Epidemiology, Haematology and Molecular Characterization of Haemoprotozoon and Rickettsial Organisms Causing Infection in Cattle of Jammu Region, North India

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Abstract

Background: The present study was aimed to establish the prevalence, epidemiology and molecular characterization of major haemoprotozoons (*Babesia* and *Theileria*) and rickettsia (*Anaplasma*) of cattle in Jammu region (North India) using microscopy and Polymerase chain reaction (PCR). Hematology, Microscopy and PCR based prevalence studies were undertaken with 278 blood samples from cattle. Molecular prevalence studies were followed by genetic characterization of the isolates of *Babesia*, *Anaplasma* and *Theileria* spp. based on 18S rRNA, 16S rRNA and Tams1 gene, respectively. The data related to metrology and epidemiological variables like temperature, rainfall, season, age and type of livestock rearing was analyzed and correlated with disease by statistical methods.

Results: The study revealed prevalence of *Babesia* spp., *Anaplasma* spp. and *Theileria* spp. to be 14.02%, 23.74% and 1.079% respectively. The metrological and epidemiological variables made inroads for the propagation of vector ticks and occurrence of infection. Haematological alterations predominantly related to haemoglobin, red blood cell count and packed cell volume were evident in diseased animals and collaterally affected the productivity. Further the genetic characterization of *Babesia* spp. (MN566925.1, MN567603, MN566924.1), *Anaplasma* spp. (MH733242.1, MN567602.1) and *Theileria* spp. (MT113479) provided a representative data of the isolates circulating in the region and their proximity with available sequences across the world.

Conclusions: Despite holding much significance to the animal sector, comprehensive disease mapping has yet not been undertaken in several parts of India. The present study provides a blue print of disease mapping, epidemiological correlations and genomic diversity of *Babesia* spp., *Anaplasma* spp. and *Theileria* spp circulating in the region.

Background

India is primarily an agricultural country and the losses incurred as a result of tick borne diseases (TBDs) to livestock in India are huge, estimated to be 8.7 million USD, predominantly affecting the small and marginal farmers. Among the various tick transmitted diseases babesiosis, theileriosis, anaplasmosis and ehrlichiosis are the significant ones that drive attention. Babesiosis is considered as economically important tick-borne haemoprotozoan disease in tropical and subtropical countries, the most prevalent species being *Babesia bovis* and *Babesia bigemina* [1]. *B. bigemina*, transmitted by brevivirostrate tick, *Rhipicephalus* (*Boophilus microplus*) is usually associated with high parasitemias. Anaplasmosis is a rickettsial disease transmitted biologically through ticks mainly by *Rhipicephalus* (*Boophilus*) *microplus* [2]. It is endemic in some parts of the world [3, 4] while in India incidence of *A. marginale* infection has been recorded in livestock [5, 6, 7]. The *Theileria* species that infect bovines in India are *T. annulata* and *T. orientalis*, and both species are transmitted by *Hyalomma anatolicum*. The most common clinical signs observed in TBDs are fever, inappetence and mortality, with peculiar hemoglobinuria and anemia for bovine babesiosis, and enlarged lymph nodes for theileriosis.

The gold standard for diagnosis of these diseases is undoubtedly conventional microscopy but, cryptic cases and carrier states are often misdiagnosed or not diagnosed at all, besides low sensitivity and lack of species level differentiation [8]. Similarly, sero-diagnosis suffers from the problem of cross-reactivity and sometimes low antigen output. Microscopy along with sensitive and specific molecular tools like PCR may help in mapping the diseases and various risk factors for the transmission of major TBDs of livestock *viz.* babesiosis, theileriosis and anaplasmosis. In the light of the fact that 39 million crossbred cattle of India, meant to boost productivity, are at potential risk of contracting TBDs; it becomes imperative to model the prevalence, identify the risk factors and devise control strategies for the control of tick and TBDs of livestock.

The present study was aimed to generate baseline data about the prevalence of tick borne haemoprotozoan and rickettsial infections in cattle in Jammu province of North India, based on conventional and molecular techniques, assess the risk factors and haematological alterations associated with the disease. Moreover, phylogenetic relatedness and genetic diversity of the isolates infecting cattle was also investigated to aid in epidemiological surveillance.

Methods

Study area and sample collection strategy

The study was conducted in Jammu province of Union Territory of Jammu and Kashmir, India, located at 32.73°N 74.87°E. To collect maximum number of samples, the expected prevalence of 20% with confidence limits of 95% and a desired absolute precision of 5% was considered. The number of samples thus calculated was adjusted for finite population [9] and correlated with 278 samples between October, 2017 and September 2019. At the time of blood sample collection, data related to clinical signs, age, sex, breed and type of farm were also recorded. Blood was collected from the jugular vein into EDTA-coated vacutainer tubes, transported to the laboratory on ice and thin blood smears were examined after Giemsa staining. Blood was stored at – 20 °C until further analysis.

Disease prevalence and correlation with environmental variables

According to Indian meteorological department Pune, Ministry of earth sciences, Government of India, seasonal variability in the region for the year can be classified into summer (March to June), monsoon/rainy (July to September), postmonsoon/post rains (October to November) and winter (December to February). Hence study period was divided as per climatic variables and correlations were drawn with environmental variables like temperature, relative humidity and rainfall. Metrological data was obtained from the metrology observatory installed at Division of Agrometeorology, SK University of agricultural sciences and technology of Jammu.

Haematological Parameters

The various blood parameters of clinically suspected blood samples were measured with automatic blood analyser (Mythic™ 18 Vet, Orphee, Switzerland). The various attributes included white blood cell count (WBC) ($\times 10^3/\mu\text{l}$), lymphocytes (%), monocytes (%), granulocytes (%), red blood cell count (RBC) ($10^6/\text{mm}^3$), haemoglobin (Hb) (g/dl), platelet count ($\times 10^3/\mu\text{l}$), mean corpuscular haemoglobin (MCH) (picogram), mean corpuscular haemoglobin concentration (MCHC) (g/dl), mean corpuscular volume (MCV) (femtolitre) and packed cell volume (PCV) (%).

Genomic DNA extraction and Polymerase chain reaction

Genomic DNA was extracted from 200 μl of the whole blood using DNA extraction kit (DNeasy blood kit, Qiagen) following the manufacturer's protocols. Concentration of isolated DNA was measured using spectrophotometer (Eppendorf, India) and purity was checked in agarose gel electrophoresis (1.5% gel). Aliquots of extracted DNA were stored at -20°C until further use.

Each blood sample was subjected to three PCR reactions for detection of *Babesia* spp., *A. marginale* and *T. annulata* targeting 18S rRNA, 16S rRNA and Tams1 gene, respectively [10] with some minor modifications. The PCR reaction was carried out in 25 μl total volume containing 4 μl of template DNA, 2.5 μl of $10\times$ PCR Green buffer (Thermo Scientific, USA), 0.5 μl of 10 mM dNTP, 0.5 μl of each forward and reverse primer (20 pmol/ μl), 0.2 μl Dream Taq DNA polymerase and nuclease free water to make the volume 25 μl . Amplification was performed using a S1000 thermal cycler (Bio-Rad, USA) under following conditions: For *Babesia* spp. initial denaturation at 94°C for 5 min, followed by 35 amplification cycles (94°C for 1 min, 57°C for 1 min and 72°C for 1 min). For *A. marginale*, initial denaturation at 94°C for 5 min, followed by 35 amplification cycles (94°C for 45sec, 58°C for 45sec and 72°C for 45sec). For *T. annulata*, initial denaturation at 95°C for 5 min, followed by 37 amplification cycles (95°C for 30sec, 55°C for 30sec and 72°C for 30sec). A final extension step at 72°C for 15 min was followed after amplification cycles in all three reactions. Known positive genomic DNA of *Babesia* spp, *A. marginale* and *T. annulata* was used as positive control, while nuclease free water was used as negative control. The PCR products were electrophoresed in 1.5% agarose gel (Tris-borate-EDTA), incorporated with ethidium bromide (0.5 $\mu\text{g}/\text{ml}$) and visualized under transilluminator (Eppendorf, India).

Sequencing and phylogenetic analysis

PCR amplified product of each selected isolate was gel purified using PCR clean up system (Promega, USA). The eluted product was commercially sequenced in an automated DNA sequencer at Agrigenome Pvt. Ltd., Kochi, Kerala. Nucleotide sequences ($n = 6$) generated in the study were primarily analysed using BioEdit software and submitted to GenBank. The sequences were compared with the available sequences in GenBank using BLAST program of NCBI. The sequences were aligned with clustal W programme of MEGA 6 software using gap opening penalty of 10 and gap extension penalty of 0.1 and 0.2 for the pair wise and multiple alignments, respectively. Phylogenetic trees were constructed using maximum parsimony (MP) with the tree-bisection-regrafting (TBR) algorithm and tested at 1000 bootstrap replications. The sequences were initially truncated at both ends, so as to obtain sequences that started

Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js *nodium falciparum* (JQ627149), *Rickettsia*

rickettsii (U11021.1) and *Theileria lestoquardi* (KY965146.1) sequences were used as outgroup members to root the respective trees.

Statistical Analysis

Data obtained were compiled and tabulated for frequency and finally converted into percentage to draw inferences. Chi-square test and multivariate logistic regression models were used to draw inferences from the data. Haematological parameters were analysed by one way ANOVA. [11]

Results

Prevalence studies

Giemsa staining revealed infection in 12.94% animals (36/278), whereas 30.21% samples (84/278) were diagnosed as positive through PCR assay. The PCR assay could effectively diagnose cryptic and sub clinical cases and was found significantly sensitive (χ^2 23.475, odds ratio, OR = 2.911, 95% CI = 1.886–4.491) as compared to Giemsa staining. The overall prevalence of *A. marginale* was 23.74% (66/278) followed by *Babesia* spp. (14.02%, 39/278) and *T. annulata* (1.079%, 3/278). Concurrent infection of *Babesia* spp. and *T. annulata* was detected in 12 animals (4.31%).

Attribution of clinical signs to the disease

The main clinical signs exhibited were pyrexia, decreased milk production, pale mucous membrane, tick infestation and anorexia. Haemoglobinuria was observed in babesiosis and lymph node enlargement manifested in theileriosis. Tick infestation was recorded in 76.19% of animals. Decreased milk production was found in 85.71% animals while pica was characteristic of anaplasmosis (96.77%).

Assessment of risk and impact of environmental variables

Evaluation of risk factors for occurrence of disease in the present study was undertaken by modelling effect of age, season, breed, sex and type of farm. A significant effect of season and host age on occurrence of diseases was indicated. The highest prevalence of haemoprotozoan and rickettsial infection was found in animals having > 3 years of age (37.50%) followed by 1–3 years (28.57%) and < 1 year of age (16.98%). Statistical analysis revealed 2.75 times higher probability of disease occurrence in animals > 3 years than < 1 year of age (χ^2 =5.502, 95% CI = 1.230–6.149). The overall prevalence did not vary significantly (p = 0.629) between male and female animals. Unorganised farms without proper flooring, cracked walls and poor sanitation showed significantly (p = 0.007) higher prevalence of infection (36.87%) as compared to organised farms (21.18%). Cross bred animals revealed higher infection (33.62%) compared to indigenous animals (15.38%). The odds ratios (χ^2 =5.836, OR = 2.787, 95% CI = 1.249–6.216) reiterated the fact that cross bred animals are at higher risk than native breeds. The correlation of environmental variables with the prevalence presented a clear picture of epidemiological interventions in the disease occurrence (Fig. 1a and b). The probability of occurrence of infections in monsoon was 16.56 times higher than winter (χ^2 =26.315, 95%CI = 4.752–57.742).

Haematological alterations and pathology of the diseases

Haematological findings of infected animals (n = 84) were compared with healthy control (n = 10) animals. A significant decrease in the Hb (p = 0.013), PCV (p = 0.001) and RBC (p = 0.027) count of animals with babesiosis was recorded (Table 1). The RBC count was also found significantly decreased in animals harbouring mixed infection.

Table 1
Haematological alterations in animals with haemoprotozoan and rickettsial infection

Haematological Parameter	Non infected control (n = 10)	<i>Babesia</i> spp.(n = 27)	<i>Anaplasma</i> spp.(n = 46)	<i>Theileria</i> spp.(n = 2)	Mixed infection (n = 9)
Hb (g/dl)	10.65 ± 1.02 ^b	6.16 ± 0.48 ^a	7.41 ± 0.55 ^{ab}	7.08 ± 2.07 ^{ab}	7.22 ± 1.09 ^{ab}
PCV (%)	28.79 ± 1.32 ^c	17.67 ± 1.39 ^a	22.91 ± 1.26 ^{bc}	19.50 ± 4.5 ^{ab}	18.22 ± 2.22 ^{ab}
RBC (10 ⁶ /mm ³)	5.82 ± 0.56 ^b	3.07 ± 0.22 ^a	4.27 ± 0.43 ^{ab}	3.40 ± 0.70 ^{ab}	3.28 ± 0.84 ^a
WBC (x10 ³ / µl)	10.52 ± 0.96 ^{ab}	6.39 ± 0.31 ^a	11.98 ± 1.07 ^b	9.00 ± 2.0 ^{ab}	9.83 ± 1.41 ^{ab}
LYM (%)	62.96 ± 3.13	59.93 ± 2.76	60.24 ± 2.26	73.50 ± 7.5	59.89 ± 4.40
MON (%)	1.54 ± 0.1	1.55 ± 0.21	1.56 ± 0.14	1.00 ± 0.5	1.60 ± 0.36
GRA (%)	36.77 ± 1.54	40.62 ± 2.12	40.19 ± 1.3	27.50 ± 6.5	39.67 ± 3.46
MCH (pg)	15.82 ± 1.30	14.54 ± 1.25	14.63 ± 1.01	14.50 ± 3.5	13.22 ± 2.22
MCHC (g/dl)	34.62 ± 1.23 ^a	45.69 ± 2.54 ^b	32.02 ± 1.0 ^a	36.50 ± 7.5 ^{ab}	34.67 ± 2.62 ^a
MCV (fl)	46.42 ± 1.97	47.54 ± 2.47	48.21 ± 1.21	51.50 ± 5.50	45.11 ± 3.35
Values of Hb (g/dl), PCV (%), RBC (10 ⁶ /mm ³), WBC (x10 ³ / µl), and MCHC (g/dl) with different superscripts a,b,c differ significantly in a row. Hb: Haemoglobin; PCV: Packed cell volume; RBC: Red blood cells; WBC: White blood cells; LYM: Lymphocytes; MON: Monocytes; GRA: Granulocytes; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; MCV: Mean corpuscular volume.					

Polymerase chain reaction and phylogenetic analysis

In the PCR assays, specific amplifications of 504 bp, 270 bp and 751 bp products were obtained for 18S rRNA gene of *Babesia*, 16S rRNA gene of *A. marginale* and Tams1 gene of *T.annulata* (Fig. 2a, b and c).

Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js *marginale* and one *T. annulata*, representing

different isolates of Jammu region were sequenced. The sequence similarity searches in BLAST revealed that Jammu isolate (MN566925.1) of *Babesia* spp. was 99.8% identical to Kathua (MN567603), Udhampur (MN566924.1) and Meghalaya (KF606864.1) isolates of *Babesia bigemina* and had marked similarity with *B. bigemina* from Argentina (HQ688688.1) and USA (MH050387.1). Two sequences of *A. marginale* from our study, submitted to GenBank (MH733242.1, MN567602.1), revealed 100% nucleotide identity with published sequences of *A. marginale* of French (MN317256.1), Iranian (MK310488.1) and Vietnamese (MH686047.1) origin. The only sequence of *T. annulata* from Jammu region was found to be 99% similar to Indian isolates from Izatnagar (MF346013.1) and Hissar (AF214840.1). Furthermore, the Jammu isolate showed 99.07% sequence homology to United Kingdom W1_2 isolate (KX981026.1).

In the phylogenetic tree, our sequences clubbed together with Mizoram isolate (MH407694.1) forming separate Indian subclade (Fig. 3a). The phylogenetic tree of *A. marginale* (Fig. 3b) showed species level diversity in which Kathua isolate branched away from Ludhiana isolate (India, KF696858.1). The only *T. annulata* sequence from the present study formed a separate clade with Indian isolate of *T. annulata* (MF346013.1) on the phylogenetic tree (Fig. 3c).

Discussion

In developing countries like India considerable economic losses occurs in large and small holding livestock productivity farming system due to TBDs [6]. Prevalence studies are of immense importance for disease mapping and investigating the epidemiological triad. The agro-climatic conditions of the Jammu region are highly favourable for growth and multiplication of ticks which act as natural vectors of theileriosis, babesiosis and anaplasmosis. In the present study, high prevalence of *Babesia bigemina* (14.02%, 39/278), *A. marginale* (23.74%, 66/278) and low prevalence of *Theileria annulata* (1.079%, 3/278) can be attributed to *Rhipicephalus (Boophilus) microplus* being the only tick which infests the bovines of Jammu region [12]. A thorough review of literature reveals varying incidence of babesiosis ranging from 0.76 to 18.50% in India [13, 14, 15]. *Anaplasma* is also associated with a long term/life long carrier state [16] and so the probability of detecting positive animals, particularly in areas of endemic instability, is increased, primarily with use of molecular tools [6, 17]. The animals found positive for theileriosis had a history of importation from the neighbouring state, Punjab, having high prevalence of *H. anatolicum* [18] and *T. annulata* [5]. Owing to meagre presence of vector tick (*H. anatolicum*), considerably low prevalence of theileriosis was found in union territory of Jammu and Kashmir [19]. Livestock owners import high yielding animals from bordering states of Punjab and Haryana without proper quarantine and thus carrier cattle get transported to non endemic areas, increasing the likelihood of transboundary outbreaks. PCR assay has been employed for sensitive detection of haemoparasites in many diagnostic laboratories and is considered as an alternative to microscopy [20] particularly in latent infections. In our study as well, PCR was able to identify more than twice the number of cases as detected by microscopy.

There were no uncharacteristic clinical signs in the animals found positive for haemoprotozoan and
 Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js branes and decrease in milk production were

however prominent signs. The endogenous pyrogens released in the blood due to cellular lysis lead to fever and consequently, inappetence [17]. Anemia occurs due to erythrophagocytosis, lysis of RBCs due to parasite multiplication and subsequent removal by reticuloendothelial system. [21, 22] Consequently, the vital blood parameters which include level of haemoglobin, PCV, MCHC, MCV, TLC and TEC get deranged and further aggravated by the continuous loss of blood sucked out by the ticks[23]. The impact of haemoprotozoan and rickettsial infection on the milk production is a major economical set back. Our results reflect the deviations in the blood parameters following the same cascade and are in agreement with [15, 17, 24].

The study suggests that outbreaks are likely to occur in the rainy and post rainy season due to interplay of epidemiological factors. During rainy season the epizootiological determinants such as ambient temperature and atmospheric humidity and microclimate of grazing lands are favourable for growth and development of ticks. This is unlikely to differ in other parts of country as well, based on earlier reports viz 29.31% [25] and 58.55% [26]. The vector population was observed as a major differential factor in the prevalence of TBDs in organised and unorganized farms ($p = 0.007$), latter showing increased prevalence. These farms are Kuchha houses (non cemented) or made of bricks only, having cracks and crevices, with improper drainage and poor ventilation, favouring survival and breeding of vector ticks. Moreover, the owners of unorganized farms are comparatively uneducated, so lack of awareness regarding use of acaricidal and sustainable managemental practices against ticks was observed at the time of sample collection. The other determinants recorded for disease occurrence are age and breed of animal. Inverse age resistance shields the young population from clinical outbreak of diseases [27]. The highest prevalence (37.50%) was observed in animals > 3 years of age which is supported by studies from [26, 28]. Also, the adult cattle are predisposed to various stresses due to cycling heat, production, vaccination and reproduction which may augment the pathogenesis of diseases. Cross bred animals revealed higher prevalence of infection (33.62%) than indigenous breeds (15.38%), in agreement with earlier reports [26, 29]. Some workers have attributed susceptibility to the difference in the immune response to produce pro-inflammatory cytokine, which is higher in exotic animals or native breeds harbouring genetic loci for greater tolerance [30, 31].

Intracellular haemoprotozoons are under constant pressure from the immune system of their hosts, leading to emergence of genetic variants [32]. The genetic polymorphism of *Babesia* spp. has been reported earlier using sequence information of 18S rRNA gene [1, 33, 34]. In the phylogenetic tree, the isolates of the present study formed a separate sub clade but clustered with an isolate from Iraq. However, these distinguished itself from South African and American isolates, simulating reports from Kerala [1], wherein the isolates appeared in two different clades. In another study from India [34], close genetic relatedness was observed between *B. bigemina* isolates from North Eastern India with Argentina and Kenya rather than with China. Sequence information of *A. marginale* isolates from the present study revealed a marked divergence from *A. capra*, *A. central* and *A. ovis*, although, small length of nucleotide sequence couldn't reveal any marked genetic heterogeneity from isolates of *A. marginale* across the world. In a study from south India, minimal heterogeneity was revealed within 16S rRNA and *msp4* genes

The key mechanism responsible for genetic diversity among *Theileria* spp., is recombination during sexual reproduction [35]. The Tams1 gene has been shown to be a promising candidate for carrying antigenic diversity studies in *T. annulata* parasites [36, 37], however some studies have suggested no geographic specificity and other showing region specificity based on the gene polymorphism [36, 37, 38]. In the present study, *T. annulata* showed 99% nucleotide homology with Indian isolates and clustered together with an Indian isolate in the phylogenetic clade. Parasite diversity reports from India suggests that the Indian isolates were distributed into two groups along with other countries like Spain, Italy, Tunisia, Iran, Bahrain, Turkey and Iraq [39, 40]. The genetic diversity among the parasite strains can be one of the reasons for vaccination failures and inability to constraint the disease.

Conclusions

In conclusion the study provided a holistic picture about the prevalence of haemoprotozoon and rickettsial infection in cattle and its correlation with the environmental variables and epidemiological determinants. In addition, the hematological data may be presumed as a marker of health status of animal and its implications on the productivity of animal. The present study is probably the first report of molecular characterization of the haemoprotozoon and rickettsial infection from Jammu region of North India. It provides pioneering information about the circulating genotypes and possible diversities which are quintessential for developing vaccine and diagnostic strategies in future.

Abbreviations

TTBD: tick and tick borne diseases, RNA: ribonucleic acid DNA: deoxyribonucleic acid, PCR: polymerase chain reaction, Hb: Haemoglobin; PCV: Packed cell volume; RBC: Red blood cells; WBC: White blood cells; LYM: Lymphocytes; MON: Monocytes; GRA: Granulocytes; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; MCV: Mean corpuscular volume.

Declarations

Ethics approval and consent to participate

The cattle owners graciously permitted animal examination and blood collection through verbal consent. The permission for blood collection was granted by institutional animal ethics committee (IAEC, FVSc & AH, SKUAST-Jammu) registered with ID: 862/CPC/SEA as per order no. AU/FVSJ/AGB/17-18/415.

Consent for Publication

Not applicable

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest

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Authors contributions

All authors have read and approved the manuscript. RK undertook literature search, clinical studies, experimental studies and data acquisition. AY conceptualised and designed the study, aided in manuscript preparation, editing and review. SIR performed the experiments, interpreted the results, prepared and edited the manuscript. RG did the data analysis, statistical analysis and manuscript editing. VS performed data analysis and manuscript editing. DC did statistical analysis of data. RK conceptualised the study and reviewed the final manuscript.

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Figures

Figure 1

a: Graph depicting rainfall (in mm) and temperature (0C) during different seasons of the year in Jammu region
 region b: Graphical representation of morning and evening relative humidity and prevalence during different seasons of the year in Jammu region

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Figure 2

a: PCR amplification of Babesia 18S rRNA, Lane 1, 2 negative control, Lane 3, 4, 5 : test samples M: 100 bp ladder, Lane 6: positive control
 b : PCR amplification of Anaplasma marginale 16s rRNA, Lane 1-4 test samples, M1: 100 bp ladder, M2: 50bp ladder, Lane 5: positive control, Lane 6 negative control
 c : PCR amplification of Theileria annulata Tams1 gene, Lane 1: positive control, M: 100 bp ladder, Lane 3,4,5,6, : test samples at gradient , Lane 7: negative control

Figure 2

a: PCR amplification of *Babesia* 18S rRNA, Lane 1, 2 negative control, Lane 3, 4, 5 : test samples M: 100 bp ladder, Lane 6: positive control b : PCR amplification of *Anaplasma marginale* 16s rRNA, Lane 1-4 test samples, M1: 100 bp ladder, M2: 50bp ladder, Lane 5: positive control, Lane 6 negative control c : PCR amplification of *Theileria annulata* Tams1 gene, Lane 1: positive control, M: 100 bp ladder, Lane 3,4,5,6 : test samples at gradient , Lane 7: negative control

Figure 3

a: Phylogenetic relationship of *B. bigemina* isolates based on 18S rRNA gene. All accession numbers correspond to different *B. bigemina* isolates followed by their country of origin. The sequences generated in the present study are marked as red triangle. b Phylogenetic relationship of *A. marginale* isolates based on 16S rRNA gene. All accession numbers corresponds to different *Anaplasma* isolates followed by their country of origin. The sequences generated in the present study are marked as red triangle. c. Phylogenetic relationship of *T. annulata* isolates based on Tams1 gene. All accession numbers corresponds to different *T. annulata* isolates followed by their country of origin. The sequence generated in the present study is marked as red triangle

Figure 3

a: Phylogenetic relationship of *B. bigemina* isolates based on 18S rRNA gene. All accession numbers correspond to different *B. bigemina* isolates followed by their country of origin. The sequences generated in the present study are marked as red triangle. b Phylogenetic relationship of *A. marginale* isolates based on 16S rRNA gene. All accession numbers corresponds to different *Anaplasma* isolates followed by their country of origin. The sequences generated in the present study are marked as red triangle. c. Phylogenetic relationship of *T. annulata* isolates based on Tams1 gene. All accession numbers corresponds to different *T. annulata* isolates followed by their country of origin. The sequence generated in the present study is marked as red triangle

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