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SEROPREVALENCE OF *BABESIA BIGEMINA* IN CATTLE IN WEST-CENTRAL REGION OF BHUTAN

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ABSTRACT: Ticks are considered to be the most important vector of disease-causing pathogens in animals and humans and are therefore of major animal health and public health importance globally. In livestock, it causes significant economic losses in terms of morbidity, mortality, loss of production, control and treatment related costs. In the West-Central region, *Rhipicephalus (Boophilus) microplus*, the principal arthropod vector for *Babesia bigemina*, is the most common ixodid tick species found on cattle across five agro-ecological zones (AEZs). Therefore, a survey was conducted to determine the seroprevalence of *B. bigemina* and its associated risk factors in apparently healthy cattle in the five AEZs of the region. A total of 445 local and crossbred cattle of both sexes and different age groups were sampled using a multi-stage sampling method. Serum antibodies against *B. bigemina* were estimated using indirect ELISA and expressed as percent positivity. 86% of the cattle in the region were found seropositive for *B. bigemina*. Using a binomial logistic regression model to predict the likelihood of sero-positivity, the study found that cattle aged 3 years and above (p<0.001) and free-ranging cattle (OR 2.14; 95% CI 1.14, 4.02; p=0.018) had higher odds of *B. bigemina* seropositivity. Besides, the odds of *B. bigemina* seropositivity was higher in cattle in wet subtropical, humid subtropical, dry subtropical and warm temperate AEZs as against those in cool temperate zone (p<0.001). However, sex (p=0.369) and breed (p=0.560) did not show statistically significant effect on the seroprevalence of *B. bigemina* antibodies in cattle. This study found high seroprevalence of Bovine Babesiosis caused by *Babesia bigemina* in the region indicating high carrier status and endemic stability. Age of cattle, type of grazing system and agro-ecological zones were the predictors that significantly determined *B. bigemina* seropositivity in cattle in the region. Since the prevalence of other tick-borne pathogens (TBPs)/tick-borne diseases (TBDs) of veterinary significance or zoonotic potential including their distribution in the region are not known, further investigations are recommended to understand the epidemiology of TBPs/TBDs in the region to aid in developing a holistic framework to decrease the burden of ticks and TBPs/TBDs.

Keywords: *Babesia bigemina*; determinants; seropositivity; ticks.

1. INTRODUCTION

Livestock plays an integral role in the lives of farmers in the country, with majority of the households in the farming community owning cattle. The total number of cattle (*Bos taurus*) in the West-Central region in 2018 was 66372; of which, 66% were local/indigenous cattle breed and the remaining 34% were pure or cross breed of Jersey, Holstein Friesian or Brown Swiss cattle (Department of Livestock [DoL] 2019). Local

cattle breed is predominantly managed under free grazing system during the day and stall fed on the homestead at night. On the other hand, pure bred and high producing cross bred cattle are primarily stall fed and tethered grazed. Owing to the cattle rearing system in practice, ticks are extensively found in cattle in the region. Ticks are considered to be the most important vector of disease-causing pathogens in domestic and wild animals; and are also considered to be second to mosquitoes as vectors of human diseases globally (de la Fuente et al. 2008). Ticks, therefore, are known to be of major animal health and public health importance. Besides serving as a host, wildlife also acts as reservoirs for many of the most important tick-borne pathogens (Hurtado and Giraldo-Rios 2018; Schischke 2015). Ticks and tick-borne diseases are known to cause significant economic losses in terms of morbidity and mortality; control and treatment related costs; loss of production in livestock; and transmission of zoonotic diseases causing high morbidity and mortality in people (Schischke 2015). In the West-Central region alone, approximately 1million out of the total 6.5 million (in BTN) budget is spent annually on synthetic acaricides to control ticks and acaricides are also considered as one of the most sought-after medicines that are often in short supply in the field (Regional Livestock Development Centre [RLDC] 2019). Despite the known undesirable effects of synthetic acaricides on host and environment, such as, its non-biodegradability, environmental toxicity, issues related to residuals in animal body and development of resistance in ticks Jaiswal and Mishra (2015), synthetic acaricides have been the only tick control method that has been in use in the country.

In order to establish the baseline information on the prevalence of ticks and tick-borne pathogens, a systematic survey was carried out in the fiscal year 2018-2019. The survey was mainly aimed to morphologically identify ticks in healthy cattle in different AEZs in the region, study its distribution as well as its determinants. Five genera of ixodid ticks were found prevalent on cattle in the region; and no argasid ticks were encountered in the tick specimens examined (RLDC 2019). A follow up laboratory study conducted in fiscal year 2019-2020 to morphologically identify ticks till the species level revealed the presence of seven species of ixodid ticks in the West-Central region viz., *Amblyomma integrum, Haemaphysalis (Allocerae a) aponommoides, Haemaphysalis* (*Kaiseriana*) *bispinosa, Haemaphysalis* (*Kaiseriana*) *spinigera, Ixodes ovatus, Rhipicephalus (Boophilus) microplus and Rhipicephalus haemaphysaloides* (Pem et al. 2020). *Rhipicephalus (Boophilus) microplus*, a one-host tick, was documented as the most prevalent ixodid tick species infesting cattle across the five AEZs in the region (RLDC 2019).

Rhipicephalus (Boophilus) microplus is the principal arthropod vector for *Babesia bigemina* and *Babesia bovis*, both of which are protozoan parasites that causes haemoprotozoan disease in cattle known as bovine babesiosis (World Organisation for Animal Health [OIE] 2018). *Babesia bigemina* and *Babesia bovis* are known to be widely distributed and of great economic importance in many tropical and sub-tropical regions in the world viz. Asia, Africa, Australia, Central America and South America (OIE 2018; Schischke 2015). Bovine babesiosis is a disease that is clinically manifested as a haemolytic anemia and the infected animals develop a life-long immunity against re-infection with the same species and some cross-protection in *B. bigemina*-immune animals against subsequent *B. bovis* infections (RLDC 2019).

Given the wide distribution of *Rhipicephalus (Boophilus) microplus* tick species across the five AEZs in the West-Central region, the study was conducted to determine the seroprevalence of tick-borne pathogens of veterinary significance (*Babesia bigemina, Babesia bovis & Anaplasma marginale*) known to be harbored by *Rhipicephalus (Boophilus) microplus* including their determinants in cattle. However, because of logistical reasons, in particular due to lack of access to most of the diagnostic kits, only seroprevalence of *Babesia bigemina* and its determinants in cattle could be assessed in this study.

Therefore, the objective of this study was to determine the seroprevalence of *Babesia bigemina* in cattle across five AEZs of the West-Central region, the zones where cattle (*Bos taurus*) are predominantly reared; and also, to identify risk factors associated with the seropositivity so as to understand the risks and assess the potential of controlling bovine babesiosis in the region.

2. MATERIALS AND METHODS

2.1 Study area

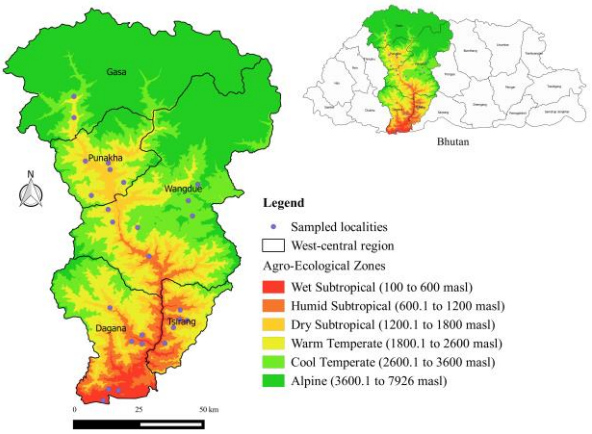
This study was carried out in five Dzongkhags (districts) viz. Dagana, Gasa, Punakha, Tsirang and Wangdue of the West-Central region of Bhutan. The region is subdivided further into 56 Gewogs (sub-districts). The total geographical area of the region is 10618.6 km^2. AEZs in the region extends from wet subtropical in the south to alpine in the north with an elevation ranging from 100 to 5400 masl. Except in the alpine zone, cattle (*Bos Taurus*) are reared in other AEZs viz. wet subtropical, humid subtropical, dry subtropical, warm temperate and cool temperate zones in the region.

2.2 Study design

A cross sectional survey was carried out from September 2018 to June 2019 in the five AEZs of the West-Central region using a pre-designed semi-structured questionnaire to record information. In absence of published prevalence data available either at national level or in the region, the sample size was determined based on the following: the assumption of 50% prevalence to get the maximum sample size as the seroprevalence for *B. bigemina* was not known prior to this study; indirect enzyme-linked immunosorbent assay (ELISA) test with 96% sensitivity and 97.5% specificity employed for laboratory analysis of sera samples (SVANOVA n.d.) and 95% confidence level and 0.05 absolute precision. Based on the above, a total of 445 cattle were sampled for this study.

Stratified random sampling at multiple stages was employed. The Gewogs in the West-Central region were classified according to the six agro-ecological zones (AEZs) in the country. Of the six AEZs, alpine zone was not included in this study as cattle (*Bos taurus*) are not reared in this zone. The altitude at which the gewog extension centers are located was taken as a basis for classification of Gewogs into various AEZs. At the most, seven Gewogs from each AEZs were selected by generating random numbers. In the selected Gewogs, at least five herds were randomly sampled. The selection criteria of the herds for this survey were the availability of cattle of three different age groups (between 6 months and 1 year, between 1 and 3 years, and >3 years). In each herd, one animal from each of these three different age groups was randomly sampled.

The locations of the herds sampled were geo-coded to ensure cattle from right AEZs were sampled and then later mapped using QGIS software (Figure 1).



2.3 Sample collection and laboratory analysis method

Blood sample was collected from each sampled cattle using a 10 ml plain vacutainer tube. Sera was separated by centrifugation and then stored at -80^{o}C until laboratory analysis was conducted. The ELISA technique was used for sero-diagnosis of *B. bigemina* using a commercial indirect ELISA kit which had bovine-specific diagnostic antigens (SVANOVA n.d).

2.4 Data analysis

The data recorded in the predesigned semi-structured questionnaire were keyed into Microsoft excel 10 and cleaned before analysis. Descriptive statistics was generated for baseline characteristics of the sampled animals and presented as frequencies and percentages. Serum antibodies against *B. bigemina* in cattle were estimated using a commercial ELISA following protocols provided with the kit. The estimated antibodies levels were expressed as a percent positivity which was computed from the optical density values. Univariate analysis and multivariate analysis were conducted using logistic regression models to examine association between seropositivity for *B. bigemina* in cattle and potential risk factors. Those potential risk factors that had univariate $\chi 2$ test p-value <0.25 were included in the final regression model.

Adjusted odds ratio, 95% confidence intervals and p-values were calculated to measure the effect of determinants on *B. bigemina* seropositivity in cattle. Levels of significance in univariate and multivariate analysis were obtained using Likelihood ratio tests. A two-sided *p*-value of <0.05 was considered as indicating statistical significance. All the statistical analyses were performed using statistical software Stata version 11.0 (StataCorp, College Station, TX, USA). GPS machine was used to capture the geo-coordinates of the sampled localities. QGIS software was later used to map the study area to visually illustrate the localities sampled across the five AEZs and also to determine the spatial distribution of *B. bigemina* in cattle in the West-Central region.

3.RESULTS AND DISCUSSIONS

The overall seroprevalence of *B. bigemina* in cattle in the West-Central region during the study period was found to be 86% (383/445). Table 1 shows the descriptive statistics presented as frequencies and percentages of the baseline characteristics of the sampled cattle. The univariate analysis $\chi 2$ test *p*-value for sex (p=0.369) as well as breed (p=0.560) of cattle were >0.25 and hence not included in the final multivariate logistic regression model. The *p*-value >0.25 indicates that the effect of sex and breed of cattle on *B. bigemina* seropositivity is not statistically significant. Similar studies conducted in other countries (Salih et al. 2009; Schischke 2015; Swai et al. 2007) also found *B. bigemina* seropositivity to be independent of sex and breed of the cattle.

Table 1: Baseline characteristics of sampled cattle (n=445)

Characteristics	Sub-groups	Frequency (%)
Sex	Male	108 (24.27)
	Female	337 (75.73)
Age group	Calf (6mo-1 yr)	148 (33.26)
	Young(>1yr-3yrs)	143 (32.13)
	Adult (>3yrs)	154 (34.61)
Breed	Local	227 (51.01)
	Cross bred	218 (48.99)
Type of grazing system	Mix of stall feeding & tethered grazing	130 (29.21%)
	Mix of stall feeding & free grazing	315 (70.78%)
No. of animals sampled from five agro-ecological zones	Cool Temperate	74 (16.63)
	Warm Temperate	90 (20.22)
	Dry Subtropical zone	110 (24.72)
	Humid Subtropical	90 (20.22)
	Wet Subtropical	81 (18.20)

Through multivariate analysis (Table 3), it was found that cattle aged 3 years and above had higher odds of *B. bigemina* seropositivity as compared to cattle below 3 years of age (p<0.001). A similar study conducted by Swai et al. (2007) in Tanzania also found a trend of increased seropositivity for *B. bigemina* infection with age. Several studies (Magona et al. 2008; Schischke 2015; Zintl et al. 2005) observed the presence of inverse age-resistance to *Babesia* spp. infection and disease, wherein young cattle are less susceptible than older cattle.

Furthermore, Zintl et al. (2005) confirmed the existence of good evidences of innate resistance of calves against bovine babesiosis being antibody-independent, as antibodies in early immune sera appear late in the primary infection and long after parasites have been cleared from the periphery. The above probably explains the present study findings of lower *B. bigemina* seropositivity in younger cattle as compared to adult cattle.

It was also found that free grazed cattle had higher odds of *B. bigemina* seropositivity than those that were tethered grazed (OR 2.14; 95% CI 1.14, 4.02; p=0.018). This finding concurs with results of several studies conducted elsewhere in the world which also concluded that restricted grazing or zero grazing were amongst the most important ways to decrease *B. bigemina* infection (Schischke 2015). On the other hand, communal grazing led to high tick burdens and higher occurrence of TBDs in cattle (Rubaire-Akiiki et al. 2004; Swai et al. 2007). Besides, cattle in wet subtropical zone, dry subtropical zone and warm temperate zone were found to have higher odds of *B. bigemina* seropositivity as compared to the cattle in cool temperate zone (p<0.001).

A study on distribution of ticks on cattle in the West-Central region found that tick burden on cattle increased as altitude decreased; and that amongst the tick species, the prevalence of

Table 2: Univariable association between odds of *Babesia bigemina* seropositivity & potential risk factors

Determinant	Estimated Odds Ratio	95% Confidence Interval	p-value
Sex	0.73	0.37, 1.44	0.369
Breed	0.85	0.49, 1.46	0.560
Age group			
Calf (6mo-1year)	1.00		0.065*
Young (1-3 years)	1.37	0.73, 2.58	0.325
Adult (>3 years)	2.23	1.11, 4.45	0.023
Type of grazing system	2	1.15, 3.48	0.014
Agro-ecological zones			
Cool Temperate	1.00		<0.001*
Warm Temperate	2.38	1.01, 5.59	0.045
Dry Subtropical	3.34	1.40, 7.99	0.007
Humid Subtropical	0.82	0.40, 1.68	0.587
Wet Subtropical	23.86	3.09, 184.45	0.002

**p-value from Likelihood ratio test*

Table 3. Multivariable associations between odds of *Babesia bigemina* seropositivity and potential risk factors

Determinant	Adjusted Odds Ratio	95% Confidence Interval	p-value
Age group			<0.001*
Calf (6m-1year)	1.00		
Young (1-3 years)	1.36	0.70, 2.64	0.366
Adult (>3 years)	2.42	1.18, 4.50	0.016
Type of grazing system	2.14	1.14, 4.02	0.018
Agro-ecological zones			
Cool Temperate	1.00		<0.001*
Warm Temperate	2.42	1.02, 5.76	0.045
Dry Subtropical	4.73	1.85, 12.08	0.001
Humid Subtropical	0.98	0.46, 2.08	0.960
Wet Subtropical	24.15	3.11, 187.79	0.002

**p-value for Likelihood ratio test*

Riphicephalus (Boophilus) microplus was significantly higher than any other tick species (RLDC 2019). Therefore, the higher odds of *B. Bigemina* seropositivity in cattle from AEZs lower than the cool temperate zone as found in this study possibly correlates to the geographical variation in distribution of its vector *Riphicephalus (Boophilus) microplus*. However, this study did not examine management-related practices such as methods and frequency of acaricide application as these practices were found mostly similar across the five AEZs in the West-Central region.

There are no known surveys conducted in the past to study the prevalence of *B. bigemina* in cattle either in the country or in the West-Central region. Appropriate study design and sampling method, adequate sample size and OIE recommended laboratory analysis techniques were employed in this study to ensure validity of the results. ELISA technique had been employed in this study for detection of *B. bigemina* antibodies as it is the most widely used test for the detection of antibodies to *Babesia* spp. due to its processing efficiency and objectivity in interpretation of results (OIE 2018). Besides, ELISA is also recommended by World Animal Health Organization for the purpose of Babesiosis surveillance in cattle (OIE 2018). Furthermore, indirect ELISA using a recombinant immunodominant antigen used in this study is bovine specific and has sensitivity of 96% and specificity of 97.5% for *B. Bigemina* (SVANOVA n.d.). Although the reported clinical incidence of Babesiosis has been very low in the West-Central region so far, the present study found high serological prevalence indicating high carrier status and probable attainment of endemic

stability in the region. It has been evidenced that the exposure of young cattle to sufficient ticks infected with *Babesia* during their life, without clinical babesiosis, can ensure a strong protection (Monique and Henri 2002; Schischke 2015). An endemic stable situation is known to arise wherein many cattle develop immunity to the parasite, without causing clinical babesiosis and this is known to occur in herds in which over 75% of cattle are seropositive (Monique and Henri 2002).

The low incidence of clinical disease in the West-Central region and the extensive distribution of *Rhipicephalus (Boophilus) microplus* ticks further substantiates this study finding of 86% seropositivity indicating endemic stability for *Babesia bigemina* in the region. This also indicates that the apparently healthy animals could be a potential source of infection to naive animals.

4. CONCLUSION

It can be concluded that antibodies against *Babesia bigemina* are widely present in cattle in the West-Central region and that the region is endemically stable for Babesiosis. The findings of the present study points that the apparently healthy animals could pose as a potential source of infection for naïve animals brought into the region. This study found that age of the cattle, type of grazing system and agro-ecological zones were the predictors that significantly determined *B. bigemina* seroprevalence in cattle in the West-Central region. However, further investigations for other tick-borne pathogens of veterinary significance and or zoonotic potential are also recommended to understand the epidemiology of TBPs/TBDs in the West-Central region to develop a holistic framework to decrease the burden of ticks and TBPs/TBDs.

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