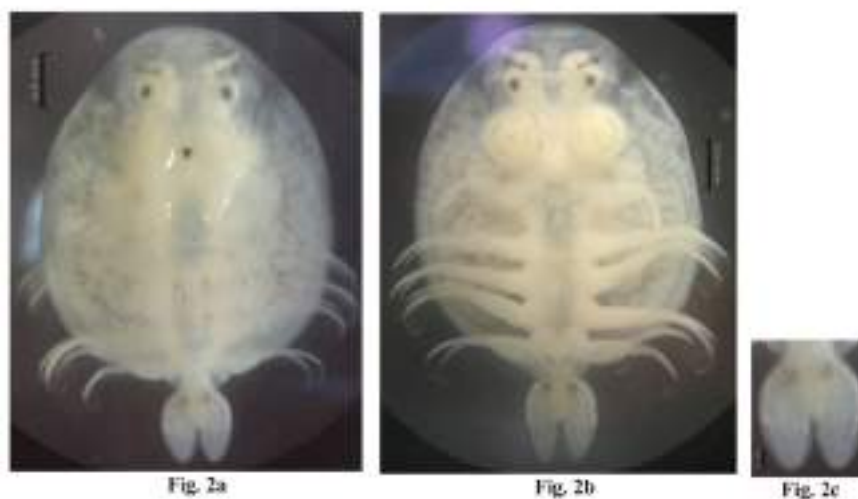


Fig. 2: Female of *Argulus japonicus* Thiele 1900 viewed under a light microscope (4x) (2a dorsal view; 2b ventral view; 2c abdomen with a pair of spermathecae (4.05 mm TL, 0.91 mm AL))

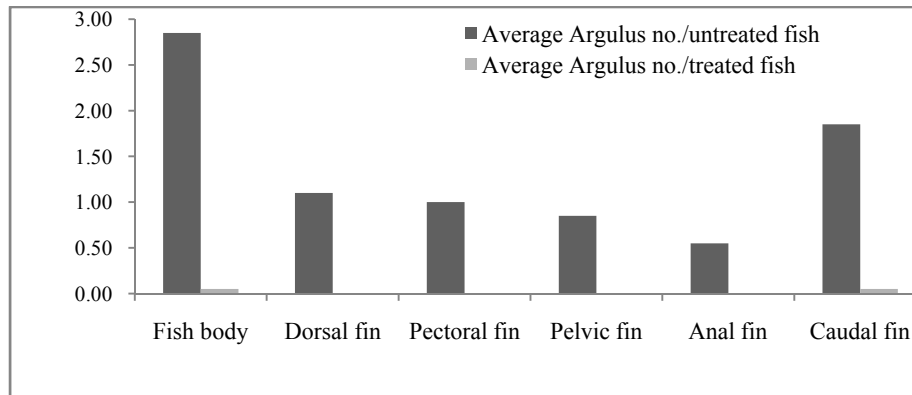


Fig. 3: Average *Argulus* no. assembled in body and fins of common carp before and after treatment

Specific difference between male and female of sampled *Argulus japonicus* included that the abdomen of the male contained the elongated a pair of testes, which were milky white with sperm in live specimens and dark in preserved specimens (Fig. 1a, b, c), while the abdomen of the female contained a pair of round spermathecae which was dark in color (Fig. 2a, b, c).

A total of 164 specimens of *Argulus japonicus* were isolated from one summer old common carp (Table 2). Prevalence rate of this parasite was 80% in infected fish. Argulids were assembled in several regions of the fish. *Argulus* mean intensity count was averaged 10.2 per infected fish with relatively high infestation on body surface (2.85 ± 3.9) followed by caudal fin (1.85 ± 4.5), dorsal fin (1.1 ± 1.6), pectoral fin (1.0 ± 1.92), pelvic fin (0.85 ± 1.87) and anal fin (0.55 ± 1.23) (Table 2, 3; Fig.

3). Fish infected with *Argulus japonicus* showed sluggish movements, jumping and poor growth in winter at water temperature 15.5 °C and pH 7.4.

Table 2: Individual number of *Argulus* parasite attached in different body parts of common carp fish, n=20 in December 2015

SN	Total weight of Common carp (g)	Total length of common carp (cm)	<i>Argulus</i> counts on body surface	<i>Argulus</i> counts on Dorsal fin	<i>Argulus</i> counts on Pectoral fin	<i>Argulus</i> counts on Pelvic fin	<i>Argulus</i> counts on Anal fin	<i>Argulus</i> counts on Caudal fin	Total <i>Argulus</i> no/infected fish
1	470	29.1	4	0	8	7	3	2	24
2	190	23.8	2	1	3	5	3	2	16
3	280	25.5	0	0	0	0	0	0	0
4	302	25.9	5	5	0	0	0	20	30
5	268	26.1	18	2	3	1	1	2	27
6	360	29.5	5	3	1	0	4	6	19
7	266	26.2	1	0	0	0	0	0	1
8	380	11.8	1	0	0	0	0	0	1
9	172	21.2	0	0	0	0	0	0	0
10	168	26.3	1	0	0	0	0	0	1
11	150	19.5	0	0	0	0	0	0	0
12	262	23.6	1	2	0	0	0	0	3
13	325	10.4	2	0	1	2	0	0	5
14	260	25	4	0	0	0	0	2	6
15	142	19.1	0	0	0	0	0	0	0
16	295	25.1	2	0	1	0	0	0	3
17	248	22.1	3	1	2	0	0	0	6
18	250	23.1	3	4	1	0	0	1	9
19	148	20.5	2	3	0	1	0	1	7
20	300	25.2	3	1	0	1	0	1	6
Total	20		57	22	20	17	11	37	164
Av	261.80	22.95	2.85	1.1	1.0	0.85	0.55	1.85	8.2
SD	85.12	4.93	3.91	1.55	1.92	1.87	1.23	4.51	9.63

Table 3: Infestations value of *Argulus* parasite on the common carp fish

Season	No. of fish examined	No. of fish infected	No. of collected parasites	Prevalence %	Mean Intensity	Abundance
Winter	20	16	164	80	10.25	8.2

Besides, heavily infected fishes had some clinical symptoms as brown-grey to reddish points throughout their body surface, especially on the caudal fins and on the body portion. As a control measure, Duroclean was found effective against this parasite. Prevalence rate of this parasite in common carp reduced by 95% and Argulids were assembled in several regions of the fish that was 0.1/treated fish by feeding Duroclean treated feed. Although assembled argulids was 8.2/infected

fish (Table 2, 3; Fig. 3). The effective dose of the drug was 0.3g per kg fish feed and fed for 3 days consecutively with one day off and then again for 2 days.

DISCUSSION

Argulus japonicus was originally described by Thiele (1900) locality from China and further discovered in Europe, North America, Australia and Africa (Taylor *et al.*, 2005). Poly (2008), mentioned that *Argulus japonicus* Thiele, 1900 has been introduced from east/southeast Asia to all other continents, except Antarctica as well as currently discovered from central Nepal. *Argulus japonicus* has been studied by several authors such as Ikuta and Makioka (1997a) (described the structure of adult ovary and oogenesis); Gresty, *et al.*, (1993) (described the structure and function of cephalic appendages); Tokioka (1936) and Lutsch and Avenant-Oldewage (1995) (described the larval stages); Tam (2005) (elucidate the pathogenecity of *Argulus japonicus*); Avenant-Oldewage and Swanepoel (1993) (described the male reproductive system and mechanism of sperm transfer) and Ikuta *et al.* (1997b) (described the eggshell in *Argulus japonicus*). Rushton-Mellor (1994) described the shape of respiratory areas and abdomen as species specific of Genus *Argulus*. Additionally, Rushton-Mellor (1994) has mentioned the carapace lobes covers the all four pairs of swimming legs particularly in *Argulus japonicus*.

During current study, the morphometric comparison of collected *Argulus japonicus* was made with its closely related congeners; *Argulus coregoni* and *Argulus foliaceus*. The specimens of *Argulus japonicus* are distinguished with *Argulus coregoni* (Thorell 1866) by nearly rounded abdomen lobes (vs. sharply pointed abdomen lobes), by posterior lobes of cephalothoracic carapace extended beyond the beginning of abdomen (vs. posterior lobes of cephalothoracic carapace not extended beyond the beginning of abdomen), by the posterior incisures of abdomen reaching the mid-line (vs. posterior incisures of abdomen reach beyond middle of the abdomen) and by the body length 3.35-4.75 mm (vs. 12 mm body length) (Yildiz and Kumantas, 2002; Saha and Bandyopadhyay, 2015). *Argulus japonicus* distinct with *Argulus foliaceus* (Linne, 1758), by nearly rounded abdomen lobes (vs. rounded lobes of abdomen), by the posterior incisures of abdomen reaching the mid-line (vs. the posterior incisures not reaching the mid-line), by posterior lobes of cephalothoracic carapace extended beyond the beginning of abdomen (vs. posterior lobes of cephalothoracic carapace not extended beyond the beginning of abdomen) (Yildiz and Kumantas, 2002; Saha and Bandyopadhyay, 2015).

Argulus japonicus is an opportunist parasite (Shafir and Oldewage, 1992; Avenant-Oldewage, 2001). Infestation with this parasite can reach severe proportions in a very short time which result in catastrophic fish deaths (Menezes *et al.*, 1990; Northcott *et al.*, 1997; Avenant-Oldewage 2001; Taylor *et al.*, 2006). Bower-Shore, 1940; Shimura *et al.*, 1983; Ahne, 1985; Moravec, 1994 and Bandilla *et al.*, 2006 has mentioned that *Argulus* causes increase the susceptibility of secondary infections to its host. Against *Argulus* parasite, several chemicals such as salt (NaCl) (Singhal *et al.*, 1986), formaldehyde (Rydlo, 1989), potassium permanganate (Singhal *et al.*, 1986; Jafri and Ahmed, 1994), quicklime (Jafri and Ahmed, 1994; Ahmed, 2004), organophosphorus (Chandra *et al.*, 2004) and Gammexane (Singhal *et al.*, 1986) have been found applied in aquaculture industry. In the current study efficacy of Duroclean was found promising to minimize *Argulus* number/infected fish (*Argulus* reduced from 8.2 to 0.1/ infected fish). Studies are warned to identify

the interaction between environmental conditions and outbreak of *Argulus japonicus* for developing better management practices of carp aquaculture.

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PLANT BIO RESOURCES USED IN ETHNO- VETERINARY PRACTICES IN JUMLA DISTRICT

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ABSTRACT

Jumla is one of the districts in high mountain region of Nepal from where the medicinal and aromatic plants, their parts and products are exported annually. A study was carried out from March to June 2014 to document the plant resources used in ethno veterinary practice by local people. Focal group discussions and key informant interviews were carried out to enlist plant bio resources used as ethno veterinary medicines. It was revealed that people used 42 plant resources to correct 28 different ailments in animals. Among the listed plant resources, about 87% were the home products of the district. Hence, the study led to the conclusion that there is opportunity to explore the locally found medicinal plants as ethno veterinary medical practice and commercial veterinary herbal medicine.

Keywords: Ethnoveterinary, Plant resources, ailments

INTRODUCTION

The use of plants as medicine is widespread throughout the world. Indigenous knowledge systems are not only for the cultures from which they evolve, but also for scientists, researchers and planners striving to improve conditions in rural societies. This existing valuable information is needed to be documented before it gets lost or disappeared. In many cases this knowledge is transmitted orally from generation to generation and confined to certain people (Subedi 2000). In Nepal, ethno botanical studies of various ethnic communities started from 1950's decade. Banergi began the systematic documentation of medicinal plants in Nepal. Since then, many works have been done on the documentation of medicinal resources throughout the country. Manandhar (2002) mentioned the ethno-medical uses of 1002 species of plants as used by various ethnic communities (Gurung, Newar, Majhi, Raute, Tamang, Tharu *etc.*) of Nepal. These plants are identified and used as traditional medicine (Bhattraai and Karki 2006; Shrestha *et al.*, 2000; Hasan *et al.*, 2013). Besides, out of total flora reported in Nepal, up to 1796 species of medicinal plants occur in Nepal (Anonymous, 2007).

Jumla is a historic place of situated in the center of Karnali region, where the Nepali language 'Khas' was originated. A tremendous knowledge of those historic people and huge natural stock of medicinal plants found in Karnali region is being utilized for both human and veterinary medical purposes. Not only for ethno veterinary practices, medicinal plants are also used to prepare herbal veterinary medicine and feed supplement and most of its quantity is imported from India, whereas their raw materials are exported from Karnali region of Nepal. About 85 % of Nepal's Medicinal and Aromatic Plants (MAP) are collected from the mid-western and far-western regions (GIZ,2011). Only 5% of the land in mountain region is suitable for cultivation and for that reason

livestock farming and collection of MAP are the main sources of house hold income. Transhumance animal rearing system is well established in the region and livestock such as sheep, goat, horse and other animals are the major source of cash. In this system, almost for half of the year farmers keep their animals far from their human settlements in rangeland areas. These areas lack veterinary service but are rich in medicinal herbs. People use these medicinal plants as veterinary medicines for the treatment of their livestock in the rangelands as well as in permanent settlements. Rokaya *et al.*, (2006) recorded a total of 274 species and 5 varieties of medicinal plant belonging to 63 families and 172 genera from upper Dolpo region of Karnali. Similarly, Sapkota (2001) has reported 58 medicinal plants from Humla used in the treatment of animals and human diseases traditionally. Earlier 39 plant species were listed by Joshi (1991), particularly used as the traditional veterinary medical purpose in Himalayas of Nepal. But specific to Jumla, there is no documented information on the local traditional practices on veterinary medicines using the plant resources. Regarding the medicinal plants found in Jumla, Paudel (2013) started the systematic documentation of medicinal plants and has reported 12 species of important wild medicinal plants with some notes on cultivation technology, uses and sustainable management. So, documentation of plant materials used as ethno veterinary medicine, in this study will assist to plan for their conservation and to promote both the livestock and veterinary herbal enterprises in future particular to the Karnali region and in general all over the country.

MATERIALS AND METHODS

To document the plant species used in ethno- veterinary medicine, focal group discussion was organized at Sinja and Chaudhabis areas of Jumla between March to June 2014. During the field visit, traditional healers were interviewed and gathered information one by one. In focal group discussions, a check list was designed considering the local name of plants, parts of plants used against the ailments or signs and symptoms of domestic animal diseases to enlist them cautiously and correctly. To validate the information obtained from the field, a half day workshop was organized in district head quarter where traditional healers, livestock/veterinary technicians, forest technicians, veterinary medical shopkeepers and livestock farmers were included. All the issues after consensus were taken as outputs of the workshop. The plants species and photographs were collected and taken respectively for the cross checking of the knowledge with different healers. Finally, plants were recorded with their ethno botanical notes, used as in particular disease, along with botanical names, with the citation of their plant families and local name. Quantitative data obtained were analyzed using simple statistical tools of frequency and percentage where as a descriptive analysis was carried out based on consensus made during discussions.

RESULTS

Medicinal plant bio diversity and their use in ethno veterinary medicine

Altogether 42 species of medicinal plants were recorded either heard or used by focal group participants and traditional healers (Table 1). The table indicates that all the plants and plant products were available in Jumla but some of them were not locally produced like *Bennincasa hispida* Linn., *Citrus medica* Linn., *Cucumis melo* Linn., *Curcuma longa* Linn., *Litsea cubeba* (Lour.) Pers. and *Piper nigrum*. and brought from outside. About 87% of the plants stated by the participants were produced in Jumla either cultivated or natural.

Traditional Knowledge on conservation of plants and their utilization

Among the medicinal plants and plant products listed, about 65% were harvested in a sustainable way, because the leaf, fruits, bulbs, bark, seed and by product of these plants were used to prepare the medicine. But harvesting roots for the preparation of medicine recipes was also found in remaining species. The methods of utilization found more or less similar in both the sites where the focal group discussions were held. The study shows that livestock farmers and local healers used the mentioned plant resources on different 28 types of common problems and ailments in domestic animals and poultry. Gastro intestinal, respiratory, reproductive, urogenital, muscular, skeletal, physical trauma and parasitic diseases were found to be corrected by utilizing the given plant bio resources. Not only they applied the plants, plant parts and extracts for the correction of diseases but also applied to increase the milk production. It was interesting that more than 50% of the plant species were utilized to correct the problems related to digestive system and parasitic infestation (internal and external). People used more than one species for the treatment of same disorder, for example *Acorus calamus* Linn, *Anemone obtusiloba* D. Don, *Artemisia dubia* Wall. exBesser, *Juniperus recurva* Buch. - Ham. ex D. Don and *Nicotiana tobaccum* Linn. are used to get rid of external parasite. Similarly, few species of plants were used to correct multiple diseases, for example *Acorus calamus* Linn. for respiratory problems (cough) and external parasite as well (Table 1).

Table 1: List of medicinal plants and plant parts used for the treatment of various ailments in animal in Jumla

S.N.	Botanical name	Family	Common name	Parts used	Therapeutic use
1	<i>Acorus calamus</i> Linn.	Araceae	Bojho	rhizome/ leaf	External parasite/Cough (respiratory problem)
2	<i>Allium cepa</i> Linn.	Liliaceae	Pyaj	bulb	Indigestion/anorexia
3	<i>Allium sativum</i> Linn.	Liliaceae	Lasun	bulb	Indigestion/anorexia
4	<i>Anemone obtusiloba</i> D. Don	Ranunculaceae	Maulomuro	root	External parasite
5	<i>Artemisia dubia</i> Wall. ex Besser	Asteraceae	Titepati	leaf	External parasite
6	<i>Bennincasa hispida</i> Linn.	Cucurbitaceae	Kupindo*	fruit	Abortion
7	<i>Bergenia ciliata</i> (Haw.) Sternb.	Saxifragaceae	Simpadi	root	Diarrhea/dysentery
8	<i>Betula utilis</i> D. Don	Betulaceae	Bhojpatra	bark	Sprain/internal pain
9	<i>Bistorta milletti</i> H.Lev.	Polygonaceae	Myakuro	root	Diarrhea/dysentery
10	<i>Brassica sp</i>	Brassicaceae	Choto	root	Bloat/tympani
11	<i>Cannabis sativa</i> Linn.	Canabinaceae	Ganja	leaf	Diarrhea/cold
12	<i>Cedrus deodara</i> (Roxb.ex D.Don) G.Don	Pinaceae	Diyar	oil/resin	External parasite
13	<i>Citrus medica</i> Linn.	Rutaceae	Chuk*	fruit	Poisoning
14	<i>Cucumis melo</i> Linn.	Cucurbitaceae	Aulekaddu*	seed	Abortion
15	<i>Curcuma longa</i> Linn.	Zingiberaceae	Besar*	rhizome	External wound
16	<i>Ocimum sanctum</i>	Lamiaceae	Tulasi	leaves	Cough

17	<i>Fagopyrum esculentum</i> Moench	Polygonaceae	Phapar	seed flour	Cataract/ Retention of placenta
18	<i>Ephedra gerardiana</i>	Ephedraceae	Somlata	stem	Cough
19	<i>Foeniculum vulgare</i>		Sonf	leaves	Indigestion/anorexia
20	<i>Gerbera gossypina</i> (Royle) Beauverd	Asteraceae	Jhullo	root	Internal parasite (Liver fluke, worms)
21	<i>Hippophae salicifolia</i> D. Don	Rhamnaceae	Dalechuk	fruit	Anthelmintic
22	<i>Juniperus recurva</i> Buch.-Ham. ex D. Don	Pinaceae	Dhupi	leaves	External parasite
23	<i>Lavatera kashmiriana</i> Cambess.	Malvaceae	Majaino	leaves	Agalactia
24	<i>Litsea cubeba</i> (Lour.) Pers.	Lauraceae	Siltimur*	seed	Colic
25	<i>Neopicrorhiza scrophulariifolia</i> (Pennell) Hong	Scrophulariaceae	Tito- Kutki	root	Internal parasites/Cough (respiratory problem)
26	<i>Nicotiana tobaccum</i> Linn.	Solanaceae	Kachopat	leaf	External parasite
27	<i>Oryia digyna</i>	Polygonaceae	Ragatmudo	whole plant	Swelling/internal wound
28	<i>Phaseolus radiatus</i> Linn.	Leguminosae	Mass	seed flour	Fracture of limbs/Foot and mouth disease (FMD)
29	<i>Pinus wallichiana</i> A.B.Jackson	Pinaceae	Salla	resin/stem	Sprain/internal pain
30	<i>Piper nigrum</i>	Piperaceae	Marich*	fruit	Cataract
31	<i>Pleurospermum benthamii</i> (DC.) C.B.Clarke	Apiaceae	Ganyaino	root	Colic
32	<i>Pyrus pashia</i> Buch.-Ham. ex D. Don	Rosaceae	Mayal	fruit	Constipation/ Cataract
33	<i>Rheum australe</i> D. Don	Polygonaceae	Padamchal	root/stem	Sprain/fracture
34	<i>Rhus wallichii</i>	Anacardiaceae	Bhakimlo	fruit	Constipation
35	<i>Rubia cordifolia</i>	Rubiaceae	Majitho	stem	Liver diseases
36	<i>Sesamum indicum</i> Linn.	Pedaliaceae	Tel	seed oil	External wound
37	<i>Stephania gracilenta</i> Miers	Menispermaceae	Biralkano	root	Swelling
38	<i>Swertia chirayita</i> (Roxb. ex. fleming) Karstrn	Gentianaceae	Chirayito	root/stem	Anthelmintic
39	<i>Tanacetum dolichophyllum</i> Kitam.	Asteraceae	Baijadi	root	Indigestion
40	<i>Trillidium govanianum</i> (D.Don) Kunth	Liliaceae	Satuwa	root	External wound/Poisoning (plant poisons)
41	<i>Zanthoxylum armatum</i> DC.	Rutaceae	Timur	fruit	Leeches in nostrils
42	<i>Zingiber officinale</i>	Zingiberaceae	Kachur	root	Appetizer

(Source: Field survey, group discussion, 2014); * indicates the resources not produced in the district

DISCUSSION

Ethno botanical and ethno veterinary knowledge and practice of local people

Traditional knowledge and practice on correcting various ailments in domestic animals utilizing plants, their parts and extract are more relevant where modern medicines and veterinary services are not available. Documentation of those plants and traditional knowledge and skills not only transfers the useful information to successors but also enriches the cultural values. Some of the medicinal plants among 58 species recorded from Humla districts by Sapkota (2001) were also among the present study. These facts indicate that Karnali region is an important habitat with meaningful utilization of medicinal plants as traditional skills for ethno veterinary practice. Furthermore, Bharati and Sharma (2008) mentioned 6 plants out of 42 from the present study used as ethno veterinary medicine in Sikkim. Again, Moreki, 2012 mentioned 3 species of medicinal plants within the list of present study, commonly used for the treatment of poultry ailments in Botswana. So, an ethno veterinary medical value of 42 plant species mentioned by Jumli people can be established easily.

Harvesting skill for medicinal plants demonstrated by local people of Jumla in this study strongly supports the sustainable use of medicinal plants. Harvesting leaf, fruit, bulb, seed and bark in most of the documented species, was more sustainable method than harvesting roots or whole plant. Ethno veterinary practice using plant bio resources in Jumla leads to the positive impact of those resources. In another hand, harvesting roots from natural habitats of remaining 35% of the enlisted plants challenges the sustainable way of conservation. The present finding on harvesting method supports the findings of Bhattarai & Croucher (1996) where they mentioned that harvesting of non-timber forest products (NTFP) could have a positive or negative impact on the conservation of biodiversity. Present study indicates the needs of awareness campaign to local people on scientific conservation and harvesting technologies on harvesting the medicinal plants.

Knowledge on ethno veterinary practice not only explores the common ailments found on domestic animals of the district but also gives the choice or alternatives for using plant species on traditional treatment of those ailments. Problems of digestive system including internal parasite and external parasites were found to be more common with respect to the treatment applied by local people. General observation on health service delivery pattern of local agro vet shops also indicates that there is high demand of anti-parasitic medicines (Neupane, 2014. personal communication). District Livestock Service Office, Jumla also allocated about 66% of its total medicine budget for anti-parasitic medicines in fiscal year 2071/72 (DLSO, 2014). Ethno veterinary medical knowledge expressed by local people suggests that, one type of disease can be cured by different types of plants and one type of plant cures more than one type of diseases. This practice is also confirmed by Manandhar (2001) and reported that, 16 species were used to treat wounds, 5 for dislocation of bone whereas 9 species were valued for diarrhea and dysentery. It explores the possibilities of utilizing different types of plant resources found in different geographical locations. To fulfill the gap between scientific validation and present knowledge on ethno veterinary practice of local people is need of the hour.

CONCLUSION

It is concluded that there are many plant resources from which farmers prepare medicines to correct various ailments of domestic animals in Jumla as ethno veterinary medicines. Jumla is rich not only in flora and fauna but also in traditional knowledge and practice in ethno veterinary medicine and ethno botanical as well. This traditional knowledge to treat the common diseases of animals is important where the modern veterinary services are not in access. The scope of documented plant species from this study not only limits to ethno medicine, they can be used to prepare the herbal veterinary medicine commercially. Harvesting sustainably and validate the ethno veterinary medical use of the medicinal plants scientifically are the challenging issues coming up. So, it is the need of hour to conserve and promote such useful and valuable traditional knowledge, practice and local resources for livestock development and related value chains.

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IMPACT OF CLIMATIC VARIABILITY ON SHEEP HUSBANDRY PRACTICES AT LAMJUNG DISTRICT

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ABSTRACT

This study was done with 70 households of Uttarkanya and Bhujung VDC of Lamjung district to assess the impacts of climatic variability on sheep production and to document their adaptation practices to mitigate this variability. Household interview with structured-questionnaire were used to collect primary information, and secondary data was collected from District Livestock Service Office, Lamjung, Central Bureau of Statistics and Department of Hydrology and Meteorology. This study found that more than 80% of the farmer's perception about climatic parameters matched to those recorded by department of hydrology and meteorology. Similarly, the trend of sheep rearing in Lamjung district was found in decreasing whereas youths are least attracted to sheep farming, posing serious threat to the sustainability of sheep farming in the area. The study found that the climate change has affected sheep production and livelihood of farmers since many years. Analysis of climate data of Khudi region of Lamjung district showed increase in maximum temperature from 28.32⁰C to 28.63⁰C with an average change of 0.002⁰C per year and increase in minimum temperature from 15.03⁰C to 16.38⁰C with an average change of 0.015⁰C per year in past 20 years, resulting in hotter summer and warmer winter. Similarly, rainfall has shown decreasing trend associated with erratic patterns. Most important risk factor affecting sheep farming was increase in occurrence of different types of diseases due to climate change which was significantly different ($P<0.01$). Farmers perceived decreased availability of grasses and pasture species in most cases in comparison to 10 years ago which was statistically significant ($P<0.01$). Farmers have started adaptation measures that include shifting grazing zones to increase in access to pasture; preserve the forages and grasses for dry season; using regular vaccination and deworming against diseases and parasites; and constructed dipping tank to remove external parasites. These activities have helped sheep farming to adapt to climatic variability.

Key words: adaptation, climate change, Lamjung, sheep, survey

INTRODUCTION

Climate change is a global issue and Nepal is also facing problems related to climatic variability. For example, Nepal's temperature is rising faster than the global average, with a higher rate in the Himalayas (Shrestha *et al.*, 1999). According to the definition of IPCC, "Climate Change is the change in climatic condition over time occurred either due to anthropogenic or nature induced causes, which remains for decades or even longer period showing distinct variation in its mean" (IPCC, 2007). Nepal's share in climate change is negligible. The population of Nepal is less than 0.4% of the world population and is responsible for only about 0.025% of annual greenhouse gasemissions (MOE, 2010). However, Nepal is highly vulnerable to climate change impacts.

Nepal hosts different types of feedstuff in different agro-ecological zones, which are used to feed large number of animals and birds. Animal industry is suffering from many problems which are responsible to hinder the productivity and production level in Nepalese farming system. Among these, feed related problems are also equally responsible (Upreti & Shrestha, 2006). Shortage of green forage in dry winter period is one of the pronounced problems in livestock sector (Upreti & Shrestha, 2006), whereas the nutrition condition in the available feedstuff has not been given much attention which is equally important in livestock sector.

Livestock sector is an integral part of livelihood of Nepalese farmers. Most of the indigenous people living in higher altitude are highly dependent on migratory livestock system. Sheep, which has high importance in terms of wool and meat production in Nepal, are mainly being raised by indigenous people in mid and higher region in migratory system. In recent years Nepal has witnessed a deterioration of sheep production due to dwindling resources, an increase in disease prevalence and parasites, and changing climate is likely to accelerating the problems (Poudel *et al.*, 2012). Vulnerability of the livestock sectors is high with climate variability (Thornton *et al.*, 2007). Sheep population had declined by more than 6% between 2001/02 to 2013/14 (MoAD, 2014). This study focuses on determining how the sheep health and nutrition in the forage are being affected by changing climate and what interventions are needed to increase the resiliency of the sheep to adapt to the changing climate and strengthen sheep production systems.

MATERIALS AND METHODS

The study was carried out at Uttarkanya and Bhujung Village Development Committees (VDCs) of Lamjung district of Nepal from April 2015 to October 2015. Both the Uttarkanya and Bhujung VDCs are nearer to each other so there is little variation in climatic pattern.

Thirty-five households from each VDC were selected by simple random sampling. Out of 634 households, only 70 households for the questionnaire survey were randomly selected for the study. 12.45% households of Uttarkanya and 9.91% households of Bhujung VDC were taken for the survey. Careful attention was paid to make the sample more inclusive (i.e. inclusion of farmers from different wealth categories, different ethnic groups and involvement in sheep farming etc). Inclusion criteria for the respondents for the questionnaire survey were that the respondent should be of age above 30 years and who have lived in the region for at least 10 years. The reason behind setting these inclusion criteria was because they could provide valuable and useful information regarding the effect of climate change in their region.

The primary data was collected through questionnaire survey, key informant interview, systematic field observation, structured and semi-structured interview with sheep keepers. To develop further idea of the study site, informal discussion and interview with key informant was done. Local leader, model farmers, teachers, social worker and other knowledgeable persons were taken as key informants. The interview was mainly focused on change in climatic parameters, impact on sheep production and possible adaptation measures. The data on weather parameters such as precipitation and temperature were collected from Khudi station of Lamjung district given by DHM, Nepal. Data on precipitation and temperature from 1994-2014 A. D. was analyzed and presented (Fig. 1, 2, 3, 4, 5).

Data entry was done in Microsoft Excel 2010. For statistical analysis R ver. 3.2.5 (R Core Team, 2016) was used. Cross tabulation, Chi-square test and Fisher's exact test was performed in R using package "gmodels" (Warnes *et al.*, 2015). Analysis of variance was calculated and Post hoc analysis for ANOVA was done by Tukey-Kramer method using PhStat2 ver 3.0.4. For meteorological data, 2d-line graph was created by Microsoft Excel 2010, and linear regression trend line was constructed, and equation was plotted on same graph.

RESULTS

Perception on climate change by local people

About Ninety percent of the respondents perceived that change in microclimate since last 10 years. The findings revealed that more than 87% of respondents experienced increased hotness compared to 2.85% respondents that they did not feel so. Increased climate extremes (e.g. warm and cold temperature) were experienced by more than 90% of the respondents. Similarly, decrease in water resources was experienced by more than 85% of the respondents whereas very few respondents also felt about increase in water resources by 5.70% of the respondents and 7.15% of the respondents experienced not difference in water resources in comparison to last 10 years. Similarly, 88.57% of the respondent's experienced increased occurrence of drought compared to less than 5% respondents perceived that they did not experiencing increasing occurrence of drought (Table1). Similarly, greater than 91% of the respondents experienced that there was irregular in the time of monsoon whereas more than 84% of respondents perceived that delayed starting of monsoon and only less than 10% of the respondents felt earlier starting of monsoon and no difference in the time of monsoon comparison to since last 10 (Table2).

Table 1: Change in microclimate as perceived by the respondent (% response) since last 10 years

Responses	Increased (%)	Decreased (%)	No difference (%)
Hotness	87.15	2.85	10.00
Climate extremes	91.43	1.42	7.15
Water resources	5.70	87.15	7.15
Occurrence of drought	88.57	2.86	8.57

Table 2: Change in time of onset of monsoon perceived by the respondent (% response) since last 10 years

Onset of time of monsoon	Response (%)
Early monsoon	7.15
Delayed monsoon	84.30
Irregular monsoon	91.45
No difference	8.55

Trend of climatic variables on Lamjung district

From the climatic data it was seen that the July and August were the wettest months and the November and December were the driest months (Figs. 1, 2, 3, 4). The trend line indicates that the maximum temperature (Tmax) is increasing compared to past, from regression equation we can say

that Tmax is increasing at the rate of 0.002°C per year. Likewise, minimum temperature (Tmin) is increasing at the rate of 0.015°C per year.

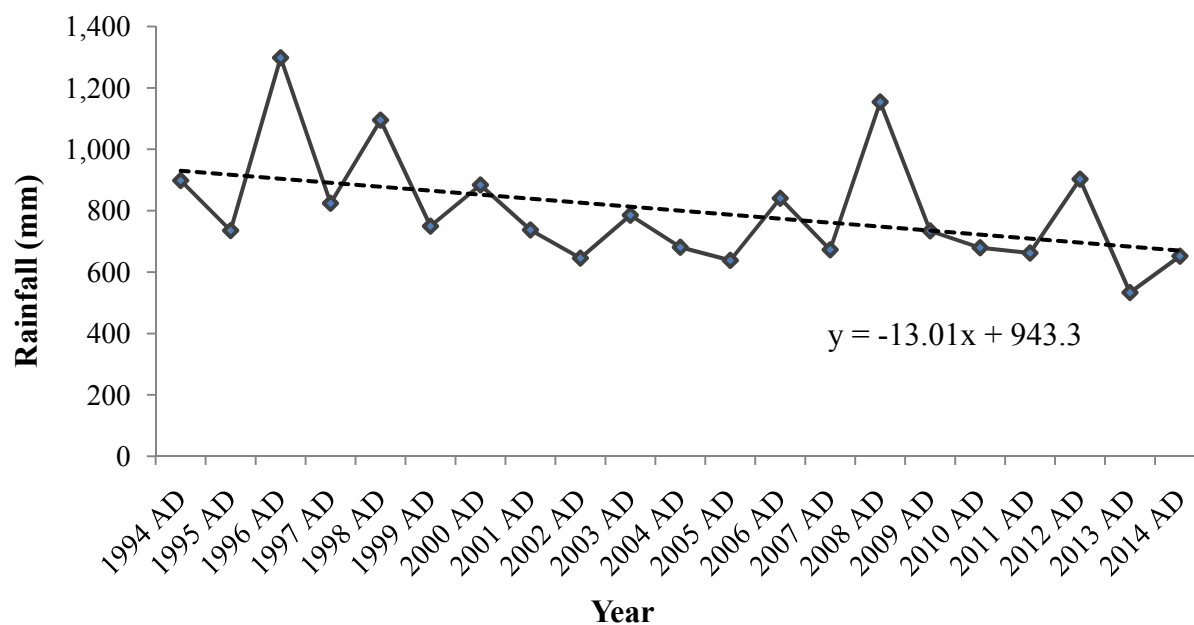


Fig. 1: Rainfall (mm) during July from 1994 to 2014 AD. Dotted line shows the trend line

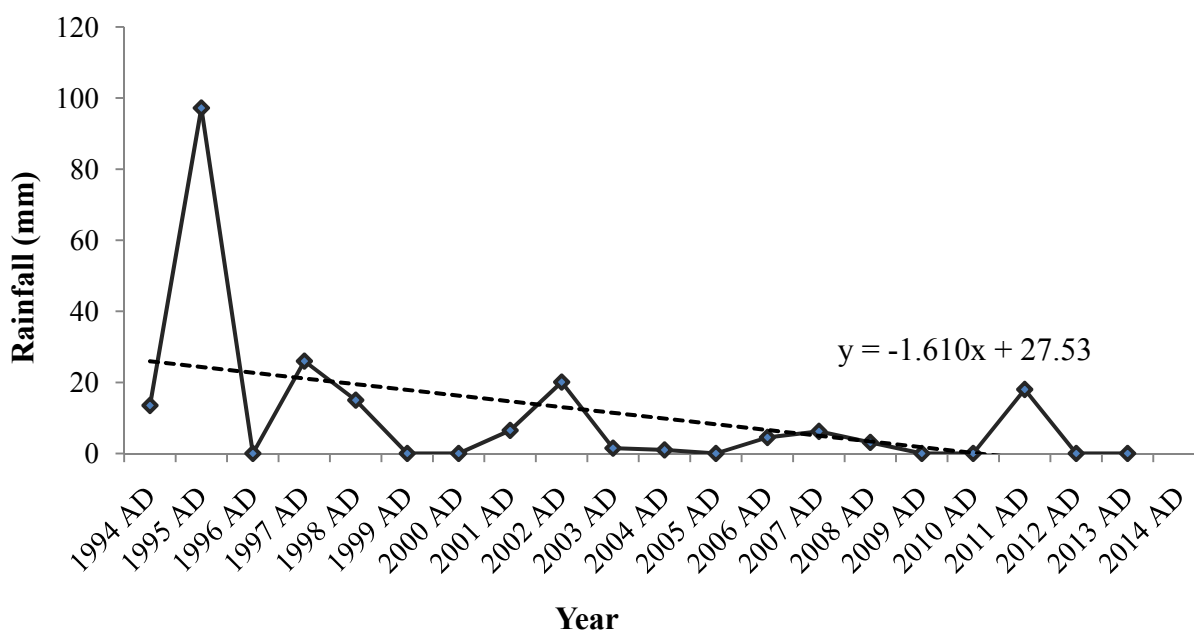


Fig. 2: Rainfall (mm) during August from 1994 to 2014 AD. Dotted line shows the trend line

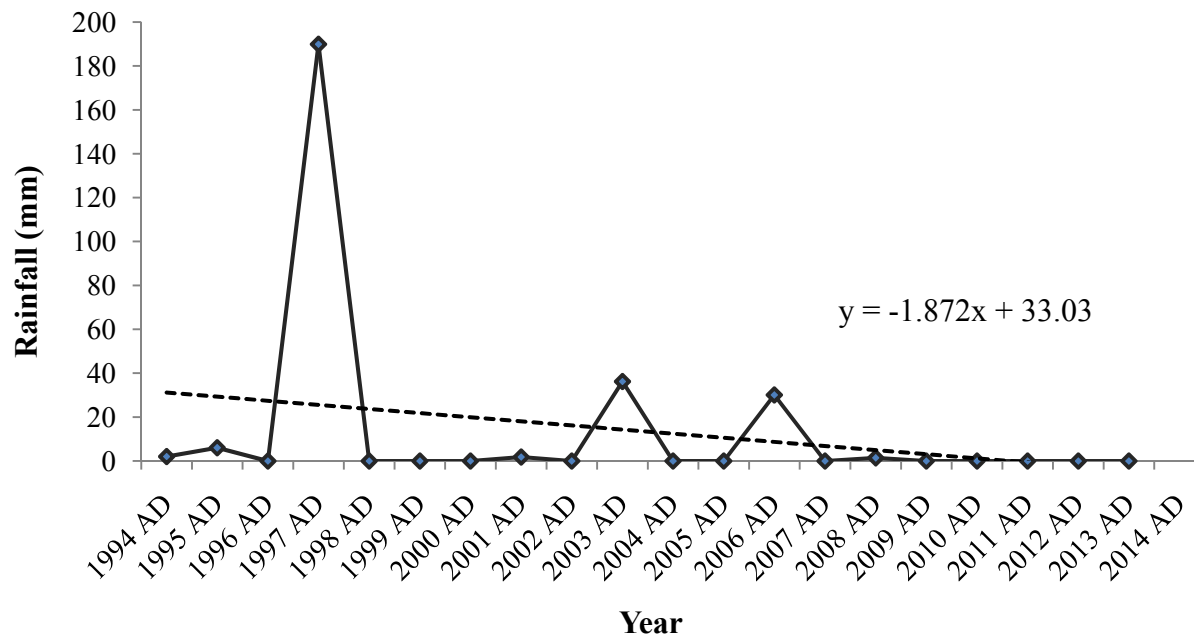


Fig. 3: Rainfall (mm) during November from 1994 to 2014 AD. Dotted line shows the trend line

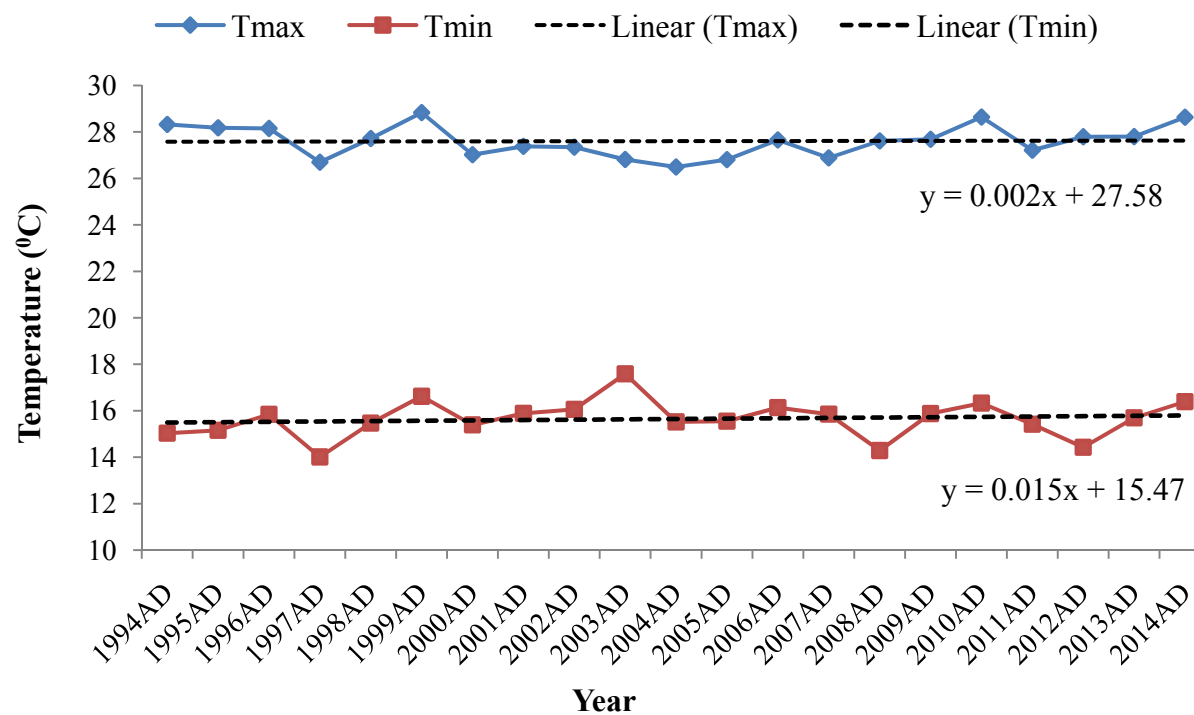


Fig. 4: Temperature pattern of Khudi region

Factors affecting sheep farming

Based on farmers perception climate change and others factors such as labor scarcity, poor livestock service, poor availability of public land, poor availability of pastures due to community forest was statistically similar in both the VDCs of Lamjung district. It was found that more than 40% of the respondents in Bhujung VDC and about 30% of Uttarkanya VDC perceived that sheep farming is affected by climate change. Similarly, about 30% of the respondents in Bhujung VDC and 40% respondents in Uttarkanya VDC felt that sheep farming was affected by labor scarcity. Likewise, about 30% of the respondents in Uttarkanya VDC perceived that poor livestock service could be one of the factor affecting sheep farming whereas only about 10% of the respondents in Bhujung VDC realized that poor livestock service. Poor availability of pastures due to community forest was found as one of the important factors affecting sheep production recorded by the 34.29% and 28.57% in Uttarkanya and Bhujung VDC respectively. However, lower percentage of the respondents in both Bhujung and Uttarkanya VDC 5.71% and 8.57% respectively perceived that poor availability of public land was a factor affecting sheep farming. (Table3). However, Chi-square test indicates that there was no significant difference in the response on the factors affecting sheep farming.

Table 3: Percent responses factors affecting sheep farming at Bhujung and Uttarkanya VDCs

Factors affecting sheep farming	Bhujung	Uttarkanya	Probability	χ^2 -value
Climate change	42.86	29.41	NS	1.349
Labor scarcity	28.57	38.24	NS	0.725
Poor livestock service	11.43	28.57	NS	3.214
Poor availability of public land	5.71	8.57	NS	FET
Poor availability of pastures due to community forest	28.57	34.29	NS	0.265

* and ** = significance levels at $P < 0.05$ and $P < 0.01$ respectively, NS= not significant. FET: Fisher's Exact Test

Major risk factor in sheep farming

Most important risk factor affecting sheep farming was increase in occurrence of different types of diseases due to climate change which was significantly different ($P < 0.01$) as compared to other risk factors. Thereafter, lack of labors, water scarcity due to drought and decrease in pasture land were second important risk factors, there was no significant difference ($P > 0.05$) among these risk factors. Lack of market was third important risk factor. Decrease in agriculture production due to climate change was found as significantly lesser risk factor for sheep farming as compared to other risk factors (Table 4).

Table 4: Mean risk for various risk factors in sheep farming

Risk factors	Mean	SEM	Probability	F-value
1. Occurrence of disease due to CC	4.04 ^a	0.16	**	44.698
2. Decrease in agriculture production due to CC	0.78 ^d	0.15		
3. Decreased in pasture land due to community forest	2.65 ^b	0.19		
4. Lack of market	1.70 ^c	0.16		
5. Water scarcity due to drought	2.79 ^b	0.17		
6. Lack of labors	3.00 ^b	0.19		

^{a,b,c} Indicate significant differences ($P < 0.05$) between the means of the risk factors. SEM= Standard error of mean. * and ** = significance levels at $P < 0.05$ and $P < 0.01$ respectively, NS= not significant.

Adoption of new methods to minimize effects of climate change

Preserve forage and grasses for dry season, make shed to protect the animals from heat, cold, and windstorm, regular vaccination, deworming, consulting with technician, provide nutritious diet in cold season are the measures adapted by the farmers. Percent responses on adoption of new methods to minimize effects of climate change at Bhujung and Uttarkanya VDCs was shown in table 5. Most respondents (77.14% in Bhujung and 82.86% in Uttarkanyana VDC) have adopted new methods to minimize effects of climate change. The statistical test indicates that there was no significant difference in the adoption of new technology in two different VDCs.

Table 5: Percent responses on adoption of new methods to minimize effects of climate change at Bhujung and Uttarkanya VDCs

Adoption of new methods	Bhujung	Uttarkanya	Probability	χ^2 -value
Don't know	14.29	14.29	NS	FET
No	8.57	2.86		
Yes	77.14	82.86		

* and ** = significance levels at $P < 0.05$ and $P < 0.01$ respectively, NS= not significant. FET: Fisher's Exact Test.

DISCUSSION

Perception on climate change by local people

Statistical analysis of climatic parameters has shown changing scenario of Lamjung District. Most of the residents especially elder people think that trend of temperature and rainfall has changed from the last 10 years. Increasing trend of temperature rise and precipitation extremes have been observed in Nepal along with the incidences of heavy precipitation at many places (Baidya *et al.*, 2008). Manandhar (2009) reported a gradual increase in temperature in different ecological zones of the western development region of Nepal.

Major risk factor in sheep farming

Most important risk factor affecting sheep farming was increase in occurrence of different types of diseases due to climate change which was significantly different ($P < 0.01$) compared to other risk factors. Due to the availability of the pastureland sheep and goat rearing is the main source of income generation in this region. According to key informants' interview with chief of DLSO, major disease and parasitic infestation of sheep population in this region are diarrhea, PPR, tympani, flea, lice, leeches, and different skin infestation has been increased in recent years.

Both the sites of respondent perceived the prevalence of the internal parasite and external parasite is high as compared to the last 10 years. Most common types of disease include the skin infections caused by the external parasites. It may be due to the presence of suitable environment for the parasite to grow, develop and adapt with the changing climatic pattern.

New parasites and diseases in all altitude especially skin diseases in livestock have been reported to be emerging due to climate change (Thakur, 2011). As suggested by Thoranton *et.al.*, (2007) that vector borne disease could be affected by the expansion of vector population into cooler areas and change in pattern during wetter years, which could also lead to expanding vector population and large-scale outbreak of disease. Helminth infections are greatly influenced by change in temperature.

Adoption of new methods to minimize effects of climate change

Most of the respondents (77.14% in Bhujung and 82.86% in Uttarkanya) have adopted new methods to minimize effects of climate change. And, the response was not significantly different ($P>0.05$) in both places. Both the sites are nearer to each other and their socioeconomic and demographic characters are like each other. Sheep farmers have traditionally adapted to various environmental and climatic changes by building on their in-depth knowledge of the environment in which they live. Transhumance system is generally practiced by sheep herders is one of the successful examples of seasonal adaptive mechanism. Introducing mixed livestock farming system such as stall fed system and pasture grazing, modifying stock routing and distances to adjust with the changed environment, cultivation of drought tolerant varieties of fodder and forage, creation of awareness among the farmers about climate change, improve the capacity of sheep herders by providing training, make shed to protect the animals from heat, cold, windstorm etc., preserve the forage and grass for dry season in some extent, regular vaccination, deworming, consulting with technician, provide nutritious diet in cold season, deep boring for ground water and rain water harvesting against erratic rainfall/drought are the some management strategies have been adopted by the farmers.

CONCLUSION

The vulnerability of sheep farming to climate change is an important concern in the study sites of Lamjung District where many rural households, small and marginal farmers depend on sheep production. Findings revealed that a significant number of respondents in the survey sites perceived an increase in temperatures together with a decline in rainfall affecting sheep productivity and overall status of sheep production. Because of change in temperature and rainfall pattern, there has been increased in infectious diseases and parasitic infestation; along with decrease availability of feeds and pasture is affecting the productivity of sheep. Decreased productivity and food insufficiency have forced the farmers to adopt other occupations. Farmers have been using their indigenous knowledge and experiences in implementing adaptation measures against climatic variability at local level. They have adopted adjustments in livestock management practices and some external technologies to cope with the impacts of climatic variability. The autonomous, short term adjustments in livestock help them to cope to some extent but if changes progress in the same manner and appropriate adaptation strategies are not developed, the sheep farmers will be at greater risk in the future.

ACKNOWLEDGMENT

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MILK MARKETING AND DAIRY VALUE CHAIN DEVELOPMENT IN NEPAL IN RELATION WITH CLIMATE RESILIENCE EFFORT IN THE PRESENT CONTEXT

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ABSTRACT

Nepal is agricultural country with a special focus on dairy sub-sector. Almost 80% of milk in market, which have been produced by small holder farmers with a well- developed dairy value chain. Ministry of Livestock Development (MoLD) has given due importance for milk production providing support on feed and forage programs to the farmers as well as introducing crossbreeding programs to uplift the genetics of local cattle and buffaloes. By 2018, the country shall be self-sufficient in milk production, where the requirement of 91 lit of milk per person per year is expected to be achieved. Foot and Mouth Disease (FMD) emerges as a major health hazard in dairy sector apart from Hemorrhagic Septicemia (HS) and black quarter (BQ). The incidence of clinical and subclinical mastitis is still high in dairy pocket areas. Moreover, the seasonal fluctuation of milk production is hampering the growth of dairy sector. The need of product diversification and powder milk production are of immense important in long run to sustain dairy sub-sector in Nepal. MoLD has given priority for four pillars for livestock development namely animal feed, animal breeds, animal health and livestock marketing. That sector on balanced development program will lead to increase on milk, meat, eggs, nutrition, and food security in the nation.

Keywords: dairy sub-sector, dairy value chain, milk production, pocket areas

BACKGROUND

Agriculture contributes 31.6% on GDP and the share of livestock sector on agricultural GDP is 26.62% (Sharma, 2007). The contribution together with production of food (meat, milk, and eggs), fibre, hides/skins and transportation amounts to about 15% of GDP which is 31% of Agricultural GDP (LMP, 1993) and 51 per cent of this contribution is derived from the hills, 38 per cent from Terai and 9 per cent from the mountains (APP, 1995). Therefore, livestock forms an integral part of agricultural production system in Nepal. Among different livestock, dairy breeds of cattle and buffaloes are popular in Nepal. In case of small ruminants, meat breeds of goat and sheep reared in mountains, hills and Terai region of Nepal (Sharma, 2002). Livestock population, in relation to arable land and animals per person, are large by Asian standards, with approximately 7.2 million cattle, 5.2 million buffalo, 9.7 million goats, and 0.8 million sheep (MOAD, 2011/2012). Animal feed has been one of the major production inputs drawing attention of the producers as it alone shares nearly 50-60 percent of the production cost of milk and meat from ruminants (Sharma, 2007).

Agricultural by-products and forages are the major feed resources in Nepal. Winter forage crops (oat, vetch, berseem and fodder pea) and perennial forage species (Napier, Stylo, Molasses, molato

etc.) on marginal lands are increasing each year. About 0.1 percent of the cultivated land brought under forage cultivation and over 4,000 ha of pasturelands have already been improved (Pande, 1994). Among the cultivated forage crops, oat is the main fodder crop that can fit with the existing mixed crop livestock farming systems in Terai and hill zones (Upreti, 2005). However, rice production is the Nepal's most important crop amounting to about half of the total cereal grains produced in the country (Ghimire *et al.*, 2013). Rice by-products like straw plays a vital role in livestock production system in Nepal as an alternative to the green forages during the lean season. Apart from rice straw, there has been use of husk, rice polish, and de-oiled rice polish in cattle feed. The production of rice has been increased by 21.66% as compare of 2015. At present, the production of rice is 52, 30000 MT on an area of 15, 52469 hectre (MOAD, 2016 report) yielding 5,972,823 MT straw (Source: Rice Varietal Mapping in Nepal: Implication for Development and Adoption Published by Crop Development Directorate, August 2015). The cost of rice straw alone in animal feed is NRs 53.75 billion which is a huge amount that has been spent on animal feed (MoLD, 2016).

Agriculture is mostly rain-fed and dominated by subsistence farming systems. In recent years, increase in both flood and drought conditions have been observed. Undoubtedly, changes in climatic factors have substantial impact at the local level as they change the agro ecosystem, resulting in loss of land, livestock and household assets (Pant 2011). The warmer climate is expected to lead to a higher intensity of extreme weather events increasing the risk of flash floods in parts of Nepal. Available studies offer differing estimates of the impact on crop production in Asia from increasing temperature and water stress due to unobserved characteristics of cross-sectional units, lack of information on agricultural production, and ignorance in price variation and carbon fertilization effects (Mendelsohn, 2000).

The low production of high nutritious grass in dry spring season may lead to less nutrition to the farm animals. The pattern of calving season in buffaloes may have impact on milk production. Availability of water is less in dry warm season and hence effects on dairy value chain.

INTRODUCTION

Livestock is an integral part of agricultural production system in Nepal. Animal feed forms the major part in any production system as it covers the major share in the overall production cost (Sharma, 2007). There is excess green forage available during the monsoon period, but for the remaining six months, over the winter and spring, there is a lack of feed. The commercialized farms compensate these shortages by supplementing livestock with expensive concentrate feeds. Moreover, adoption of livestock insurance by farmers has started gaining pace. In insurance premium collection, livestock has 80% contribution and remaining 20% is obtained from crops and vegetable sector. Government of Nepal (GoN) has provided 75% subsidy on premium for insurance program, which benefited to dairy farmers. However, the poor transport network round the year is still one of the major hurdle for dairy farmers in Nepal. There is immense need of product diversification of dairy such as establishment of milk powder plant of 100,000 lit per day capacity, which will be beneficial to the farmers to lower milk holidays during the flush season. There is also a need to establish milk collection schedule twice a day. It will enhance the quality of milk. The Ministry of Livestock Development (MoLD) has decided to launch four pillars for betterment of livestock practices in Nepal i.e. animal health, livestock breed, animal feed and livestock market

and it is of utmost importance to maintain balance between four pillars for robust growth in livestock sector.

Present status

At present, the milk production per year in Nepal is 1800000 MT (MOLD, 2016) of which 20% goes to market from formal sector and rest 80% is sold in local markets and used for household consumption. Of the total milk produced, 70% is obtained from buffaloes and 30% comes from cows. The milk production from buffalo is very much seasonal, whereas cow milk is available round the year in similar pace. Currently, the availability of milk is 71 lit / head/year. The MoLD has 40 points commitments to increase the milk production to reach 91 litre per head per year within 3 years' time (MOLD, 2016).

Based on four pillars, the present status of dairy sector can be described as follows:

1. Animal health- The herd health is important for producing optimum level of milk from improved cattle and buffaloes. Foot and Mouth Disease (FMD), Haemorrhagic Septicemia (HS), Black Quarter (BQ) are some of the important infectious diseases in dairy cattle and buffaloes. Therefore, there has been national FMD control project focusing on vaccination of animals in dairy pockets of the country. HS and BQ vaccination has been done by district livestock services office under devolved program. Similarly, liverfluke is the major problem in low lands, hills and Terai of Nepal. Periodic de-worming campaign and awareness program has been implemented in the country. There is a need for enhancing bio-security of the farms along with improved laboratory facilities.

2. Livestock breeds: Murrah buffalo is the major exotic breed in Nepal. There are 38% of cross-bred buffaloes in Nepal. Similarly, Lime, Parkote and Gaddi breeds of buffaloes are also doing well in western part of Nepal giving good yield of milk. Jersey is the major exotic breed in Nepal followed by Holstein-Friesian. There are 25% of cross-bred cattle in Nepal. DLS focus on cross breeding with Jersey in hill and mountain regions. There is cross breeding with Holstein-Friesian in Terai and hill region. The local cattle have potential of 300 litres of milk per lactation and exotic cross bred cows give 2400 litre per lactation whereas improved buffaloes give 1500 litres of milk per lactation (MoLD, 2016).

3. Animal feed: There is excess green forage available during the monsoon period, but for the remaining six months, over the winter and spring, there is a lack of feed. In commercialized farming situations, farmers compensate shortages of forages with supplementation of expensive concentrate feeds. As concentrates are expensive, animals are not fed to their requirement which increases costs without significant increase in production. This has serious implications for competitiveness of the local products against imported products and for the sustainability of livestock production systems. There has been forage mission program focusing on improving forage production in farm level which promotes implication of the strategies to produce forage round the year and emphasizes on hay and silage making. Currently, the program covers 50 districts of the country. There is production of 1400 MT to 1800 MT of feed per day in Nepal of which 80% has been used in poultry feed and rest 20% has been utilized for cattle feeding. Rice bran has been used 30% in cattle feed and 10% in poultry feed generally (personal communication with animal feed association of Nepal).

4. Livestock market: Livestock market promotion directorate (LMPD) has mandate for dairy marketing program under DLS. LMPD distributes 500 litres, 1000 litres and 2000 litres chilling vat on 70% subsidy based on the need of the milk cooperatives. There are 1855 milk marketing cooperatives in Nepal. In the policy of dairy sector, there is National Dairy Development Board (NDDB). It relays on policy guidelines of MoLD and Dairy policy-2064 BS.

Future Potentials

Based on four pillars, the future course of action on dairy sector can be described as follows:

1. Animal health: The herd health is important for producing optimum level of milk from improved cattle and buffaloes. Nepal is focusing on progressive control and eradication of FMD from Nepal and regional cooperation is required to eliminate FMD from South Asia. National FMD control projects are focusing on vaccination of animals in dairy pocket areas. FMD vaccination campaign shall be continued which shall cover major dairy pockets of the country so that the number of outbreaks will be reduced. HS and BQ vaccination has been done by district livestock services office under devolved program. The program will be continued in major livestock pocket areas to reduce number of outbreaks. Similarly, liverfluke is the major problem in low lands, hills and Terai of Nepal. Periodic de-worming campaign and awareness program has been implemented in the country and shall be continued.

2. Livestock breeds: Murrah buffalo is the major exotic breed in Nepal. There is a need of increasing up to 70% of cross bred buffaloes in Nepal. Similarly, Lime, Parkote and Gaddi breeds of buffaloes should be preserved and the best of those animals shall be identified, and semen collection shall be done for artificial insemination (AI) in native areas of such buffaloes in western part of Nepal. Local buffaloes are also giving good yield of milk with higher fat %. Jersey is the major exotic breed in Nepal followed by Holstein-Friesian. There should be 50% of cross bred cattle in Nepal. DLS will focus on more milk production from cows so that self sufficient mark of 260,0000 MT of milk can be produced in Nepal. Dairy cattle and buffaloes resource centre establishment program has been created in potential districts of Nepal.

3. Animal feed: There has been forage mission program focusing on Forage Resource Centre (FRC) one in each district. Propagation of forage seeds, saplings, slips are very much important. There is need for fodder tree cultivation program in mid hills, hill and mountain regions. The raw materials of feed shall be harvested by Ministry of Agriculture Development (MoAD) in close collaboration with MoLD. Therefore, Nepal can save millions of scarce foreign currencies. The consumption of milk is increasing, and it creates large demand of forage and feed which shall be provided from agricultural land and agro-forestry policy will also focus on animal forage and pasture issues.

4. Livestock market: There are 1855 milk marketing cooperatives in Nepal. 45% of dairy marketing has been done by Dairy Development Corporation (DDC). Private dairy has 55% share in the market. The quality milk production and consumers' choice is very much limited due to unavailability of milk in required amount round the year in city and metropolitans. Therefore, the quality of milk has been compromised from farm level itself although MoLD has initiated quality milk production and better supply chain from farm to table. Moreover, NDDB has limited role and

responsibilities. There is more transparent role of NDDB dairy market research. The LMPD shall focus on milk transportation vehicle support, focus on processing of milk and clean milk production for marketing.

CONCLUSIONS

1. Agriculture makes up 31.6 percent of the GDP and livestock accounts for 26.9 percent of agricultural GDP Nearly 70 percent of the population engaged in agriculture keeps livestock. The Agriculture Development Strategy(ADS), 2015(2015-2035), which will guide Nepal's agriculture program for the next 20 years, assigns livestock a key role, both as an engine of agricultural and economic growth and as a source for reducing poverty and improving food and nutrition security. The ADS aim at achieving "self-reliant, sustainable, competitive and inclusive agriculture sector that drives economic growth, and contributes to improved livelihoods and food and nutrition security". To achieve the vision, the ADS will accelerate agriculture sector growth through good governance, productivity, profitable commercialization and competitiveness. The ADS recognize that the productivity and competitiveness of the agricultural sector in the long run will depend on the access and adoption to improved technology, smart advisory services, and engagement of private sector and institutional capacity building to achieve total factor productivity increase. The robust livestock and dairy marketing along with value chain are in high importance.

2. Demand for livestock and livestock products, and particularly milk and meat, has outstripped supply in Nepal, causing increasing reliance on imports. This mounting dependence on imports diverts scarce foreign exchange. At the national level, urban demand for milk and meat are met by average annual imports of nearly 10,500 metric tons of dairy products (\$14 million value), 476,000 live goats and 150,000 buffalo annually (DLS, 2010); representing NR6 billion a year on meat imports and NR8.61 billion a year on milk imports.

3. Limited institutional capacity, extension and outreach services which are limited in their capacity to reach smallholder producers, and underdeveloped value chains have undermined the livestock sector in its potential to capitalize on local demand and to both substitute livestock-based imports and generate export revenue. Weak extension and outreach services are a serious impediment to livestock sector growth in Nepal. Extension services tend to end at the District or village Development Committee level, leaving a huge gap between farmers and extension centers. The Livestock Service Centers have remained largely ineffective due to the lack of basic diagnostic facilities, budget, and trained human resources. While the use of ITs have been gaining momentum in the region, Nepal has yet to capitalize on it despite a very well established mobile phone service network and internet facilities in the country. Modernized services and competitive production systems have been key to vitalize the livestock sector by the ADS (2015).

4. The (2015-2035) Agriculture Development Strategy of the Government of Nepal has identified institutional capacity building as one of the key instruments to lead livestock development. The newly established Ministry of Livestock Development announced by the government on 24 December 2015 will be responsible for delivering an evidence-based sector strategy, policies, and acts. Fulfilling this mandate will require significant capacity on the part of the Ministry, as well as the development of an appropriate legal and regulatory framework such as the food quality and safer commerce and trade, and public enterprise reform. The Nepalese products

have been facing non-tariff barriers in the form of sanitary and phyto-sanitary and technical standards in the export market such as Yak cheese products into the bordering town in India and China.

5. Livestock remains a predominantly smallholder enterprise in Nepal, with most of the production geared toward subsistence or sub-commercial purposes targeted at local markets. The ongoing analytical work has identified meat and dairy production as potential drivers of economic growth in Nepal. While several private companies operate in the dairy sector the lack of road access to urban areas has discouraged private investment in areas where the bulk of milk and meat is produced, preventing most producers from linking into value chains. Lack of access to affordable financing, credit, and information services have made it difficult for the private sector to establish and operate milking centers, processing plants, and other commercial operations. The lack of live livestock markets/auction sites and hygienic slaughter houses are additional impediments to livestock and livestock product development. Transforming dairy and meat production into a modern, economically viable and environmentally sustainable sector will require a value chain approach that aggregates and mainstreams smallholder producers into the supply chain. In addition, there is a need for restocking to replace livestock losses during the earthquake. This provides an opportunity to introduce improved-breed animals. Restocking will be done after carefully assessing the carrying capacity to ensure a good balance between the number of animals and environmental considerations.

6. Nepal is vulnerable to climate related risk. Nepal has an extremely diverse and complex climate with niche weathers in various regions. The GCM projections shows a multi-model mean of 1.4 °C increase in temperature in Nepal by the 2030, with the range of 0.5-2.0 °C. The models also predict a -14 to 40% change in precipitation in monsoon and increase in heavy precipitation event by the 2030s. Some of these predictions have already observed in terms of increased rainfall variability, increased winter drought, increased number of dry days in pre- and post-monsoon seasons. Climate change has and will continue to affect the largely rain-fed and traditional agriculture sector, leading to large annual variations in crop and livestock production. In addition, climate change is likely to increase the frequency of the weather-related hazards, which will also have its toll on agriculture production. Efforts to increase climate-resilience in rural communities and the agricultural sector more broadly must now be scaled up along with earnest operationalization of climate adaptation strategies at the local levels. The livelihood of more than 80 percent of local populations in the hilly regions is heavily dependent on climate sensitive areas such as agriculture and livestock. Lack of access to climate risk insurance, climatic information and agricultural techniques have denied the farmers opportunities to use climate smart innovations and thus exposing them to greater risks. At a global scale livestock is a significant contributor to climate change, and for Nepal, given its exposure to climate related events, modernization of the livestock sector must necessarily consider opportunities to attain production efficiencies which will positively affect the sector's GHG emissions by tapping into the wide range of climate-smart practices and technologies.

7. Developing the livestock sector offers opportunities to address gender disparities and tackle nutrition challenges. FAO estimates that nearly 70 percent of the population engaged in agriculture keep livestock and women provide more than 70 percent of the labor. With a rapidly

growing market for meat, dairy and dairy products, the livestock sector provides significant scope and opportunity to engage/retain men, women and youth. In the high mountains, potential for development of cashmere fiber production exist which has an established brand name in the global market. Furthermore, the malnutrition rate in Nepal is alarmingly high with stunting of children under five at 37.4 percent, underweight at 30.1 percent, and wasting at 11.3 percent. Consumption of animal source food during the first 1000 days of a child's life is critical to the physical and mental wellbeing of the child. Thus, the investment in the livestock sector will not only help increase income, employment and import substitution, as well as opportunities for returnee migrant workers, it will also help address nutrition deficiency.

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ALTERNATIVES TO FIGHT AGAINST COCCIDIOSIS: A REVIEW

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ABSTRACT

Coccidiosis is one of the most important parasitic diseases of poultry with economic losses. The loss is mainly due to the poor feed conversion and increased mortality. Nowadays, farmers rely heavily on prophylactic and therapeutic use of chemical compounds for the management of coccidiosis. The conventional anti-coccidials, however, are associated with residual effects and increased resistance. Similarly, increasing consumer awareness and their pressure for natural food has increased governmental pressure basically. Because of regulatory change, there is increasing pressure to phase out the chemical anticoccidials in diet of farm animals. Moreover, there are renewed management practices as alternatives that are safe, little/no residual effects, and economical. Non-conventional compounds such as Mannan-oligosaccharides (MOS), organic acids, phyto-chemicals, probiotics and essential oils have shown encouraging result against coccidian growth in intestinal mucosa of chicken and turkey. But, comprehensive studies on chemical structure, modes of action and residual effects is needed to unravel their applicability and effectiveness in the field conditions.

Keywords: anticoccidials, resistance, coccidiosis, alternatives

INTRODUCTION

Avian coccidiosis is a common widespread disease associated with considerable economic losses to poultry farmers worldwide. It can affect birds raised in any production systems and for any production purposes. The parasites that cause coccidiosis in poultry consist of a wide range of single-celled protozoans of the genus *Eimeria*. Seven species of *Eimeria* (*E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella*) are recognized as affecting chickens. Each of the seven species occurs in a single host species or a group of closely related hosts and they invade the lining of the intestine or ceca, causing death or reduced productivity in poultry. Infection by coccidian parasites in sufficient numbers produces clinical manifestations of the disease (Conway & McKenzie, 2008). Furthermore, subclinical infections, which significantly impact on productivity and food security, are common due to widespread drug resistance, high parasite prevalence, and environmental persistence (Blake & Tomley, 2014).

Methods for avian coccidiosis prevention and treatment has been studied and developed for several years. It includes good husbandry practices and use of anti-coccidial drugs. However, extensive use of anti-coccidial drugs has resulted in the development of resistance. With recent advances in technology and immunology, vaccines against poultry coccidiosis have been developed still have not met farmers' expectation as an effective, safe and economical way of control. Specifically, they

are expensive and may result in subclinical coccidiosis in the birds. This can be disadvantageous as it reduces performance momentarily (Gussem, 2006).

Although there are several molecular methods in use for diagnosis and development of vaccines, these techniques have not solved many practical questions on what kind of prevention is adequate for a certain poultry production unit (Gussem, 2006). Recently, there are increased number of studies going on in search of alternatives for avian coccidiosis control such as acids, vitamins, probiotics, mushrooms, amino-acids, non-steroidal anti-inflammatory agents, natural feed additives, essential oils or botanicals having anti-coccidial properties etc. Besides factors like production system, disease prevalence, resistance level etc., the use of prophylactic and therapeutic methods varies between countries due to differences in regulations.

With stringent regulations and bans on the use of anti-coccidial drugs together with the associated costs in developing new drugs, the live vaccines reinforce the need for the development of novel approaches and alternative control strategies for coccidiosis. This work seeks to review the current methods used in the control of coccidiosis in diverse regions of the world.

Control measures

A) Conventional control measures (With application of anticoccidials)

Anticoccidials are drugs used for the prevention and control of coccidian infections. They can either be coccidiocidal or coccidiostatic. The former kills the coccidial parasites whereas latter prevents the replication and growth (Quiroz-Castaneda & Dantan-Gonzalez, 2015). In general, coccidiocidals are more effective than coccidiostats because when coccidiostatic medication is withdrawn, halted parasites may resume their life cycle and go on to contaminate the environment with infective oocysts (McDougald *et al.*, 2008). Efforts on the development of anti-coccidial compounds have focused on drugs that attack both the sexual and asexual stages of the parasites (i.e. stages that occur within the host) rather than targeting the most infectious stage i.e. the oocyst (Dantán-González *et al.*, 2015).

Two categories of drugs are employed in the poultry industry. The first are chemicals or synthetic drugs which affect parasite metabolism by inhibiting certain biochemical pathways. Examples are halofuginone, clopidol, decoquinate and amprolium (Dantán-González *et al.*, 2015), (Tewari and Maharana, 2011). The other group is polyether ionophores such as lasalocid, monensin, narasin, maduramycin, and salinomycin, which interferes ion transport and disrupt the osmotic balance of the parasite (Tewari & Maharana, 2011). Ionophores are considered as coccidio-cidal because of their ability to preferentially move ions, usually sodium, which results in highly toxic conditions to the cell. They are basically by-products of bacterial fermentation and now predominant in coccidiosis control (Quiroz-Castaneda and Dantan-Gonzalez, 2015).

It is however, seems quite worrying that there is some degree of resistance to all these anti-coccidial drugs, including ionophores. To overcome this problem, shuttle and rotation systems of drugs are employed. In the shuttle program, different drugs are used within a single growing season, whereas in the rotation program, the type of drug used is switched after one or several grow-out periods or seasonally (Gussem, 2006), (Tewari and Maharana, 2011). However, even with the shuttle and rotation programs there is no method reported to fully prevent drug resistance. This has been

observed when strains of *E. maxima* showed resistance to ionophores, such as Monensin or Lasalocid (Martinet *et al.*, 1997).

(B) Integrated control program

This involves the use of alternative control methods like; use of botanicals, vaccine, pre- and probiotics and immune-modulatory compounds in order to reduce the use of chemical compound (Abbas, Colwell, & Gilleard, 2012).

(a) Immuno-prophylaxis

Protective immunity in chickens can be induced by deliberate infection with low numbers of *Eimeria* parasites and it is established that after two to three consecutive infections the birds develop solid immunity. All commercially available coccidiosis vaccines are based on this principle. Depending on the characteristics of the vaccine strains used, they are divided into two main groups; Live attenuated and Live non-attenuated (Price, 2012).

The Live, non-attenuated vaccines include a variable number of wild type strains (Shulman, 1987). For broiler-breeders up to eight *Eimeria* species are included in these products (Coccivac_ D, Immucox C2), whereas for use in the broiler industry the number is restricted up to four species (Coccivac B, Immucox C1). The mode of application is with drinking water for all the four live vaccines (Tewari and Maharana, 2011). The attenuated lines of *Eimeria* parasites can be developed through repeated selection for early maturation (precociousness) or by serial passage through embryonated eggs (Long, 1971), (Jeffers, 1976). The most important feature of these attenuated lines is their reduced proliferative capacity resulting in less damage to the intestinal epithelium after one passage through the gut. Paracox (precocious strains) and Livacox (precocious: TA strains, (Bednicket *et al.*, 1989) are the example of such attenuated vaccine and their performance is comparable to anti-coccidial drugs (Williamset *et al.*, 1999).

However, uses of therapeutics or feed additives that interfere with development of the oocysts are prohibited during the period of immune system maturation. For this reason, live vaccine that can be used with different ionophores has been introduced (Nobilis_Coxatam) to the market (Vermeulen, Schaap and Schetters, 2001). The vaccine comprises strains of three different *Eimeria* species, namely *E. acervulina*, *E. tenella*, and *E. maxima* which are relatively tolerant to ionophores. The advantage of these specific vaccines is that they allow the use of ionophores during the first 3–4 weeks when immunity is immature and the birds are still susceptible (Tewari and Maharana, 2011). In large organic farms, vaccination is a common means of coccidiosis control as vaccines are currently recognized as the only practical alternative to anti-coccidial drugs (Chapman *et al.*, 2002). Presently, two vaccines are available EU-wide (Paracox® and Paracox® 5) and one other (Livacox® T) is available in a limited number of countries (Czech Republic, Italy, Latvia and Slovak Republic)

(b) Biosecurity and management practices

Generally, in animal production, bio-security is a key program to prevent or minimize the number of organism entering and spreading within the farm. Bio-security measures could be different depending on the production system but basically has three main components: isolation, traffic control and sanitation.

In avian coccidiosis prevention, bio-security program and management practices also play an important role besides other prophylactic methods including ionophores, chemical drugs or vaccines. Many studies indicated that the risk of coccidiosis and the severity of infection is attributable to environment and management factors (Graat *et al.*, 1998; Chapman *et al.*, 2002; Hafez, 2008).

The basic idea of these practices is to minimize the entry and spread of oocysts in production environment. There are two notable events in life cycle of *Eimeria* sps. for which appropriate bio-security and management practices should be focused. (1) The shedding of oocysts in droppings and (2) their sporulation, or becoming infective, under conducive temperature, humidity and oxygen conditions.

The production area such as water, feed, litter, soil or facilities can be contaminated by fecal matters. Oocysts are highly resistant to tough conditions (only freezing or very high temperatures can kill them) or disinfectants, and can survive in soil or litter for several months. To minimize number of oocysts and prevent them from spreading, these practices are usually applied:

Housing design

- Maintaining appropriate bird density. High stock density increases the number of infective oocysts ingested by the birds, thus commercial farms have higher risk for coccidiosis outbreak than backyard/free-range or organic system. A typical commercial broiler production may contain between 20–50,000 chickens at a stocking density of one bird per 0.08 m² (Chapman, 2014). (Drouin & Toux, 2000) and (Elson, 2010) recommended small flock producer to maintain a stock density of one square foot of floor space per chick. Some backyard and organic broiler producers keep birds in floorless pens and move daily.
- Feeders and drinkers should be kept at the level that birds cannot defecate infected litter into them. Also, preventing birds from picking up oocysts from the floor/litter by keeping the feeders always full is another preventive practices (Fanatico, 2006).

Good hygiene

- Feeder and waterer should be kept clean because they can be contaminated with fecal matter.
- Daily removal of litter is important to get rid of infected droppings. The litter may be replaced after every flock or after several successive flocks, in the USA it is common to change litter every six flocks (Chapman, 2014; Drouin and Toux, 2000 and Elson, 2010) indicated that in built-up litter system, microbes in the litter can develop and destroy oocysts. Therefore, removing only half of the litter can keep its anti-coccidial effect.

Bio-security program

- All in-all out principle. This practice is often used and highly recommended to break the cycle of disease by providing time to clean-up and disinfection. Additionally, separating birds in different ages or keeping older birds away from chicks are also helpful practices to prevent coccidiosis.
- Disinfection of materials, people and equipment entering the farm and poultry house.
- For visitors and employers, it is essential to take complete head-to-toe shower, wearing new protective footwear and clothing before entering the production area.

- Entry of other animals into the farm or premises should be limited. Several researches have been done to study the optimal environmental conditions for sporulation of different *Eimeria* strains. Generally, sporulation of the oocyst depends on environmental factors like temperature, humidity and aeration (Duszynski & Conder, 1977; Paterson and Desser, 1982; Graat *et al.*, 1998; Rind and Brohl 2001; Waldenstedt *et al.*, 2001; Schmid *et al.*, 2012). Management practices aimed to prevent the sporulation of oocysts is mainly about litter management including:
- **Dry litter:** In fact, research have shown that higher sporulation rate was observed in dry litter condition (Graat *et al.*, 1998), and the occurrence of ammonia and bacteria in wet litter can suppress oocyst sporulation (Williams, 1995). However, it is not feasible to increase humidity of litter due to the increase in footpad lesions and skin burns among poultry herd (Peek & Landman, 2011).
- **Proper ventilation**
- **Leakage free water system:** Water leak, that increase the humidity in litter, should be avoided. Provision of balanced diet and uncontaminated feed to the poultry. Rations with excessive protein or excessive salt can result in wet litter (Fanatico, 2006).

(C) Alternative control measures

(1) Acid

Acids are known to have antimicrobial activity particularly at low pH. Many acids like formic acid, butyric acid, anacardic acid, acetic acid and hydrochloric acid are found effective in controlling avian coccidiosis (Garcia, 2007), (Zamanet *et al.*, 2012). A work by Garcia (Garcia, 2007) reported that formic acid has positive effects on growth of villi such as increase in height of villi, depth of crypt and surface area of villi in broiler chickens experimentally challenged with *E. tenella*. Organic acids like acetic acid have also been reported to have anti-coccidial effects. (Abbas *et al.*, 2011) reported that acetic acid (ethanoic acid) at 3% concentration produced almost equivalent result as amprolium administered at 125ppm in suppressing the pathogenic effect and negative performance associated with *E. tenella* infection.

Some strong acids like hydrochloric acid, in low concentrations, are being used for the control and treatment of avian coccidiosis (Abbas *et al.*, 2012a). Additionally, the acids when used at low concentrations resulted in better performance by improving the solubility of the feed ingredients, digestion and absorption of nutrients but at higher concentrations resulted in negative impact on weight gains and feed intake (Owingset *et al.*, 1990 and Verstegen and Williams, 2002).

The exact anti-coccidial effect of acids is not fully known to the date but it is believed that after entering the microbial cell, the acids ionize to release hydrogen ions, resulting into decrease of intracellular pH. This reduced pH negatively affect microbial metabolism by inhibiting the action of important microbial enzymes and forces the cell to use energy to export the excess of protons H⁺, ultimately resulting death by starvation. Additionally, the protons H⁺ denature the acid sensitive proteins and DNA of the microbial cell (Abbas *et al.*, 2011).

(2) Probiotic and Prebiotics

Probiotic are live microbial feed supplements given to the birds to boost the intestinal microflora. Prebiotics on the other hand are a non-digestible food ingredient that improves the host health by selectively stimulating the growth and activity of beneficial micro flora in the colon (AFRC, 1989).

Currently, little is known about the anti-coccidial mechanisms of probiotics. However, the following modes of action have been proposed: maintaining healthy balance of bacteria by competitive exclusion and antagonism, promoting gut maturation and integrity, modulating immunity and preventing inflammation, altering metabolism by increasing digestive enzyme activity and decreasing bacterial enzyme activity and ammonia production, improving feed intake and digestion, and neutralizing entero-toxins and stimulating the immune system (Muthamilselvan *et al.*, 2016a and Lee *et al.*, 2007).

Reports have shown that *Pediococcus* and *Saccharomyces*-based probiotic, when included in the broiler diet at concentration of 1%, may improve the resistance against *E. tenella* and *E. acervulina* by enhancing humoral immune response as well as reducing oocyst production in birds (Lee *et al.*, 2007). Lactobacillus-based probiotic has positive influence on cellular immunity. They have been used in diet to stimulate the gut-associated bacteria in neonatal chicks, thereby protecting them from disease without decreasing growth performance (Sato *et al.*, 2009). A probiotic containing *Pediococcus acidilactici* and *Saccharomyces boulardii*, was evaluated as an alternative control method and it was found that chickens fed with the probiotic, at concentrations of 0.1%, and challenged with *E. tenella* or *E. acervulina* had shown an enhanced humoral immunity and significant changes in body weight gain and fecal oocyst shedding rates (Lee *et al.*, 2007). Probiotics, in combination with vaccines, were used to observe the immune response in broilers. (Stringfellow *et al.*, 2011) Stringfellow *et al.* observed an increase in lymphocyte proliferation on day 14 in addition to higher levels of heterophil oxidative bursts at day 7. These results confirm probiotics to be very useful in modulating immune response. In addition to probiotics, many nonspecific immune-modulatory agents have been used against several pathogens in the poultry. Heat-killed *Mycobacterium phlei* exhibited an immunotherapeutic potential in broiler chickens infected with *E. tenella* by improving weight gain and cecal lesions score (Bera *et al.*, 2010).

The most common prebiotics, used in poultry, include inulin, arabinoxylo-oligosaccharides (AOS), fructo-oligosaccharides (FOS), mannan-oligosaccharides (MOS), xylo-oligosaccharides (XOS), isomalto-oligosaccharides (IMOS), soy oligosaccharides (SOS), and pyro-dextrins (Muthamilselvan *et al.*, 2016a). Mannan-oligosaccharides (MOS), derived from the cell wall of the yeast *Saccharomyces cerevisiae*, are to promote gastrointestinal health and performance. MOS is believed to block the binding of pathogens to mannan receptors on the mucosal surface and stimulate immune response (Springer *et al.*, 2000). In poultry, MOS enhance the development of *Bifidobacteria* spp. and *Lactobacillus* spp. in the intestinal tract of chickens and suppress the number of enterobacteriaceae members. According to (Elmusharaf *et al.*, 2007) dietary supplementation of MOS, at a concentration of 10g/kg feed, reduced the oocyst excretion and diminished the severity of lesions caused by *E. acervulina*. This anti-coccidial effect was also observed against light infection induced by subclinical doses of sporulated oocysts. However, further research is required to validate whether MOS has anti-coccidial activity when used at higher concentrations in feed in combination with higher challenge doses.

Arabinoxylans derived from wheat (*Triticum aestivum*) have also been shown to have immune-stimulatory and protective effects against coccidiosis in broiler chickens (Akhtar *et al.*, 2012). Some fungi, *Lentinula edodes* and *Tremella* and plants *Aegle marmelos*, *Eclipta alba*, *Olea europaea*, *Pinus radiata*, and *Echinacea purpurea*, have been shown to contain active ingredients with mode

of action that include immune stimulation, whether alone or in combination with vaccines (Abbas *et al.*, 2012a).

The yeast *Pichiaguilli ermondii* has also been used to induce immune response in chickens. The dead whole yeast cell is commercially available as citristim and is considered as a good source of mannan oligosaccharides and β -glucans known for their immune-modulatory effects (Shanmugasundaram & Selvaraj, 2012). This product contains an exclusive mixture of partially fermented yeast that is left following citric acid extraction from the yeast culture. Broilers fed with citristim showed a reduction in the number of oocysts excreted seven days after coccidial challenge. Thus, quickening the removal of the coccidian (Inovocox, Pfizer Animal Health, NY). A post-infection increased macrophage nitric oxide and inflammatory cytokine production was observed as evidence of immune response.

Basically, prebiotics operate by increasing gut probiotics to suppress pathogens and boosting immune response in chickens to inhibit gut pathogens (Muthamilselvan *et al.*, 2016a). Furthermore, (Çabuket *et al.*, 2006), (Bozkurt *et al.*, 2016) reported that prebiotics diminished coccidial infection in chickens but kept marginal oocyst production that might serve as a source of live vaccine for uninfected chickens.

(3) Natural feedstuffs

It is suggested that the destruction of *Eimeria* proteins and suppression of inflammation by pappain and vitamin A respectively found in papaya helps to suppress coccidiosis (Muthamilselvan *et al.*, 2016a). Also, diets supplemented with 2.5 to 10% fish oil, 10% flaxseed oil, or 10% linseed oil when fed to chickens from the first day significantly decreased cecal lesions (Allen *et al.*, 1996); (Allen *et al.*, 1997). These diets are known to be rich in n-3 fatty acids like docosa-hexaenoic acid, linoleic acid, eicosa-pentaenoic acid. According to (Allen *et al.*, 1996) they significantly hinder the development of both sexual and asexual stages of *E. tenella*, by inducing oxidative stress due to the high concentration of easily oxidized double bonds.

The effect of these fatty acids was only observed in *E. tenella* infected animals but not in *E. maxima* infected animals (Allen *et al.*, 1997); (Allen *et al.*, 1996). However, diets with low levels of linolenic acid do not show protection against *E. tenella* infection (Allen, Danforth, & Augustine, 1998). The results suggest that these diets induce an oxidative stress which is detrimental to sporulated oocysts and sporozoites of *E. tenella*, (Prowse, Michalski, & Fahey, 1992).

Glycine betaine or betaine

In cells, betaine build up provides protection from osmotic stress and allows the cells to perform their regular metabolic activities even during unfavorable situations (Petronini *et al.*, 1993). Reports have shown that it does not only protect the intestine against enteric diseases like coccidiosis but also improve weight gains. However, to get maximum protection, authors suggested to use betaine in combination with anti-coccidial drugs (Augustine & Jenkins, 1998), (Allen *et al.*, 1998). In an experimental tissue culture, betaine and salinomycin could significantly reduce cell invasion by *E. acervulina* by directly affecting the development of the parasite (Augustine & Jenkins, 1998). Conversely, a study by Burt *et al.* (Burt *et al.*, 2013) showed that betaine rather enhanced invasion by *E. tenella* in to a cell monolayer due to its osmotic effect.

Essential Oil (EO)

Most of the Essential Oils inhibit nitric oxide production in macrophages. Nitric oxide in macrophages helps in fighting microbial infection and this is necessary for innate immune response (Abbas *et al.*, 2012), (Abbas *et al.*, 2012). A combination of oregano EOs with some other plants EOs and extracts reduced the activities of both bacteria and *Eimeria* species (Bona *et al.*, 2012). The major components of Oregano EOs (carvacrol and thymol) are thought to impart anti-coccidial activity by maintaining the intestinal integrity (Greathead, 2003; Silva *et al.*, 2009; Da Silva *et al.*, 1984).

Additionally, unlike other conventional anti-coccidials, the use of essential oils as part of diets to control coccidiosis is focused on attacking the oocysts. In vitro destruction of *Eimeria* oocysts was reported after a 3 hours contact period with essential oils from *Artemisia*, hyme, tea tree, and clove (Remmalet *et al.*, 2011). Out of ten essential oils tested, only those four had LC50 less than 1mg/mL for oocysts. Although the mechanism of action of essential oils is still unknown, the reports are an example of the use of natural substances as agents for the destruction of the most resistant structure of the parasite, the oocyst.

Phyto-chemicals (Herbal extracts and medicinal plants)

In recent times, biologically active compound from various types of plant have been explored as sustainable alternatives for controlling coccidiosis and seen to be quite effective. In the poultry industry, the use of antioxidants from botanicals can help in restoring the balance of oxidants/antioxidants, leading to an improvement of birds infected with coccidiosis. Various fruit and herb plants such as plum cranberries, pomegranate, bearberry, grape seed extract, pine bark extract, rosemary, oregano, green tea, and other spices function as antioxidants in meat and poultry products. *Curcumin*, present in *Curcuma longa*, reduced the severity of an infection of the upper and middle part of the small intestine caused by *E. acervulina* and *E. maxima* (Allen *et al.*, 1998). Naidoo *et al.*, (Naidoo *et al.*, 2008) reported that *Tulbaghi aviolacea*, *Vitis vinifera*, and *Artemisia afra*, used in doses of 35 g/kg, 75mg/kg, and 150mg/kg, respectively, exhibit an activity similar to that observed in the control drug, toltrazuril. Selenium and polyphenolic compounds in green tea are thought to be active compounds to inactivate the enzymes responsible for coccidian sporulation (Muthamilselvan *et al.*, 2016b).

Azadirachta indica (Neem plant) and *Artemisia Annua* are examples of antimicrobial plants (Abd El-hady *et al.*, 2013)). Neem fruit in broiler feed had tremendous effect against coccidiosis similar to salinomycin. Also, the aqueous extract of neem showed significant reduction in oocysts load without negatively affecting growth (Nidaullah *et al.*, 2010). In another study, Post-infection period result of Neem (10%) or *A. annua* (5%) incorporation in broiler diet suggested that either of two was able to alleviate the adverse effect of *E. tenella* infection indicating the importance of both herbal plants as a potential cheap and natural anti-coccidial feedstuff (Abd El-hady *et al.*, 2013). The anti-coccidial effect of neem may be ascribed to some bioactive chemicals such as azadirachtin which has a significant efficacy on viruses, fungal pathogens and protozoan, parasites such as coccidian species (Dkhilet *et al.*, 2013), (Etuket *et al.*, 2004).

Artemisinin, an extract isolated from *Artemisia annua*, is effective in reducing oocyst shedding output from *E. acervulina* and *E. tenella* but not *E. maxima* infections when broiler chickens are fed

the extract at concentrations of 1 or 2.5mg/kg. Artemisinin generates reactive oxygen species (ROS) through degradation of iron-implicated peroxide complex and, therefore, induced oxidative stress. Also, ROS inhibit sporulation and cell wall formation in *Eimeria* species. Oxidative stress cause imbalance of oxidant or antioxidant species in the host due to microbial and parasitic infections including coccidiosis (Muthamilselvan *et al.*, 2016b), (Arab *et al.*, 2006).

A study to assess the effect of *Artemisia sieberi* extracts on 21-day old broiler chickens infected with *E. tenella*, *E. maxima*, *E. necatrix*, and *E. acervulina*. showed that chickens treated with *A. sieberi* extract has reduced oocysts count per gram of feces as well as improved growth performance parameters such as feed intake and weight gain when compared with the effects observed with monensin treatment (Pirali Kheirabadi *et al.*, 2014). Similar results were recorded when day old broilers infected with *E. tenella* were treated with granulated extract of *A. sieberi* obtained from petroleum ether (Kaboutari *et al.*, 2014). In addition, *A. annua* contain compounds which help birds maintain commensal microflora and take up large amounts of nitrogen. Commensal bacteria play a significant role in enhancing digestion, absorption and improvement of innate and acquired immune response in poultry (Muthamilselvan *et al.*, 2016b).

The extract from the bark of the pine tree (*Pinusradiata*), which is rich in condensed tannins, is reported to inhibit sporulation of the oocysts of *E. tenella*, *E. maxima*, and *E. acervulina*. It is suggested that the tannins enter the wall of the oocyst and damage the cytoplasm by deactivating enzymes responsible for sporulation. This was further supported by the appearance of abnormal sporocyst (Molan, Liu, & De, 2009),(Muthamilselvan *et al.*, 2016b). Grape seed pro-anthocyanidin extract was shown to reduce *E. tenella* by decreasing nitric oxide and increasing superoxide dismutase in the plasma of chickens, which suggests that pro-anthocyanidin from grape seed reduces coccidiosis via downregulation of oxidative stress (Muthamilselvan *et al.*, 2016a).

A study of two phyto-chemical combinations, (carvacrol, cinnamaldehyde, and *Capsicum* oleoresin) and (*Capsicum* oleoresin and turmeric oleoresin), proved that both combination treatments effectively protected chickens against *E. tenella*infection by showing increases in NK cells, macrophages, CD4+ T cells, CD8+ T cells, and their cytokines (IFN- γ and IL-6) and decrease in TNFSF15 and IL-17F, leading to induction and elevation of host immunity in chickens (Lee *et al.*, 2011). Similar result were observed for *Aloe excels* and *Prunus salicina* (Muthamilselvan *et al.*, 2016b). An assessment by Ola- Fadunsin and Ademola (Ola-Fadunsin & Ademola, 2013) of the anti-coccidial effect of *Moringa oleifer* acetone extract on broiler chicken naturally infected with several *Eimeria* species showed positive results in terms of oocyst output inhibition, fecal score, weight gain, and mortality

Additionally, evaluation of hematological indices showed a significant increase in packed cell volume, hemoglobin concentration, and red blood cell count of the treated birds. Also, Crude extract of *D. febrifuga*, a Chinese herb for protozoan diseases, was observed to be effective against *E. tenella*infection in chickens due to its alkaloid and halogenated derivative, halofuginone (Yang *et al.*, 2015), (Muthamilselvan *et al.*, 2016b). The results of these studies provide evidence that these phyto-nutrients and herbal extracts possess immune enhancing properties in chickens, which offers the possibility of developing effective drug free alternative strategies to control poultry coccidiosis (Lillehoj *et al.*, 2011).

Chickens fed 35mg/kg *C. longa* turmeric supplemented diet showed reduced fecal oocyst for only *E. tenella*, infection but also a decrease in intestinal lesion scores for with *E. maxima* infection. These results showed that the effect of dietary *C. longa* depends on the species of *Eimeria* which seem to indicate differential rates of phyto-nutrient absorption in the various regions of the gut (Kim *et al.*, 2013). Furthermore, chickens fed with *C. longa* showed increase in both cellular and humoral immunity, as measured respectively by enhanced spleen cell proliferation and higher serum anti-EtMIC2 antibody levels in response to microneme proteins involved in parasite invasion of host cell.

Table 1: Summary of some plant reported to have anti-coccidial effect

Name	Mode of action	Studies species	References
Essential oils (Oregano, Thyme etc)	Oocyst lysis	Oocyst	(Remmalet <i>et al.</i> , 2011)
<i>Curcuma longa</i> (Turmeric)	Anti-oxidative, anti-inflammatory, Immuno modulation	<i>E. tenella</i> , <i>E. maxima</i>	(Kim <i>et al.</i> , 2013)
<i>Galla rhois</i>	Antimicrobial	<i>E. tenella</i>	(Lee <i>et al.</i> , 2012)
<i>Echinacea purpurea</i>	Immuno-modulation Oxidative stress	Mixed species	(Burtet <i>et al.</i> , 2013) (Abbas <i>et al.</i> , 2012)
<i>Allium sativum</i> (Garlic)	Antioxidant and anti-inflammatory	Mixed species	(Pouraliet <i>et al.</i> , 2014)
<i>Aloe spp</i>	Cellular immuno modulation	<i>E. maxima</i>	(Abbas <i>et al.</i> , 2012)
<i>Artemisia spp.</i>	Oxidative stress	Mixed species	(Abbas <i>et al.</i> , 2012)
<i>Azadirachta indica</i> (Neem)	Unknown	Mixed species	(Abbas <i>et al.</i> , 2012)
<i>Beta vulgaris</i> (Sugar beet)	Maintain osmotic pressure	Mixed species	(Abbas <i>et al.</i> , 2012) (Fetterer <i>et al.</i> , 2003), (Matthews & Southern, 2000)
<i>Camellia sinensis</i> (Green tee)	antioxidant properties	<i>E. maxima</i>	(Abbas <i>et al.</i> , 2012)
<i>Yucca schidigera</i>	Saponin effect on protozoa	Protozoa	(Abbas <i>et al.</i> , 2012)
<i>Agele marmelos</i> (Bael)	Unknown	Mixed	(Khan <i>et al.</i> , 2008) (Abbas <i>et al.</i> , 2012)
<i>Ageratum conyzoides</i>	Oxidative stress	<i>E. tenella</i>	(Abbas <i>et al.</i> , 2012)

<i>Carica papaya</i> (Pawpaw)	Pappain effect on sporozoite in ceca	<i>E. tenella</i>	(Abbas <i>et al.</i> , 2012)
<i>Cyamopsis tetragonoloba</i> (Guar)	Bind with sterol molecules present on protozoal cell membrane surfaces	<i>E. tenella</i>	(Abbas <i>et al.</i> , 2012)
<i>Linum usitatissimum</i> (flax seed)	Oxidative stress	<i>E. tenella</i>	(Abbas <i>et al.</i> , 2012)
<i>Olea europaea</i> (olive tree)	Antioxidant and anti-inflammatory	<i>E. tenella</i>	(Abbas <i>et al.</i> , 2012)
<i>Pisum sativum</i> (Pea plant)	Inhibit sporozoites reproduction	<i>E. tenella</i>	(Abbas <i>et al.</i> , 2012)
<i>Commiphora swynnertonii</i>	Unknown	Oocyst	(Muthamilselvan <i>et al.</i> , 2016a)

Although the host cell invasion process by the parasite is largely understood, the limitations of defense mechanisms which promote the survival of the parasite within the host need to be explored. The genetic basis of survival of the parasite and the associated key molecules need to be identified to help in finding ways for their control.

CONCLUSION

Despite the benefits of using natural products as anti-coccidial, there are still several issues such as product efficacy, identification of active compounds, mode of action, toxicity, and cost-effectiveness of plant extracts and compounds that need to be addressed for extensive applications. Also, future studies on alternative control strategies should be focused on integration of already proven alternatives into an effective control program so that farmer could control coccidiosis in an effective manner with minimal use of drugs.

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- ☞ मुखको वरिपरि गिजामा घाउ देखा पर्ने ।
- ☞ धेरैजसो पशुहरूमा निमोनिया हुने ।
- ☞ दिशा पातलो हुदै पानी जस्तो छेँने ।
- ☞ रोगी जनावर रोगको लक्षण देखा परेको एक हप्ता भित्र मृत्यु हुने ।

पि.पि.आर. रोगबाट आफ्ना भेडा बाखालाई बचाउन किसानहरूलाई निम्न उपायहरू अवलम्बन गर्न अनुरोध गरिन्छ :

- ☞ आयात गरिएका भेडा बास्वा मार्फत पि.पि.आर. जस्तो महामारी रोग भित्रिन सक्ने संभावना भएको हुदा त्यस्ता पशुहरूको क्वारेन्टाइन जाँच गराएर मात्रै पैठारी गरौं ।
- ☞ रोगको शंका लागेमा त्यस स्थानबाट पशुको आवत जावत तथा विक्री वितरण कार्य तुरुन्त बन्द गरौं ।
- ☞ रोगी पशुलाई वथानबाट अलग्गै छुट्याएर राख्ने गरौं ।
- ☞ आफ्ना भेडावास्वाहरूलाई नियमित रुपमा पि.पि.आर. विरुद्धको खोप लगाऔं ।
- ☞ रोगी भेडा वास्वाहरूको सम्पर्कमा आएका वा प्रयोगमा ल्याइएका सबै सर सामानहरू नष्ट गरौं ।
- ☞ रोग लागेको शंका लागेमा नजिकको पशु सेवा निकाय वा जिल्ला पशु सेवा कार्यालयमा सम्पर्क राखौं ।



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त्रिपुरेश्वर, काठमाडौं
फोन : ०१ ४२६१५६९/०१ ४२६११६५

**प्राणघातक रेबिज रोगबाट बच्न
आफ्ना कुकुर तथा विरालोहरुलाई
नेपालमा उत्पादित भरपर्दो र
गुणस्तरिय एण्टिरेबिज
"NeJaRab" खोप लगाउनु होस् ।**



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रेबिज भ्याक्सिन उत्पादन प्रयोगशाला, त्रिपुरेश्वर**

जुनोटिक रोग सार्ने जिवाणु , विषाणु , किटाणु तथा परजिवीहरू अस्वस्थ जनावरमा मात्र नभएर स्वस्थ देखिने जनावरको शरिरमा समेत पाइन्छ । प्रायजसो बान्ता, छेरोटि, पेट दुख्ने , ज्वरो आउने तथा काँप छुट्ने लक्षणहरू देखिने यस्ता रोगहरूले केहि घन्टा देखि केहि दिन सम्ममा मृत्युको मुखमा पुर्‍याउन सक्छन् ।

तसर्थ

स्वच्छ तथा स्वस्थ दुध, फुल र मासु उपभोगको निम्ति निम्न कुरामा ध्यान दिऔं ।

- पशुपन्छी फार्मको नियमित सरसफाई र जैविक सुरक्षामा ध्यान दिऔं । नियमित लगाउनु पर्ने खोप लगाऔं र रोगी जनावरको तुरुन्त उपचार गरौं।
- दुवानी गर्दा रोगी जनावरको दुवानी नगरौं साथै दुवानी गर्ने सवारीलाई प्रत्येक दुवानी पछि निसङ्क्रमण गरौं ।
- रोगी जनावरको उत्पादन प्रयोग नगरौं /नगराऔं । पशु वध गर्दा छाला तथा मिर्त्र्याङ्सलाई छुट्टै राखौं उपभोग्य मासु संग लसपस नगराऔं । वध गरेको जनावरको रगत पूर्ण रूपमा निथारौं ।
- जनावरको उत्पादनहरू राम्रो संग पकाएर खाने गरौं । काँचो तथा राखरी नपाकेको बीजहरू उपभोग नगरौं ।
- पशुपन्छी जन्म उत्पादनहरूको संसर्गमा दैनिक जसो रहने व्यक्तिहरूको नियमित स्वास्थ्य परिक्षण गरौं ।



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महामारीको रूप लिनसक्ने पशुपंक्षीका
रोगहरु जस्तै: खोरेत, पी. पी. आर., बंगुरको
हैजा, बर्ड फ़्लुसंग मिल्दाजुल्दा लक्षणहरु
कहिकतै देख्नु भएमा तुरुन्त नजिकको पशु
सेवा केन्द्र वा जिल्ला पशु सेवा कार्यालयमा
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