



RESEARCH ARTICLE

Molecular Surveillance and Risk Factors of *Anaplasma marginale* in Pregnant Cows in Pakistan with The Evidence of Transplacental Transmission in Neonatal Calves

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ARTICLE HISTORY (25-700)

Received: July 19, 2025
Revised: October 07, 2025
Accepted: October 09, 2025
Published online: October 16, 2025

Key words:

Anaplasma marginale
Neonatal dairy calves
Pakistan
Punjab
Transplacental transmission

ABSTRACT

Anaplasmosis is an important tick-transmitted bacterial disease worldwide. The knowledge regarding transplacental transmission of anaplasmosis is lacking in Pakistan. The aim of the current study was to evaluate the prevalence, risk factors and to assess the transplacental transmission rate of *A. marginale* in pregnant dairy cows to their neonatal calves from the Sheikhupura and Chakwal districts of Punjab, Pakistan. A total of 796 blood samples were collected from pregnant cows and their neonatal calves (n=72). The pregnant cows were sampled at 60–90 days of gestation and subsequently just after parturition along with their neonatal calves for the detection of *A. marginale* before colostrum feeding using the *msp1b* gene-based PCR. The overall transplacental transmission rate in calves was 12.50%. District-wise transplacental transmission rate was 11.90% and 13.33% in Sheikhupura and Chakwal districts, respectively. Univariate logistic regression analysis indicated that season and area were significant risk factors ($P < 0.05$; $OR > 1$). However, multivariate logistic regression revealed that area was a significant risk factor. The sequencing and phylogenetic insights of representative isolates (OR854453 and OR738301) detected novel strains of *A. marginale* with 96–100% similarity with other countries. The *msp1b* gene-based phylogenetic tree uncovered that current isolates expressed homology with isolates from Pakistan, India, and Egypt. We can conclude that the intrauterine route of transmission should be considered while devising the prevention and control strategies for anaplasmosis as well as to prevent a significant number of neonatal mortalities.

To Cite This Article: Atif FA, Shujait MA, Haque SE and Khan I, 2025. Molecular surveillance and risk factors of *Anaplasma marginale* in pregnant cows in Pakistan with the evidence of transplacental transmission in neonatal calves. Pak Vet J. <http://dx.doi.org/10.29261/pakvetj/2025.279>

INTRODUCTION

Tick-borne diseases cause huge economic losses, and globally 80% of the cattle population is at risk of ticks and tick-borne diseases (Hussain *et al.*, 2021). Anaplasmosis, babesiosis, and theileriosis are the major tick-borne diseases causing constraints on animal health and production (Atif, 2015; Jabbar *et al.*, 2015; Atif *et al.*, 2024). Bovine anaplasmosis, a bacterial disease caused by *Anaplasma (A.) marginale* (Rickettsiales: Anaplasmataceae), is widely distributed in tropical and subtropical areas (Atif, 2016; Lerardi *et al.*, 2025).

Infected animals express pyrexia, anemia, and

weight loss; reduced milk production, retarded growth, body scores, abortion, infertility, and death (Bahia *et al.*, 2021). The incubation period of *A. marginale* ranges from 7 to 60 days. Nearly 10% to 90% of the RBCs are infected in adverse conditions of the diseases. Mostly, the cattle of all ages were infected, but the intensity of the disease depends on the age of the cattle. The young calves between two years of age suffer in acute and severe form, with mortality rates ranging from 29 to 49% (Nazar *et al.*, 2018).

Transplacental, biological, mechanical, and iatrogenic are the major routes of transmission of disease (Bahia *et al.* 2021). Transplacental transmission from

dam to neonatal cow-calf is one of the important transmission attributes of *A. marginale* (Atif *et al.*, 2021). The different studies from various scholars have depicted transplacental transmission posing huge economic consequences (Silva *et al.*, 2015; Nazar *et al.*, 2018; Henker *et al.*, 2020; Atif *et al.*, 2021 Garcia *et al.*, 2022). Earlier researchers, Potgieter and Rensburg (1987), reported a 15.6% transplacental transmission rate in *Anaplasma*-infected calves maintained under laboratory conditions in South Africa using a rapid card agglutination test. Furthermore, Salabarria and Pino (1988) from Cuba documented a higher transmission rate of 86.4% in the last month of gestation during clinical anaplasmosis. Similarly, Meldonado *et al.* (2012) demonstrated vertical transmission (20.69%) of anaplasmosis in the neonatal Brahman calves from Yaracuy State, Venezuela, using nested *msp5*-PCR and competitive ELISA (cELISA). Likewise, Pohl (2013) mentioned the prevalence of anaplasmosis (70.21%) and depicted the intrauterine transmission rate (12.5%). For instance, Grau *et al.* (2013) reported 10.5% transplacental transmission in the Braford calves from Pelotas, Brazil, using PCR. Besides, Costa *et al.* (2016) reported 25% prevalence of vertical transmission in calves. In addition, Silvestre and co-researchers reported a 10% intrauterine transmission in male Holstein calves from Minas Gerais, Brazil, using nested *msp4*-PCR (Silvestre *et al.*, 2016). Moreover, Žilić and colleagues detected *A. marginale*, *A. ovis*, *A. phagocytophilum* and other blood parasites in ruminant fetuses from Croatia using PCR; they concluded that hemoparasites might have contributed to the transplacental transmission and abortion in ruminants (Žilić *et al.*, 2025).

The transplacental transmission of *A. marginale* chiefly occurs in pregnancies ranging from the second to the third trimesters (Costa *et al.*, 2016). Additionally, Bahia *et al.* (2021) stated that chronic infection of cows can potentially transmit disease to offspring via transplacental route even though the cow never got an acute infection of anaplasmosis. The epidemiology of anaplasmosis in pregnant dairy cows, associated risk factors, and information about transplacental transmission during large-scale surveillance studies are lacking in Punjab, Pakistan. Keeping in view the importance of tick-borne diseases, the present study was designed to determine the potential of transplacental transmission of *A. marginale* from dam to neonatal cow-calves.

MATERIALS AND METHODS

Area of study and sample collection: The present study was conducted from December 2020 to November 2022 in the Sheikhupura and Chakwal districts of Punjab, Pakistan (Fig. 1). The asymptomatic pregnant cows ($n=796$) were sampled during the 60–90th day of gestation, assuming 50% prevalence, and follow-up sampling from *Anaplasma* carrier cows just after parturition ($n=83$) and subsequently from neonatal calves ($n=72$) just before colostrum feeding from PCR-positive dams. The blood samples before colostrum feeding were considered without compromising the neonatal health to avoid the possible role of milk in disease transmission. The

dams and environment were regularly sprayed with acaricide to avoid tick infestation. The pregnancy was assessed through ultrasound or rectal palpation. The cows were sampled irrespective of their breed (Holstein Friesian, Crossbred, Sahiwal, and Non-descriptive). Blood sample (5 ml) was collected aseptically from the jugular vein in properly labeled EDTA-coated tubes, stored in the icebox and transported to the Post-graduate Laboratory of Medicine, Department of Clinical Sciences, CVAS, Jhang, for further processing.

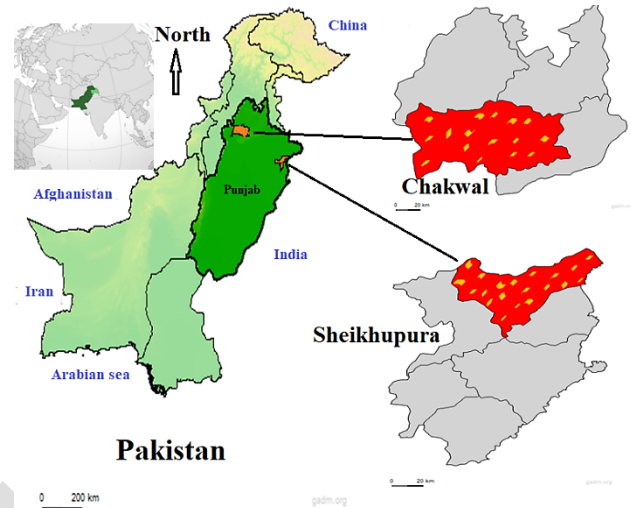


Fig. 1: Map describing study area and showing sampling sites from Sheikhupura and Chakwal districts of Punjab, Pakistan. The raw maps were retrieved from www.gadm.org. The study area is highlighted in red and sampling sites are marked in orange color.

Risk factor analysis: For the collection of data regarding risk factors, a prior-tested questionnaire was developed to gather information regarding risk factors. The data regarding the variable of the area (Sheikhupura and Chakwal), breed (Sahiwal, Holstein-Friesian, Non-descriptive, and Crossbred), reproductive experience (first lactation, second lactation, and third lactation), tick infestation load (low, moderate, and heavy), stocking density (low, medium, and high), season (winter, autumn, spring, and summer), acaricide use (regular, irregular, and never used), and grazing system (zero, semi, and free range) were collected.

PCR: The PCR was carried out for the detection of *A. marginale* by targeting the *msp1b* gene, following the procedure described by Bilgiç *et al.* (2013). In brief, the DNA was extracted from blood samples using kits following the protocol provided by Thermo Fisher Scientific, USA, following the manufacturer's instructions. To improve both the yield and quality of the extracted DNA, the elution step was repeated. The extracted DNA was either stored at -20°C or used immediately for the amplification of *msp1b* gene through PCR by using the previously published primers (Bilgiç *et al.*, 2013). The PCR was performed in a 25µL reaction mixture, which included 12.5µL of PCR Master Mix (Thermo Fisher Scientific, USA), 1µL of forward primer (MAR1bB2: 5'-GCT CTA GCC GGT TAC GCG TC-3'), and 1µL of reverse primer (MAR1bB2: 5'-CTG CTT CGG AGA ATA CAC CT-3'). Additionally, 5.5µL of nuclease-free water and 5µL of template DNA were added to complete the mixture. The PCR was

performed in a T100 thermal cycler (Bio-Rad, USA) to yield a 256 base pair product, following the conditions outlined by Bilgic *et al.* (2013). The cycling protocol included an initial denaturation at 94°C for 5min, followed by 34 cycles of denaturation at 94°C for 1min, annealing at 57°C for 1min, and extension at 72°C for 1min. A final extension step was performed at 72°C for 10min. Positive and negative (nuclease-free water) controls were included (Bilgic *et al.*, 2013). The PCR products were separated by gel electrophoresis on a 1.3% agarose gel using 1X TAE buffer. Electrophoresis was carried out at 90 volts for 30min, and DNA bands were visualized under a UV illuminator.

Sequencing and phylogenetic insights: The positive representative samples isolated from cattle were sent for nucleotide sequencing using Barcode Tagged sequencing (BTSeq™) to Celemics Inc. The amplified products were purified using a PCR purification kit (Qiagen, Germany), following the manufacturer's guidelines. The sequences were submitted to GenBank (BankIt) for accession numbers. The selected *msp1b* specie-specific sequences of *A. marginale* isolated from cattle, horses, buffalo, and donkey were employed for the creation of a phylogenetic tree and matched with isolates from diverse countries (India OR333500 and OR333501; Myanmar LC764753 and LC764754; Egypt MH796636 and MH796637; and Pakistan MK032842, MK032843, MK032846, and OQ657190) retrieved from <https://www.ncbi.nlm.nih.gov/>. The evolutionary history was assembled using the maximum likelihood method and the Tamura 3-parameter model (Tamura 1992). The evolutionary analyses were performed using bootstrap analysis with 1000 replications in MEGA11 software (Tamura *et al.*, 2021). The *Ehrlichia canis* (U26740) sequence isolated from a dog was used as an out-group for phylogenetic analysis.

Statistical analysis: The data was analyzed by univariate and multivariate regression analysis using IBM SPSS Statistics 26. The P-value less than or equal to 0.05 was considered as significant.

RESULTS

After first sampling at 60-90 days of gestation, 10.42% (83/796) pregnant cows were found positive for *A. marginale* through PCR (Fig. 2). Follow-up sampling

of these cows after parturition was 86.75% (72/83). This was the percentage of infected cows that remained infected throughout the pregnancy. Prospectively, neonatal calves (n=9) were found positive with the transplacental transmission rate of 12.50% from carrier cows (Table 1). The district-wise prevalence in relation to sampling intervals was 12.05, 89.36, and 11.90% in Sheikhpura and 8.87, 83.33, and 13.33% in Chakwal district during first, second, and third sampling intervals, respectively. The results of the current study revealed that the transplacental transmission of anaplasmosis in neonatal calves in district Chakwal [13.33% (4/30)] was higher than district Sheikhpura [11.90% (5/42)] with non-significant association based on Chi-square analysis ($\chi^2=0.327$; $P=0.86$) (Table 2).

Table 1: Prevalence of anaplasmosis in pregnant dairy cattle and transplacental transmission rate.

Sampling phases	Total samples	Positive animals	Percentage (CI)*
60-90 days of gestation	796	83	10.42 (8.30-12.54 %)
After Parturition	83	72	86.75 (79.45-94.04 %)
Transplacental transmission	72	09	12.50 (4.86-20.14 %)

* CI = Confidence interval

Table 2: District-wise prevalence of anaplasmosis in pregnant dairy cattle.

Sampling phases	Total samples	Positive animals	Percentage (CI)*
Sheikhpura			
60-90 days of gestation	390	47	12.05 (8.82-15.28 %)
After Parturition	47	42	89.36 (80.55-98.18 %)
Transplacental Transmission	42	05	11.90 (2.11-21.70 %)
Chakwal			
60-90 days of gestation	406	36	8.87 (6.10-11.63 %)
After Parturition	36	30	83.33 (71.16-95.51 %)
Transplacental Transmission	30	04	13.33 (1.17-25.50 %)

* CI = Confidence interval

Evaluation of risk factors: The overall risk factor assessment based on univariate regression analysis of both the study district revealed that area (83/796) [coefficient of regression (B)=5.98; odds ratio (OR)=393.99; probability value $P=0.00$ and confidence interval (CI)=75.49–2056.2] and season (B=3.22; OR=24.96; $P=0.00$, CI=4.94-126.08)

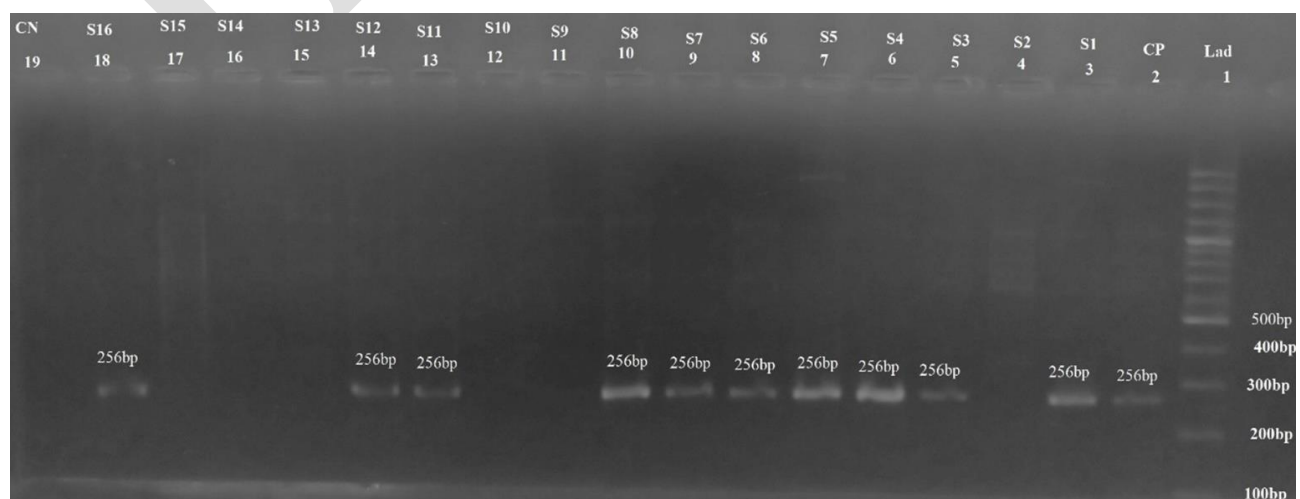


Fig. 2: The *msp1b* PCR products on 1.3% agarose gel showing amplification of *A. marginale*.

were statistically significant and at a higher risk of acquiring *A. marginale* infection with P -value <0.05 and $OR>1$ (Table 3). On the other hand, the independent variables of tick infestation and grazing system revealed non-significant association. While acaricide use, breeds, reproductive experience, and stocking density had collinearity issues and removed from analysis due to lower tolerance value (<0.1) and higher variance inflation factor (>10). Additionally, the predictors of season and area with $P<0.05$ and $OR>1$ were further processed through final multivariate analysis. Furthermore, the Hosmer and Lemeshow test, goodness of fit test model expressed a non-significant p -value ($p>0.05$), indicated the suitability of the model. Overall, the season was non-significant risk factor (Wald=3.95; $P=0.13$). It was revealed that the winter season expressed a non-significant negative coefficient ($B=-0.26$, $P=0.76$, $OR=0.04$, $CI=0.13-0.42$). The odds of the occurrence of anaplasmosis in winter were 0.76 times (24%) lesser than the reference season (summer). Whereas, autumn indicated non-significant association and positive correlation ($B=-0.63$, $P=0.10$, $OR=1.89$, $CI=0.87-4.10$). The chances of the disease in autumn are 1.89 times higher than summer. Overall, the area expressed non-significant risk factor (Wald=36.15; $P=0.00$). Chakwal district expressed a negative correlation [$B=-3.09$, $P=0.00$, $OR=0.45$, $CI=0.01-0.12$; Table 4]. The probability of the occurring of disease in Chakwal district was about 0.45 times (55%) lesser compared to the reference Sheikhpura district.

Table 3: Univariate logistic regression analysis for the evaluation of risk factors associated with anaplasmosis in pregnant dairy cattle.

Variables	B	S.E.	Wald	df	Sig.	Exp. (B)	95% C.I. for Exp.(B)	
							Lower	Upper
Tick infestation load	-8.79	1.35	42.20	1	0.00	00.00	00.00	00.00
Grazing system	-2.06	0.49	18.14	1	0.00	00.13	00.04	00.49
Season	3.22	0.83	15.16	1	0.00*	24.96	04.94	126.08
Area	5.98	0.84	50.26	1	0.00*	393.99	75.49	2056.2
Constant	-2.54	0.95	07.11	1	0.08	0.79	-	-

B= Regression coefficient; S.E. Standard error; df= degree of freedom; Sig. = Significant; Exp. (B) exponentiated value of the regression coefficient/odds ratio; C.I. = Confidence interval; * Statistically significant.

Table 4: Multivariate logistic regression analysis for the evaluation of risk factors associated with anaplasmosis in pregnant dairy cattle.

Variables	B	S.E.	Wald	df	Sig.	Exp. (B)	95% C.I. for Exp.(B)	
							Lower	Upper
Season			3.95	2	0.139			
Season (Winter)*	-0.26	0.87	0.09	1	0.76	0.76	0.13	0.42
Season (Autumn)	0.63	0.39	2.59	1	0.10	1.89	0.87	4.10
Area			36.15	2	0.00			
Area (Chakwal)	-3.09	0.53	33.41	1	0.00	0.45	0.16	0.12
Area (Sheikhpura)*	-1.72	0.34	24.91	1	0.00	0.17	0.09	0.35
Constant	-0.71	0.18	14.49	1	0.00	0.48	-	-

B = Regression coefficient; S.E. Standard error; df = degree of freedom; Sig. = Significant; Exp. (B) exponentiated value of the regression coefficient (B), it represents the odds ratio; C.I. = Confidence interval; *Reference category.

Sequencing and phylogenetic insights: The sequencing and phylogenetic insights of representative isolates in the present study (GenBank numbers: OR854453, OR738301) detected isolates of *A. marginale* with 90–100% similarity from different countries. The

representative sequences were submitted to GenBank, each from cattle and calf. We successfully got accession numbers OR738301 and OR854453O. There were four clusters in the tree. Current isolate OR854453 was included in cluster II, whereas OR738301 was present in an independent cluster. The *msp1b*-based cattle revealed the unique isolate OR738301 with higher genetic diversity, while isolate OR854453 expressed homology with isolates from Pakistan, India, and Egypt (Fig. 3).

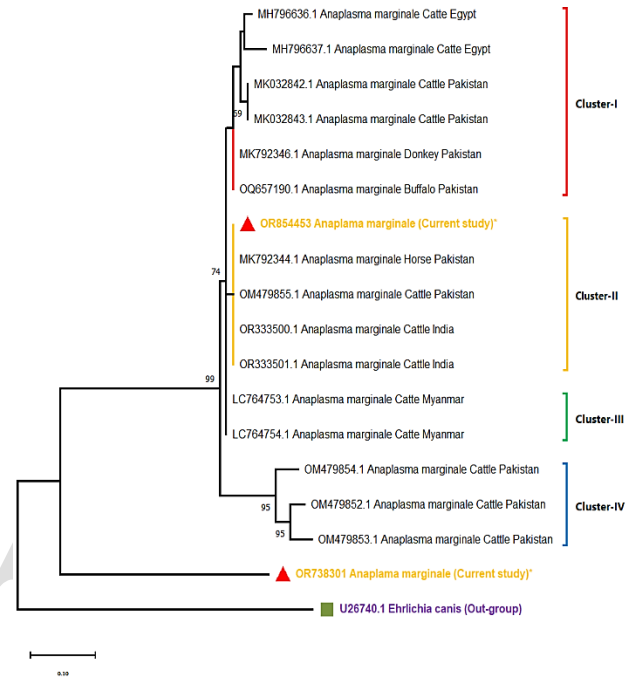


Fig. 3: Maximum likelihood based phylogenetic tree of *A. marginale* targeting *msp1b* gene with bootstrap analysis with 1000 replications using MEGA11 software. *Ehrlichia canis* (U26740) sequence was used as out-group. Our isolates are represented with red triangle in yellow color bold font with asterisks.

DISCUSSION

The ticks and tick-borne diseases are causing a serious impact on the health and production of animals. Transplacental transmission of anaplasmosis from dam to neonatal cow-calf is an important transmission attribute and poses economic consequences (Silva *et al.*, 2015; Nazar *et al.*, 2018; Henker *et al.*, 2020; Atif *et al.*, 2021). The information regarding large-scale molecular surveillance and risk factors of *A. marginale* in pregnant cows from Pakistan associated with transplacental transmission in neonatal dairy calves is lacking in Punjab, Pakistan. The current study utilized the *msp1b* gene for the detection of *A. marginale*, this gene is highly sensitive and specific as well as a conserved region within isolates, making it a reliable target using PCR. Our isolates expressed 100% similarity between cow and calf isolates. While nucleotide blast showed that cattle isolate OR738301 showed 97.6% similarity to Pakistani and isolates from other countries. Whereas OR854453 indicated 96.34% homology with *A. marginale* based on the *msp1b* gene and had higher similarity with other Pakistani isolates collected from cattle, donkey, buffalo and horse. However, the real time PCR (qPCR) is a quantitative assay that determine the degree of *A. marginale* infection (Camacho-Nuez *et al.*,

2000; Carelli *et al.*, 2007). Nevertheless, the qPCR requires expertise, higher equipment and reagent cost. Whereas, the multiplex PCR requires that the pathogens should have the same annealing temperature, with the limitation of interfering substances, and variation in GC contents; amplification of individual targets can display lower efficacy compared to singleplex PCR (Markoulatos *et al.*, 2002; Buchan *et al.*, 2019). The blood samples were taken before colostrum feeding as early as possible without compromising the neonatal health.

To date, only one report from Khyber Pakhtunkhwa (KPK), Pakistan, has documented vertical transmission of *A. marginale* in cattle at 13.7% (Nazar *et al.*, 2018). Furthermore, this study reported prevalence rates of 45.83% and 34.3% using qPCR targeting the *msp1a* gene of *A. marginale* and iELISA, respectively. However, they did not provide crucial details such as the sources of the samples, the disease status of the dams at parturition, the breeds of both the newborn calves and their mothers, as well as whether the neonates had ingested colostrum prior to blood sampling. These factors were essential for confirming transplacental transmission. However, the study of Atif and colleagues (Atif *et al.*, 2021) conducted study on a single dairy farm with a small sample size. In this farm, most of the dairy cows were carriers of the infection. The results of the present study showed that 28% (9 out of 32) and 13% (4 out of 32) of the neonatal calves had intrauterine infections, as confirmed by cELISA and PCR, respectively (Atif *et al.*, 2021). Furthermore, the difference in gene targets and study location might account for variable results.

In the current study, 83 (10.42%) cows were found positive for anaplasmosis. The positive cows were followed up for second sampling after parturition, and the disease positivity was 86.75% (72/83). It was noticed that 11 cows who were found positive during gestation revealed negative at post-partum. The cows might test negative due to lower parasitemia during persistent infection or treated during gestation after initial screening. As we know that bacteremia levels drop drastically between cyclic peaks, making detection challenging using standard PCR (Torioni de Echaide *et al.*, 1998). Our study is supported by Pohl (2013), who found the prevalence of anaplasmosis as 70.21% (66/94), which is somewhat lower than 86.75%. The difference may be due to the incidence of disease in the selected area and the diagnostic test employed. The overall transplacental transmission rate was 12.5% (9/72). Our results were supported by Nazar *et al.* (2018); they mentioned a transplacental transmission rate of anaplasmosis from dam to calves as 13.7%. The variation may remain due to demographic changes, sample size, and season. Nonetheless, Atif *et al.* (2021) found transplacental transmission at 13%, which is lower than 13.7%, as depicted by Nazar *et al.* (2018). The referred study was conducted at a dairy farm with no variation of breed (Nazar *et al.*, 2018). However, it can be said that the attachment to a farm and breed may be the reason for higher transplacental transmissions. Costa *et al.* (2016) depicted a 25% (15/60) prevalence of transplacental transmission in calves. The difference in results may be due to change in the study area, sampling strategy, breed variation, and season.

Similarly, Silvestre and co-researchers reported a

10% vertical transmission rate in male Holstein calves from Minas Gerais, Brazil, with a lower detection rate using nested *msp4*-PCR, possibly reflecting differences in regional tick control measures and management practices (Silvestre *et al.*, 2016). Likewise, Potgieter and Rensburg (1987) found a 15.6% transplacental transmission rate in *Anaplasma*-infected calves maintained under laboratory conditions in South Africa, using a rapid card agglutination test. On the other hand, Costa and colleagues observed a higher transplacental rate in crossbred neonatal calves using nested PCR (Costa *et al.*, 2016). Furthermore, Silva *et al.* (2015) reported a 41% vertical transmission rate in Rio de Janeiro, while Salabarria and Pino (1988) from Cuba documented an even higher rate of 86.4% (32/37) during the last month of gestation under clinical anaplasmosis conditions.

The district-wise transplacental transmission rate was higher in the Chakwal than in the Sheikhpura district. It is evident that district Chakwal is more prone to anaplasmosis than Sheikhpura. The district-wise comparison of transmission is also supportive to the scientific study of Atif *et al.* (2021); they described a 13% prevalence of anaplasmosis in cows. The alterations in the climatic conditions of the various agro-ecological zones have an effect on the bionomics of tick vectors, which promotes tick fecundity, dispersal, abundance, and transmission of tick-borne diseases that ultimately have an impact on the prevalence rate of tick-borne diseases in the research area (Perfilyeva *et al.*, 2020). The area factor in univariate analysis had significant effect with higher odds ratio but due to wider confidence interval, this value is statistically less reliable compared to less wider confidence interval. But in practical situation the area had an impact on the disease occurrence.

It was noted that free-grazing animals have a higher prevalence than stall-fed animals; this variable was a significant determinant. This activity resulted in higher interaction with tick-infested animals and allowed ticks to spread more easily, leading to an increase in *A. marginale* infection. However, cattle housed in stalls and kept in farms or fences rather than being free to wander have lower chances of tick exposure. Tick-infested animals, plants, and grasses in grazing fields cause the ticks to ambush and wait for a vertebrate host for attachment. Our findings are contradictory to Ullah *et al.* (2021), who mentioned that free-grazing animals were less prevalent to anaplasmosis. The therapeutics and clean pastures could have reduced the chance of infection.

In the current study the prevalence was noted highest during summer, followed by spring, autumn, and the lowest in winter. Our study is coherent with the findings of Atif *et al.* (2022) and Akhtar *et al.* (2019), while disagreeing with the results of Asif *et al.* (2022). In their study, prevalence was noted to be highest during autumn, followed by winter, summer and spring.

Different diagnostic methods with varying sensitivity levels can yield different estimates of vertical transmission rates. For instance, Grau *et al.* (2013) reported a higher seropositivity rate (100%) using the indirect fluorescent antibody test (IFAT), compared to 97% using the indirect ELISA assay. In their study, PCR-based detection revealed a 10.5% transplacental transmission rate in Braford calves from Pelotas, Brazil.

Meldonado *et al.* (2012) demonstrated transplacental transmission of anaplasmosis in the neonatal Brahman calves (20.69%; 6/29) from Yaracuy State, Venezuela, using nested *msp5*-PCR and competitive ELISA (cELISA) from the infected mother to the fetus during pregnancy. Additionally, they noted that none of the calves tested positive for anaplasmosis via ELISA, while 10% were positive with IFAT. In contrast, Atif *et al.* (2021) depicted a higher transmission rate of 28% using cELISA in their dairy farm-based study from Pakistan, likely due to a greater number of carrier animals.

These variations in transmission rates may stem from differences in diagnostic techniques, genetic diversity, regional agro-climatic conditions, and host or vector dynamics (Costa *et al.*, 2016; Žilić *et al.*, 2025). In addition, Žilić and colleagues detected *A. marginale*, *A. ovis*, *A. phagocytophilum* and other blood parasites in ruminant fetuses from Croatia using PCR, they concluded that hemoparasites might have contributed to transplacental transmission and abortion in ruminants (Žilić *et al.*, 2025). Our findings suggest that lower transmission rates may also relate to dam susceptibility and local environmental factors compared to those in district Jhang, Pakistan. This aligns with observations by Pohl (2013), who emphasized that vertical transmission in cattle is often linked to persistent infections within a population. The timing of fetal infection also plays a crucial role, with higher transmission likelihood observed later in gestation.

Conclusions: We can conclude that intrauterine is an important route of transmission for *A. marginale*. This route should be considered for devising the prevention and control strategies to prevent a significant number of neonatal mortalities. Furthermore, it is suggested that the surveillance should be expanded to additional agro-climatic regions in future research.

Acknowledgements: The authors thanked the Pakistan Science Foundation, Project No. PSF/NSLP/P-UVAS (697) and Higher Education Commission of Pakistan's project No. 9041/Punjab/ NRPU/R&D/HEC/2017 for the provision of technical support and equipments for this study.

Author's contribution: FAA played its role in conceptualization, writing—original draft, methodology, writing—review & editing, supervision and provision of resources. MAS involved in writing—original draft, data curation, methodology, formal analysis, investigation and provision of resources. S E-ul-H contributed in writing—review and editing, provision of resources and supervision. IK played his role in writing—review, editing and provision of resources.

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