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EVALUATION OF VECTOR COMPETENCE OF ADULT IXODID TICKS FOR *Anaplasma marginale, Babesia bigemina* and *Theileria annulata* IN CATTLE FROM CENTRAL PUNJAB, PAKISTAN

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ABSTRACT

Anaplasmosis, theileriosis and babesiosis, are the most important tick-borne diseases (TBDs), causing great financial losses to the livestock industry. The objective of the study was to evaluate the biological transmission dynamics of tick-borne diseases in cattle using conventional (blood smear), serological (cELISA), and cutting-edge modern techniques (PCR). For the biological transmission study, pathogen-free ticks were collected and divided into three groups. Each group of ticks was allowed for acquisition feeding for seven days on the respective diseased calves (anaplasmosis, theileriosis, and babesiosis), separately. Later, the infected ticks were allowed to feed on disease-free calves (n=15) and divided into three groups, comprising five calves in each group. After two weeks, calves were screened by serological and/or molecular assays. All five calves infested with *Rhipicephalus microplus* ticks transmitted (100%) *Anaplasma marginale* and *Babesia bigemina* infections. Whereas, calves infested with *Hyalomma anatolicum anatolicum* ticks transmitted (80%) *Theileria annulata* infection in four out of five calves. Hence, it was proved that *R. microplus* (*A. marginale, B. bigemina*) and *H. anatolicum anatolicum* (*T. annulata*) ticks are competent vectors in the region. The identification of vectors and determinants can help in the control and prevention of TBDs.

Keywords: Biological, transmission, tick-borne diseases, cattle

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INTRODUCTION

Farmers demand for genetically improved exotic and crossbred animals for higher productivity is increasing, but these breeds are highly vulnerable towards ticks and tick-borne diseases (TTBDs). The TTBDs are one of the significant issues for the cattle industry in tropical and subtropical agro-climatic regions, as more than 80% of the cattle population resides in these areas (Cooke *et al*., 2020; Omar and Albarrak, 2025) and cause estimated financial losses of about 13.9-18.7 billion US dollars every year (Hussain *et al*., 2021). The main tick genera found in Pakistan are *Haemaphysalis, Rhipicephalus, Dermacentor,* and *Hyalomma* (Karim *et al*., 2017; Rehman *et al*., 2017; Ramzan *et al*., 2020).

Anaplasmosis, babesiosis, and theileriosis are the most important tick-borne infections (TBI) that cause great losses to the livestock industry (Gong *et al*., 2025). Anaplasmosis is caused by *Anaplasma* (Anaplasmataceae: Rickettsiales), an intracellular coccoid gram negative alpha-proteobacterium (Atif, 2016). Among domestic bovines, cattle play a chief role as a reservoir host. Anaplasmosis is mostly transmitted by ixodid ticks of more than twenty different species (Kocan *et al*., 2003) and has been reported in North and South America, Europe, Asia, and Africa (Atif, 2016). The occurrence of anaplasmosis in Pakistan ranges from 4–60% (Jabbar *et al*., 2015).

Theileriosis, a hemo-protozoan infection is caused by *Theileria* (Apicomplexa; Piroplasmida) species including *Theileria annulata*, *T. parva, T. mutans*, *T. orientalis, T. velifera,* and *T. taurotragi* (Sivakumar *et al*., 2014). The *T. annulata*, causes tropical theileriosis of livestock in tropical regions and among the most prevalent TBDs in Pakistan (Abdallah *et al*., 2017; Hussain *et al*., 2022; Atif *et al*., 2024). *Theileria* is

transmitted by Ixodid ticks *viz. Hyalomma* (H.)*, Haemaphysalis, Rhipicephalus* (R.), and *Amblyomma* (Florin-Christensen and Schnittger 2009; Hussain *et al*., 2022). Bovine babesiosis (BB), a malaria-like hemo-protozoan ailment caused by *Babesia* (B.) an intracellular eukaryotic parasite, an alveolate in the phylum Apicomplexa. Babesiosis in animals is caused by *B. bovis, B. divergens* and *B. bigemina.* The BB leads to severe intravascular hemolysis with clinical manifestations of hemoglobinuria, anemia, high fever, jaundice, and respiratory distress (Bock *et al*., 2004). The BB is transmitted mainly through *Rhipicephalus* while *Hyalomma* and other genera of ixodids ticks also contribute in disease transmission (Gray and De Vos 1981; Hunfeld *et al*., 2008).

The ticks and tick-associated pathogens are endemic in Pakistan due to favorable agro-climatic conditions (Sajid *et al*., 2009; Rehman *et al*., 2017; Sajid *et al*., 2018; Rehman *et al*., 2019). Vector competencies of ticks and transplacental transmission play a pivotal role in the spread and endemic stability of these TBIs (Costa *et al*., 2016; Karim *et al*., 2017; Nazar *et al*., 2018; Rehman *et al*., 2019; Atif *et al*., 2021). Vertical transmission of anaplasmosis usually occurs during the 2nd and 3rd trimester of gestation (Potgieter and Van Rensburg, 1987; Ribeiro *et al*., 1995). As far as we know, this is the first experimental validation of vector competence of *A. marginale, T. annulata* and *B. bigemina* among cattle in Pakistan. The identification of vectors and evaluation of potential transmission routes are important for disease prevention and control standpoint. Keeping in view the above discussed facts, there is a dire need to mention the biological transmission attributes of prevalent ticks using modern diagnostic techniques.

MATERIALS AND METHODS

Ethical approval: For performing research activities on live animals, ethical approval was obtained from the Ethical Committee of College of Veterinary and Animal Sciences (CVAS), Jhang vide No. CVAS 10085; dated: 23-04-2019. All ethical guidelines were followed during the study period.

Collection and maintenance of ticks: Ticks (n=900) were collected from nearby vegetation of CVAS, Jhang, Pakistan during the months of April and May by dragging a cloth and ticks were transferred to 20 ml plastic bottles having fine holes. The ticks were recognized based on their morphology using the taxonomic characteristics utilizing key (Walker, 2003). The larvae were permitted to feed on rabbits for 15 days and developed to nymphs. The engorged nymphs were removed and induced to molt to adults at 85-92% relative humidity and 20-30°C in an incubator. For *A. marginale* and *B. bigemina* transmission, the adult male *R. microplus* ticks were separately utilized. Whereas, for evaluation of vector competence against tropical theileriosis, *H. anatolicum anatolicum* female ticks were permitted to feed separately for one week on infected carrier calves. The *R. microplus* is classified as a one-host tick; males can still spread the infection multiple times by switching between cattle. The main reason for using female *H. anatolicum anatolicum* ticks in the transmission of *T. annulata* is their capacity to effectively carry the parasite through successive developmental stages—from larva to nymph and then to adult. Pathogen free ixodid ticks were reared at Postgraduate Laboratory of Medicine, CVAS, Jhang. The scheme of experimental study is given in Figure 1.

Acquisition feeding on infected cattle: Pathogen free adult male *R. microplus* and female *H. anatolicum anatolicum* ticks were allowed to feed separately for one week on *A. marginale, B. bigemina* and *T. annulata* infected carrier calves (n=3), respectively. The persistent infection was confirmed by blood smear microscopy (after observing 100 erythrocytes), cELISA (anaplasmosis), and PCR. The engorged ticks were retained in an incubator at 85-93% relative humidity and 20-30°C with 12 hours photoperiod for 7 days.

Determination of tick infectivity: After feeding of *H. anatolicum* and *R. microplus* ticks on the infected animal, they were recollected and kept in an incubator for at least 7 days, so that the mouth part would no longer be able to mechanically transmit disease. These ticks were divided into three groups based on their respective pathogen feeding. Each species of ticks was separately collected, and DNA was extracted (Kocan *et al*., 1993) for the recognition of selected tick-borne pathogens (*A. marginale*, *T. annulata* and *B. bigemina*) (Figueroa *et al*., 1992; Bilgic *et al*., 2013).

DNA extraction from tick specimens: The 10% ticks were washed with distilled water and dried up on soft tissue paper and further subjected to DNA extraction. The DNA was extracted with the help of Tissue DNA extraction kit (Thermo-fisher Scientific, USA; catalogue No. K 0722).

Transmission feeding on healthy calves: After maintenance of tick survival, these ticks were infested on the disease-free calves (n=15), tested negative through blood smear, serological/molecular techniques for major tick-borne pathogens. Thirty positive ticks harboring the respective pathogen were placed on the ear (covered with cloth) of each calf. Ticks were allowed for transmission feeding for 7 days for evaluation of its vector competency against the respective pathogen. These calves were divided into three groups, comprising five calves for each pathogen. For biological transmission of *A. marginale, R. microplus* ticks were attached to five calves (calves no. 76A, 84A, 09B, 16A, and 18A). Similarly, for *B. bigemina* transmission experiment*, R. microplus* ticks

were attached to calves No. 25A, 29A, 31A, 35A and 39A. Whereas, for *T. annulata* the *H. anatolicum* ticks were attached to the shaved ear of calves no. 19B, 41A, 23, 34A, and 48A, and remained attached for 7 days for transmission feeding. After transmission feeding, the ticks were removed from the experimental animals (Figure 2; A, B, C, D, E, F).

Microscopic evidence of tick infectivity: For evidence of tick infectivity, the ticks were longitudinally dissected into two halves. A half of each tick was fixed, cut into sections (1µm), and observed under the microscope as described by Zivkovic *et al*. (2007).

Monitoring: After two weeks of transmission feeding, the experimental calves were screened daily for post exposure monitoring of infected ticks through blood smear microscopy, cELISA, and/or PCR (Figueroa *et al*., 1992; Atif *et al.,* 2012; Bilgic *et al*., 2013; Atif *et al*., 2013).

Blood smear microscopy: The peripheral blood samples were used for the preparation of a thin Giemsa-stained blood smears and initial screening of animals. A thin film of blood was prepared on a glass slide and fixed by submerging in pure methanol for two to three minutes. The slide was then submerged in a Giemsa stain solution (1:20) for twenty to thirty minutes, then the slide was washed under tap water and dried (Anwar and Din, 2017). Furthermore, the intra-erythrocytic parasites of *Anaplasma, Babesia,* and *Theileria* were recognized based on their morphological characteristics (Coles, 1986).

Sero-diagnosis

Competitive ELISA: Blood samples were collected in a gel containing the vacutainer, centrifuged at 5000 rpm for 5 minutes for separation of serum. An improved kit "*Anaplasma* Antibody Test Kit, cELISA v2 (catalog No. 283-2)" from VMRD Inc., Pullman, WA, USA" was used for recognizing the *Anaplasma* infected animals. This kit specifically detects serum antibodies that target major surface protein 5 (MSP5) of *Anaplasma* spp. with high sensitivity (100%) and specificity (99.7%) for the detection of early and persistently infected animals (Chung *et al*., 2014). This assay has already been validated by earlier researchers for naturally and experimentally diseased animals (Knowles *et al*., 1996; Torioni de Echaide *et al*., 1998; Atif *et al*., 2013; Chung *et al*., 2014). The cELISA was performed as described by manufacturer. The plate was read with ELISA reader (Bio-base, EL-10). The optical density (OD) was read at 630 nm wavelength.

Interpretation: The specific wells without color change were reflected as positive and wells with blue color were considered as negative. Furthermore, the outcomes were recorded with the aid of ELISA reader (Biobase-EL10A) at 630 nm wavelength (Figure 3; A-H).

The percent of inhibition was calculated according to formula given below:

Inhibition percentage = 100 [1 - (Sample OD ÷ Negative Control OD)]

The values above 0.4 and below or equal to 2.10 were considered as negative. The samples with percent inhibition of >30% were considered as positive.

Molecular techniques: Furthermore, the samples were subjected to molecular analysis for the recognition of TBIs in cattle. The DNA was collected after centrifugation and stored in -20°C freezer for further processing.

PCR: The DNA of *T. annulata* pathogen was amplified targeting cytochrome *b* gene of *T. annulata* using PCR. This was achieved using primers Cytob1F (3′-ACT TTG GCC GTA ATG TTA AAC-5′) and Cytob1R (3′-CTC TGG ACC AAC TGT TTG G-5′) (Bilgic *et al*., 2013). PCR reactions were conducted in a 50 µL volume containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, dNTP (250 µM), 1 U of AmpliTaq DNA polymerase, 10 p mol of each primer, and 2 µL of template DNA. Amplification was accomplished with the subsequent conditions: (i) initial denaturation at 94°C for 3 minutes; 35 cycles of denaturation at 95°C for 50 seconds, (ii) annealing at 55°C for 50 seconds, and (iii) extension at 72°C for 1 minute and subsequently by a final extension at 72°C for 10 minutes. The PCR products were either stored at 4°C or frozen at -40°C until further analysis. The PCR amplification of the *msp1β* gene of *A. marginale* was carried out in a total reaction volume of 25 µL. Nonetheless, the reaction mixture included 12.5 µL of PCR master mix, 1 µL of forward primer (MAR1bB2: 5'-GCT CTA GCC GGT TAC GCG TC-3'), 1 µL of reverse primer (MAR1bB2: 5'-CTG CTT CGG AGA ATA CAC CT-3'), 5.5 µL of nuclease-free water, and 5 µL of template DNA, following the protocol defined by Bilgiç *et al*. (2013). The amplification of *B. bigemina* was achieved yielding 278 bp product using forward (5' CAT CTA ATT TCT CTC CAT ACC CCT CC-3') and reverse (5'-CCT CGG CTT CAA CTC TGA TGC CAA AG-3') primers (Figueroa *et al*., 1992). The PCR reactions were conducted in a 25 µL volume containing Taq-buffer 5ul (10X), 4 µL $MgCl_2$, 2 µL (2mM) dNTPs, 1 µL (100 nmol) Taq DNA polymerase, 2 µL pmol of each primer, 5 µL of template DNA and nuclease free water. Initial denaturation was conducted at 94°C for five minutes followed by 34 cycles of denaturation at 94°C for one minute. Annealing was carried out at 57°C for one minute, and then extension at 72°C (one minute). Further, the final step of extension was accomplished at 72°C for 10 minutes. The positive and negative (nuclease free water) controls were added during the tests. The PCR was

completed using a T100 thermal cycler (Bio-Rad, USA). The resultant PCR products were resolved on an agarose gel prepared in 1 X TAE buffer and electrophoresed at 90 V for 30 minutes. Moreover, the DNA bands were observed under UV illuminator.

Statistical analysis: The difference in transmission rates were analyzed using independent t-test utilizing the statistical software SPSS-21. The *p*-value <0.05 was considered as significant. This would be the indication of significant difference between groups.

RESULTS

Acquisition feeding and tick infectivity: Three calves having natural infection without the treatment history were used for acquisition feeding. A total of 900 ticks were collected from vegetation. Out of these, 451 and 344 adult ticks were identified as *H. anatolicum anatolicum* and *R. microplus*, respectively (Figure 4; A-F). The collected ticks were assigned into three groups for acquisition feeding of the respective pathogen i.e., *A. marginale*, *B. bigemina,* and *T. annulata*. To confirm the acquisition fed ticks, about 10% of these ticks were subjected to molecular detection of pathogens. The infection percentage of *R. microplus* ticks for *A. marginale* and *B. bigemina* was 34% and 27%, respectively. While, the tick infection percentage of *H. anatolicum anatolicum* ticks for *T. annulata* was 29%.

Transmission feeding: During biological transmission trials, about 92%, 85%, and 87% *R. microplus* and *H. anatolicum anatolicum* ticks salivary glands were infected with *A. marginale*, *B. bigemina* and *T. annulata*, respectively during transmission feeding. For evaluation of the final transmission trial, a total of 15 healthy crossbred calves (8-10 months) were recruited. Thirty positive ticks harboring the respective pathogen (*A. marginale* or *B. bigemina* or *T. annulata*) were placed on the ear (covered with cloth) of each calf. All five calves infested with *R. microplus* ticks transmitted *A. marginale* and *B. bigemina* infections. Whereas, calves (n=5) infested with *H. anatolicum anatolicum* transmitted *T. annulata* infection in 4 out of 5 calves (Table 1).

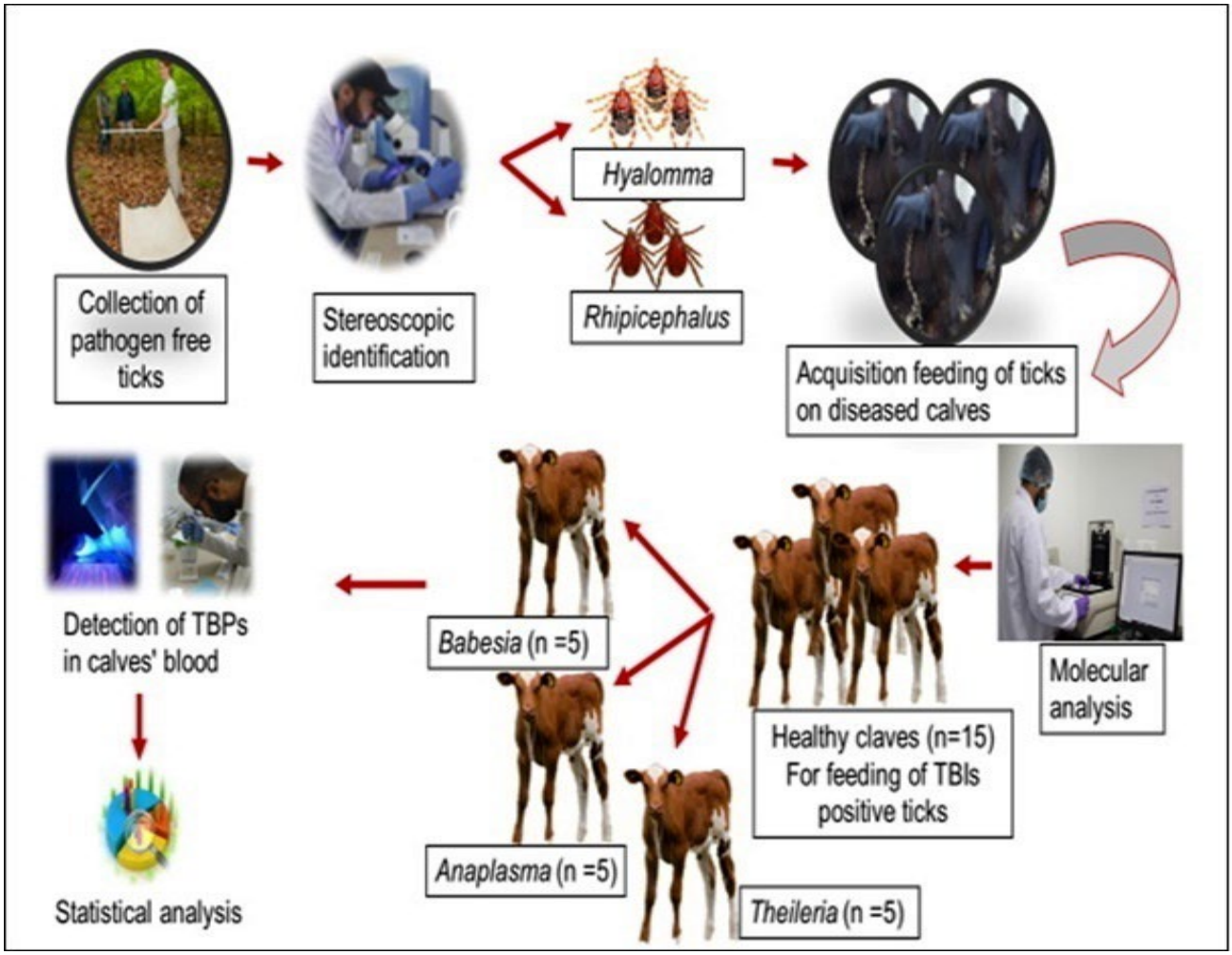

Figure 1. Experiment layout for biological transmission.

Figure 2. A. Tick collected following 7 days of incubation; B. C. Attaching ticks on ears for feeding; D. E. Covering the ear with cloth to prevent attachment of new ticks and falling of desired ticks; F. Rearing of experimental calves.

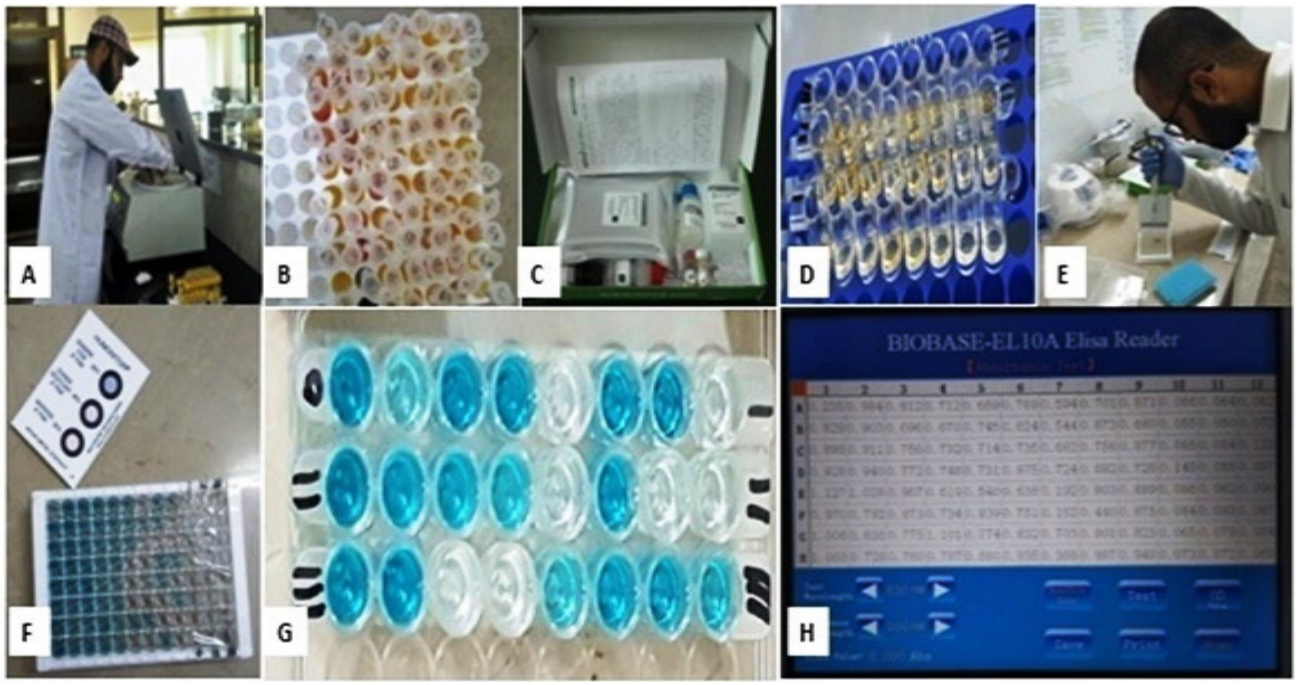

Figure 3. A. Serum extraction from blood samples of dams through centrifugation; B. Extracted serum samples; C. cELISA kit with its complete reagents and ELISA plates; D. Antigen coated ELSA plates loaded with serum samples; E. Performing the procedure of ELISA, step wise as mentioned by the manufacturer; F. G. Color difference on ELISA plate after addition of stop solution H. Results of ELISA which were observed at absorbance of 630 nm wavelength in 'Bio-Base-EL 10A, ELISA Reader'.

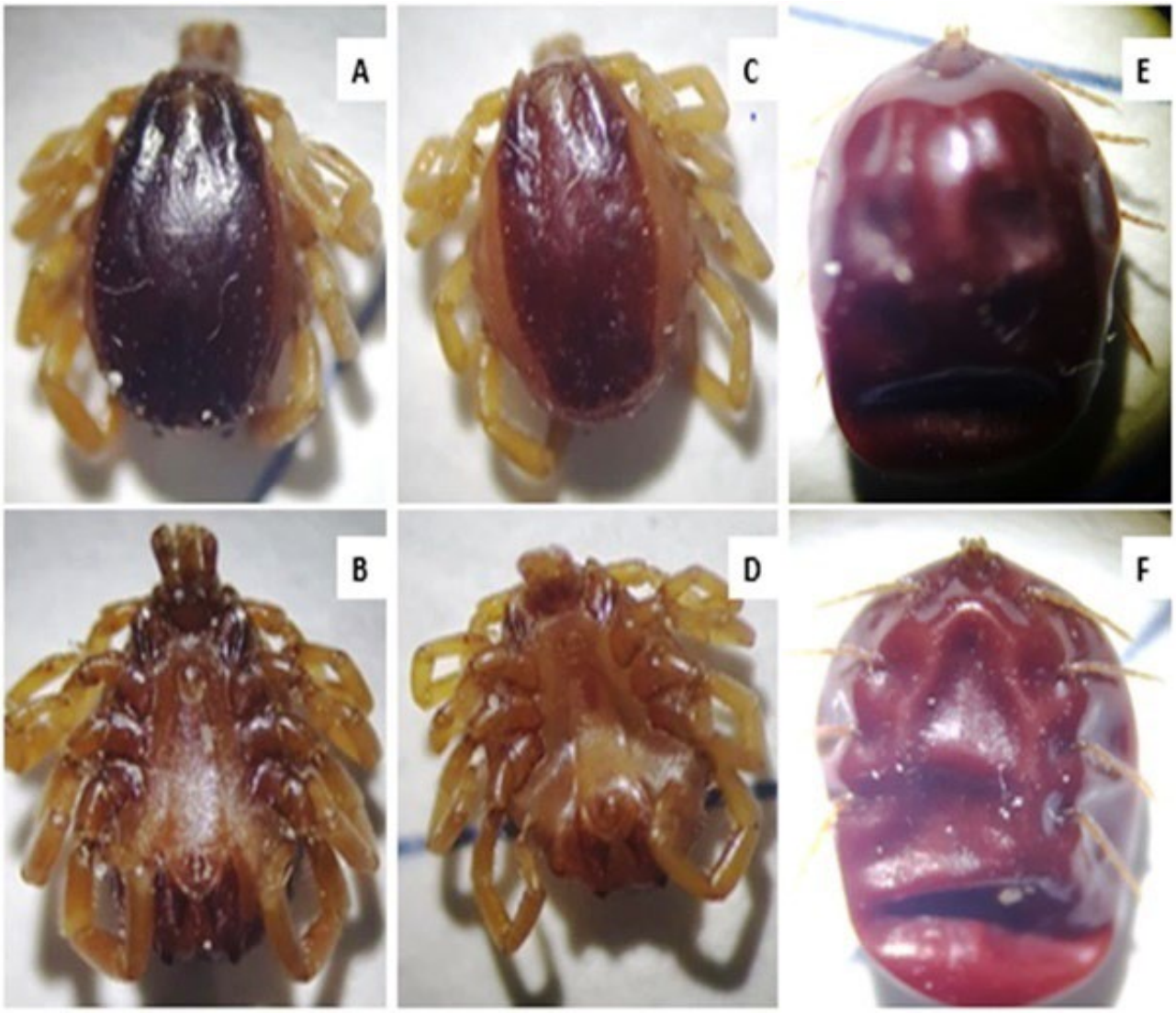

Figure 4. A. B. *H. anatolicum* dorsal and ventral view; C. D. *R. microplus male* dorsal and ventral view; E. F. *R. microplus* female dorsal and ventral view.

Table 1. Details of biological transmission of TBPs.

Details	*H. anatolicum anatolicum*					*R. microplus*				
	Group-1 (*T. annulata*)					Group-2 (*A. marginale*)				
Calf tag number	48A	34A	23A	41A	19B	18A	16A	09 B	84A	76A
Number of infested ticks	30	30	30	30	30	30	30	30	30	30
Calf positivity for TBDs*	+	+	-	+	+	+	+	+	+	+
Total positivity										

*Tick-borne diseases; TBPs = Tick-borne pathogens; *p*-value > 0.05

DISCUSSION

Tick-borne diseases are one of the limiting factors for animal health and production. Climate change and global warming is causing an alarming increase in vectors and vector-borne diseases. Limited studies have been conducted regarding vector competence during biological transmission trials. As far as we can ascertain, no controlled experimental studies have been published from Pakistan. Identification of competent vectors and transmission attributes are important from a disease control standpoint. Transmission of anaplasmosis occurs by various routes including biological vectors (ticks), mechanical vectors (Tabanus spp., biting flies,

mosquitoes, and lice), contaminated inanimate objects (needles, farm instruments etc.) and intrauterine route from placenta of mother to fetus (Aubry and Geale, 2011; Nazar *et al*., 2018; Atif *et al*., 2021; Heller *et al*., 2025). Intrauterine transmission of anaplasmosis takes place mainly during the 2nd and 3rd trimesters of gestation (Ribeiro *et al*., 1995; Grau *et al*., 2013) and subsequently death of neonatal calves may encounter (Pypers *et al*., 2011; Santarosa *et al*., 2013).

Blood smear examination is an inexpensive and extensively used technique for the recognition of hemo-parasites. The limitation associated with this technique, it has lower sensitivity and specificity than serological and nucleic acid detection assays (Sanchez-Vicente and Tokarz 2023; Anna *et al*., 2025). In Pakistan, Anaplasma was first described from (Karachi) Pakistan by Haider and Bilqees in 1988. They utilized blood smear microscopy for the demonstration of hemo-parasites and described a positivity rate of 61% (Haider and Bilqees, 1988). Competitive ELISA has 95% specificity and 96% sensitivity for serological recognition of *Anaplasma* (Torioni de Echaide *et al*., 1998; Ierardi, 2025; Gattan *et al*., 2025). This identifies the specific MSP-5 antibodies against *Anaplasma* infection.

Ticks and Tick-borne diseases are endemic in Pakistan. Various tick species have been implicated as potential vectors. During previous studies, tick-borne pathogens have been detected in *H. anatolicum anatolicum,* and *R. microplus* ticks collected from infested animals (Moumouni *et al.*, 2018; Rehman *et al*., 2019; Perveen *et al*., 2021; Makenov *et al*., 2021; Rooman *et al*., 2021; Bilal *et al*., 2025). These tick species depend on warmer temperature and humidity (Nuttall, 2022); therefore, considered as putative vectors but the scientific validation of their biological transmission requires confirmation. During biological transmission trials, it has been observed that about 92%, 85%, and 87% *R. microplus* and *H. anatolicum anatolicum* ticks salivary glands were infected with *A. marginale*, *T. annulata,* and *B. bigemina,* respectively at the time of transmission feeding. The colonization of pathogens in the salivary glands is an important factor in disease transmission (Šimo *et al*., 2017). For *A. marginale* biological transmission study, male *R. microplus* ticks were used because they have more efficient feeding pattern than their nymphs or adult females (Löhr *et al*., 2002). It is worth mentioning that *R. microplus* preferentially feeds on cattle and has greater vector potential for *A. marginale* than other ixodid ticks (Piloto-Sardiñas *et al*., 2024). The development and replication of infection inside the salivary glands initiated by tick blood feeding are the major factors in tick feeding (Löhr *et al*., 2002).

For *T. annulata* transmission study, female *H. anatolicum anatolicum* ticks were selected, owing to their higher disease transmission potential with positive acini in salivary glands than males (Sangwan *et al*., 1989). For evaluation of the final transmission trials, a total of 15 healthy crossbred calves (8-10 months) were enrolled. All calves infested with *R. microplus* transmitted *A. marginale* (100%) and *B. bigemina* (100%) infections. Whereas, calves (n=5) infested with *H. anatolicum anatolicum* transmitted *T. annulata* infection in 4 (80%) out of 5 calves.

For *B. bigemina* transmission trials, the adult *R. microplus* plays a vital role in disease transmission, while *B. bovis* transmission is mainly supported by larvae (Gray *et al*., 2019). Our findings are in accordance with the results of Rehman *et al*. (2019), they depicted *Hyalomma* to be the primary vector of *T. annulata,* while *Rhipicephalus* as the leading vector in the dispersion of *Anaplasma* and *Babesia,* respectively. On the contrary, the findings of Ruiz *et al*. (2005) mentioned that *A. marginale* lacked infectivity for Brazilian isolates. Nevertheless, most of the earlier reports have mentioned the vector potential of *R. microplus* for *A. marginale* (Zivkovic *et al*., 2007). In the present study, 100% vector competency of *R. microplus* for *B. bigemina* is supported by Chauvin *et al*. (2009) and Gray *et al*. (2019). They depicted the potential role of *R. microplus* for the transmission of *B. bigemina*. Moreover, our findings were in agreement with Rehman *et al*. (2019) and Gharbi *et al*. (2020), who described *H. anatolicum anatolicum* as the main vector for *T. annulata* transmission in cattle. Nonetheless, Rehman *et al*. (2019) they did not perform biological transmission studies rather detected tick-borne pathogens in ixodid ticks. One calf out of five infested with *H. anatolicum anatolicum* ticks did not acquire infection. The variations in vector competency may be due to wrong identification of vectors, diverse pathogenic strain, and overreliance on molecular techniques (Gray *et al*., 2019). Various researchers have supported the vector competency of *R. microplus* for *A. marginale* (Futse *et al.*, 2003; Pérez *et al*., 2025), *B. bigemina* (Oliveira *et al*., 2008; Antunes *et al*., 2015; Martins *et al*., 2022) and *H. anatolicum anatolicum* for *T. annulata* (Rashid *et al*., 2009; Gharbi *et al*., 2020).

The development of acaricide resistance to multiple acaricides is a worldwide issue. As we know that *R. microplus* is a one host tick that concludes his lifespan in a single host and develops acaricidal resistance at a greater rate than three host ticks such as *H. anatolicum anatolicum* (Rodríguez-Vivas *et al*., 2014; Shyma *et al*., 2021; Ullah *et al*., 2025). The resistance scenario is a serious risk that can change the vector distribution and disease dynamics.

Conclusions: The current study concludes that ticks are the foremost vectors for the dissemination of *Babesia*, *Theileria*, and *Anaplasma* in cattle populations. The *H. anatolicum anatolicum* is the principal vector for *T. annulata* and *R. microplus* is the primary vector for

transmission of *Babesia* and *Anaplasma* species. The difference in the disease transmission rates is likely due to the release of tick-borne pathogens during transmission feeding as well as tick-infection rates. The identification of vectors and transmission dynamics would help in prevention and control as well as minimize the economic losses incurred due to tick-borne diseases.

Author contributions: FAA, KH conceived the idea prepared the initial draft and designed the study and FAA supervised the research. KH, UI, TR, MHJ and AT carried out the analysis, sample collection and experimental designs and prepared the manuscript. All other authors contributed for interpretation, review, editing and proof reading.

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