

Molecular Detection of *Anaplasma* in Cattle of Morang, Rupandehi and Surkhet districts of Nepal

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ABSTRACT

Anaplasma, a vector transmitted rickettial parasite, infects blood cells of bovine causing asymptomatic to clinical bovine anaplasmosis. This study was conducted to estimate the prevalence of *Anaplasma* sp. in blood samples of dairy cattle in Morang, Rupandehi, and Surkhet districts of Nepal and to determine the risk factors and biochemical alterations of the respective animal. In total, 120 blood samples (40 from each district) were randomly collected from dairy cattle. The blood smears were prepared and fixed with 10% methanol on the field, and fresh blood was collected in EDTA tubes for molecular analysis and in clot activating tubes to yield serum for biochemical tests. 9 (7.5%) blood samples had produced the 577 base pairs of DNA fragments specific for the 16S rRNA gene of *Anaplasma* sp. by PCR amplification. 5 (4.16%) blood samples in the smear were found positive by microscopic examination. Serum biochemical profile was not different between *Anaplasma* sp. positive cattle and negative cattle ($p > 0.05$). The prevalence rate between Jersey and Holstein Friesian crossbred was also not different ($p > 0.05$). There was a higher prevalence among cattle aged more than 2 years compared to cattle less than 2 years ($p < 0.05$) indicating older cattle were more susceptible to the *Anaplasma* infection. In conclusion, serum biochemical alterations and risk factors should be considered in order to achieve prognosis and initiation of an appropriate therapeutic regimen for a favorable outcome of the tick-borne haemoparasitic diseases along with molecular detection for improved detection.

Keywords: Haemoparasites, Polymerase Chain Reaction, Tick Borne Disease

INTRODUCTION

Nepal has 6 indigenous breeds of cattle belonging to *Bos indicus* except Lulu (*Bos taurus*). Dairy cattle comprise 14.60% of the total cattle population in Nepal (AITC 2020). Tick borne diseases (TBDs) are among the major constraints to the livestock industry in developing countries that causes paralysis or toxicosis and physical damage to livestock

with significant economic losses (Rajput et al., 2005; Durrani et al., 2008, Zulfiqar et al., 2012). The impact is felt by large-scale production systems but is potentially even more devastating for smallholding rural farmers and communities. In addition to clinical episodes of tick borne diseases, productivity loss will also occur in animals that appear healthy but are infected, and play a critical role in the epidemiology. The local environment is also a critical epidemiological factor, because various tick species have been distributed to transmit pathogens to susceptible animals in Nepal (Shrestha et al., 2005; Thakuri et al., 1990).

Bovine anaplasmosis is also known as gall sickness and the disease causing agents are *Anaplasma marginale*, *A. centrale*, *A. phagocytophilum*, *A. bovis* and *A. platy*, among which *A. marginale* is responsible for clinical bovine anaplasmosis (Aubry & Geale, 2011; Theiler, 1910). More than seventeen tick species acts as vectors including *Hyaloma*, *Dermacenter*, *Ixodes*, *Argas*, *Rhipicephalus* (Ashraf et al., 2013; Fyumagwa et al., 2009). In tick, the infection may occur intra-stadial, trans-stadial or rarely via trans-ovarian. Sometime the bites of dipterous flies' act as mechanical transmitters. Trans-placental infection occurs during the acute phase of infection in dam about second and third trimester (Kocan et al., 2010). During diagnosis of *Anaplasma* sp., they appear as dense, homogeneously staining blue purple inclusions (0.3-1) μm in diameter. Inclusion bodies contain 1-8 initial bodies (0.3-0.4) μm known as individual Rickettiale (Merck, 2010).

Most of the research works are based on the sero-prevalence and other microscopic diagnostic techniques for screening of vector borne parasitic diseases (Yadav, 2015; Gautam et al. 2019). However, pathogen species can be misidentified and due to this, and a lack of sensitivity, the level of carrier animals can be underestimated by microscopy. The introduction of PCR based molecular techniques, the sensitivity, specificity and reproducibility for detection of *Anaplasma* sp has been improved. The analytical sensitivity of PCR based method has been estimated at 0.0001% infected erythrocytes. A nested PCR has been used to identify *A. marginale* carrier cattle with capability of identifying as few as 30 infected erythrocytes per ml of blood, well below the lowest levels in carrier. Real-time PCR assays may target one of the several genes (Carrelli et al., 2007; Decaro et al., 2008) or 16S rRNA (Reinbold et al. 2010) and are reported to achieve a level of analytical sensitivity equivalent to nested conventional PCR (Carrelli et al., 2007; Decaro et al., 2008; Reinbold et al., 2010).

As limited information is available regarding the prevalence of *Anaplasma* sp infection in ruminants from Nepal, the present study was designed to report the prevalence of *Anaplasma* sp in blood samples of dairy cattle from Morang, Rupandehi, Surkhet districts. Furthermore, the present study will provide a baseline data regarding risk factors associated with *Anaplasma* sp infection and the potential effect of the parasite on the

selected parameters of serum biochemical profile of dairy cattle in Nepal.

MATERIALS AND METHODS

Sample and data collection

Blood samples were collected from 120 cattle of different dairy farms of Morang (40), Rupandehi (40), Surkhet (40) districts and collected in Serum and EDTA vials maintaining cold chain in cool box. Blood smears were prepared at field and fixed with 10% methanol. After staining with Giemsa-Stain the slides were observed under oil immersion (100X) at NCRP laboratory. A questionnaire was filled at the spot in order to gather data of risk factors associated with bovine anaplasmosis.

DNA Extraction

DNA extraction was carried out by using protocol provided in QIAGEN tissue and blood test kits, in central biotechnology laboratory of Agriculture and Forestry University (AFU), Rampur. The quality and quantity of DNA was detected in Nano-drop-spectrophotometer. About 1 µl of extracted sample was allowed to Nano-drop-spectrophotometer.

The extracted DNA sample having the value 1.8 (260nm /280nm) were subjected to Polymerase Chain Reaction (PCR) in BIORAD T100 Thermal cycler. Mixture for PCR composed of Nuclease Free Water (NFW) = 7 µl, Master Mix (MM) = 10 µl, Forward primer (F) =1 µl, Reverse primer (R) =1 µl and Extracted DNA (60ng/ µl) = 1 µl. Fwd: 5`AGAGTTTGATCCTGGCTCAG 3` Rev: 5`GTTAAGCCCTGGTATTTTCAC 3` primer is used for *Anaplasma* sp. Initial denaturation at 95°C for 5 min Denaturation (40cycles) at 94°C for 30 sec Annealing at 55°C for 30sec, Elongation at 72°C for 90sec Final extension at 72°C for 5 min.

Gel-electrophoresis was performed using 1.5% agarose 1X TBE in gel electrophoresis tank (CLEAVER Scientific Ltd.). Ingredients for Gel-E-Run, for each well, the well mixed solution of Loading Dye = 1 µl (6X) and PCR sample = 5 µl was dropped gently in the well of gel whereas ladder of (100bp) = 6 µl was dropped into single well (generally into first and last well). The setting of current in gel-electrophoresis was 400 Amp, 80Volt for 1.5 hr. And then subjected to L.E.D. ORBIT SHAKER in the solution of EtBr2 (15min) and distilled water (5min) and the gel was observed under the clear view UV-Transilluminator.

Serological analysis for serum bio-chemistry parameters in blood samples from dairy cattle i.e. Urea Nitrogen and Serum Glutamic Pyruvic Transaminase (SGPT) were determined in *Anaplasma* sp positive and negative blood samples of cattle by using BIOBASE EL-

10A Elisa microplate reader and diagnostic kits manufactured by Tulip Diagnostics (P) Ltd. (Spain).

Statistical Analysis

SPSS version-19 was used for statistical analysis. Cattle were categorized into two age groups, less than 2 years and more than 2 years old. Association between the presence of *Anaplasma* sp and two epidemiological parameters, viz. breed and age were analyzed using the Fisher’s exact test (for 2 x 2 tables). Independent T-test was applied to compare the biochemical profile between parasite positive and negative animals.

RESULTS

Prevalence of *Anaplasma* spp

Anaplasma sp was detected in 9 out of 120 collected blood samples (7.5%) from dairy cattle during present study as they amplified a 577 base pair product of 16S rRNA gene specific for *Anaplasma* sp (Figure 1). Whereas only 5 blood samples (4.17% of total) in smear were found positive under 100X oil immersion.

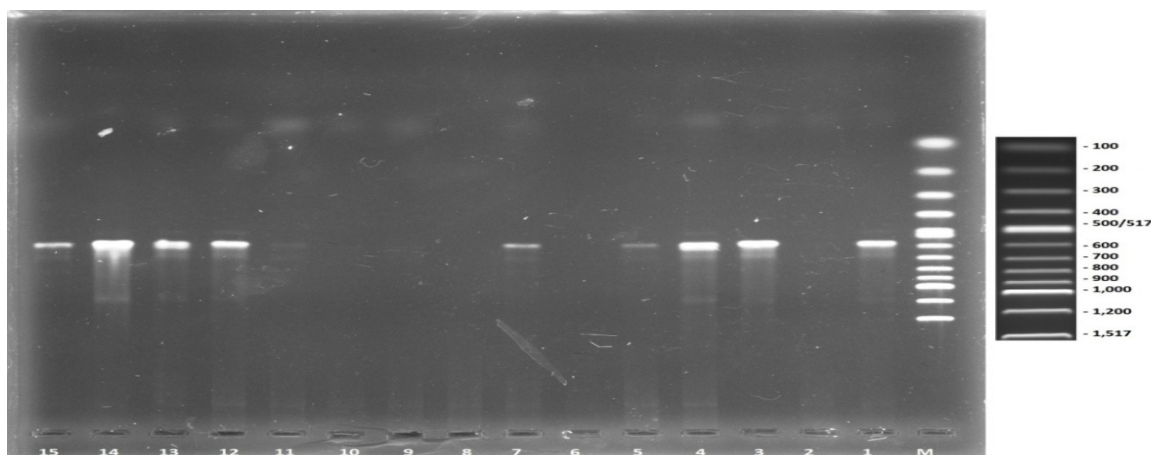


Figure. 1 Identification of *Anaplasma* sp. from blood sample of cattle by Polymerase Chain Reaction (PCR) Lane, M: DNA Marker 100bp. Lane, 1,3,4,5,7,12,13,14,15 Positive samples with base pair 577.

District wise Prevalence of *Anaplasma* spp.

Table 1. Results of *Anaplasma* sp. infection in dairy cattle of Morang, Rupandehi and Surkhet districts of Nepal by PCR.

Districts	Sample (N)	<i>Anaplasma</i> sp +ve	<i>Anaplasma</i> sp -ve
Morang	40	5	35
Rupandehi	40	3	37

Surkhet	40	1	39
Total	120	9	111

Risk factors associated with *Anaplasma spp.*

Prevalence of *Anaplasma sp* was not different by breed (Jersey Cross or Holstein Friesian Cross) ($P > 0.05$). While, cattle aged more than 2 years old were more susceptible compared to cattle less than 2 years of age ($P < 0.05$) (Table 2).

Table 2. Association between prevalence of *Anaplasma spp.*, in blood samples of dairy cattle collected from Morang, Rupandehi and Surkhet districts and animal characteristics studied during present study. P values represent the output of Fisher's exact test.

Subject		Parameters	Sample (N)	<i>Anaplasma sp</i> +ve	<i>Anaplasma sp</i> -ve	P-value
Dairy cattle of Nepal	Breed	Jersey crossed	42	5	37	$P > 0.05$
		Holstein Friesian crossed	29	4	25	
		Total	71	9	62	
	Age	>2 year	49	9	40	$P < 0.05^*$
		<2 year	22	0	22	
		Total	71	9	62	

Analysis of biochemical profile

The comparison of serum biochemical profiles found to be non-significant for all studied parameters between *Anaplasma spp.* positive and negative animals SGPT and Nitrogen Urea level remain unchanged i.e. $P > 0.05$ (Table 3).

Table 3. Comparison of various studied parameters biochemical profile between *Anaplasma sp* positive and negative cattle samples from of dairy cattle collected from Morang, Rupandehi and Surkhet districts. P-value represents the result of independent t-test calculated for each parameter

Dairy cattle of Nepal	Parameter	<i>Anaplasma sp</i> +ve	<i>Anaplasma sp</i> -ve	P-value
	SGPT (U/L)	33.30±10.47	31.48±10.00	$P > 0.05$
Nitrogen Urea(mg/dl)	17.93±1.60	17.27±0.68	$P > 0.05$	

DISCUSSION

Ticks and tick-borne diseases affect the productivity of bovines in tropical and subtropical regions of the world, leading to adverse impact on the cattle farming industry. Report of Food and Agriculture Organization (FAO) estimates, there are 50% production losses

because of their fatality and adverse effects on animal productivity in the developing countries due to different tick borne diseases including anaplasmosis (Nasir 2000; Akhter et al. 2010).

As limited information's are available in literature regarding *Anaplasma* sp incidence from Nepal, present research was designed to report the prevalence of genera *Anaplasma* in blood samples of dairy cattle from Morang, Rupandehi and Surkhet districts. The primers used in the present study can amplify the highly conserved sequence of 16S rRNA gene used to perform PCR for amplification. In the present study, the prevalence of *Anaplasma* sp. was found to be 7.5% and 4.17% by PCR and microscopic techniques respectively. This is the first research report regarding anaplasmosis adopting the molecular technique in cattle of Nepal. Pradeep et al (2019) performed a study on bovine anaplasmosis in which 7% (14/199) were positive for the presence of the *Anaplasma* sp. by successful amplification of the 16S rRNA genus-specific primers, inclusions of *A. marginale* were observed in 3% (6/199) blood smears in south India. These results are similar to our findings. The first report from central Punjab of Pakistan using molecular technique by Iqbal et al. (2019) showed the presence of two *Anaplasma* sp. with an overall prevalence rate of 10.44% which is slightly higher than our finding.

Prevalence rate of the *A. marginale* in total 211 bovines was 18.48% and 6.64% ($P < 0.05$) as per the PCR and Giemsa stained blood smear, respectively by Kumar et al. (2019) in Gujrat India. The results are higher than our findings. It may be due variation in geographical location and sample size. Shrestha SP and UM Singh (1999) conducted a research study to find the prevalence of protozoan diseases in the eastern terai by microscopic examination of blood smears of 83 crossbred dairy cattle in which 5 (6.02%) found positive for *Anaplasma* sp. which is slightly greater; this might be due to blood samples they collected were from febrile animals. Debbarma et al (2020) studied a total of 310 cattle blood smears samples and altogether 108 samples were found to harbor different blood parasites, out of which 5.8% *Anaplasma* sp. were present.

According to Anbu et al. (2020) PCR assay was significantly higher sensitivity in detection of tick borne diseases than microscopic examination in clinically suspected cattle and reported 20.78% infected cattle with *Anaplasma* sp. The infection of *Anaplasma* sp. (27.77%) was higher in less than 2 year of age group. Kaur et al (2020) reported the 23.74% (66/278) infected cattle with *A. marginale*. The highest prevalence of parasitic infection was found in animals having greater than 3 years of age (37.50%) followed by 1 to 3 years (28.57%) and less than 1 year of age (16.98%) in north India.

Debbarma et al (2020) observed that a significant increase in the activity of SGPT (32.30 ± 3.697 and BUN (23.05 ± 1.07) in infected cattle in comparison to healthy cattle. Whereas, the concentration of serum glucose level (57.94 ± 2.111) was significantly decreased

in infected group comparison to healthy group. A study conducted by Nasreldin et al (2020) found the significantly elevated serum alanine amino transferase, aspartate amino transferase and urea in blood from parasite-infected cattle compared with controls. The blood glucose level was significantly decreased in infected cattle compared with controls. In our research and the research report of Hussain et al (2015) it was found that serum biochemical profile unaffected when compared between the *Anaplasma* sp. positive and negative animals. It is because the sample collection was performed from most of the apparently healthy cattle.

Velusamy et al. (2014) found that there was a significantly ($p < 0.05$) high prevalence of haemoprotozoan diseases in Holstein Friesian (HF) and Jersey cross breeds than indigenous breed. We have included only HF and Jersey cross breed and the result was non-significant among two breeds. The occurrence of these haemoprotozoan diseases was found to be high among the age groups of 2 to 7 years in cross-bred animals and below 2 years in indigenous animals which is similar concurrent with our findings.

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DECLARATIONS

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Conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethical Approval

The publish data and results in the research were approved by NARC. The NARC Research Ethics Committee of Nepal has confirmed that no ethical approval is required.

Consent to participate

For this research all the involving animal subjects were given freely and respective animal

owners were informed prior to sampling.

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