Research Article

Seroepidemiological Investigation of Bovine Anaplasmosis and Its Associated Risk Factors in the Southern Districts of Khyber Pakhtunkhwa, Pakistan

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Abstract | Bovine anaplasmosis, caused by tick-transmitted haemoparasitic bacteria of the genus *Anaplasma*, is a major constraint on cattle productivity in the semi-arid southern belt of Khyber Pakhtunkhwa (KP), Pakistan. A cross-sectional study was conducted on 600 cattle sampled from seven southern KP districts (Kohat, Karak, Hangu, Bannu, Lakki Marwat, Tank, and Dera Ismail Khan) to estimate the seroprevalence of anti-*Anaplasma* antibodies by a commercial cELISA and to identify associated factors using the chi-square test and logistic regression. The overall seroprevalence was 10.83% (65/600; 95% CI 8.59–13.57). District-level seroprevalence ranged from 5.88% in Tank to 17.65% in Bannu, but the overall difference among districts was not significant ($p = 0.214$). Exotic and crossbred cattle had a higher seroprevalence (17.50%, 35/200) than local breeds (7.50%, 30/400; OR = 2.62, 95% CI 1.55–4.41; $p < 0.001$). Tick infestation (OR = 4.27, 95% CI 2.19–8.35), irregular or no acaricide use (OR = 2.61, 95% CI 1.43–4.77) and female sex (OR = 2.06, 95% CI 1.05–4.04) were significantly associated with seropositivity in univariable analysis; seroprevalence was lowest in winter (5.19%) and tended to be higher in herds of more than 10 animals (15.38%). Seropositivity indicates exposure to *Anaplasma* and does not distinguish current from past infection. These findings indicate that anaplasmosis is endemic in the southern KP livestock belt and support season- and breed-targeted tick control with regular acaricide use.

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Introduction

Anaplasmosis is a tick-borne haemolytic disease of cattle and other ruminants caused by Gram-negative, obligate intracellular bacteria of the genus *Anaplasma*, most notably *Anaplasma marginale*, which infects mature erythrocytes and produces progressive anaemia, fever, weight loss, reduced milk yield, abortion, and, in severe cases, mortality (Aubry and Geale, 2011). The disease is transmitted principally by ixodid ticks, and to a lesser extent mechanically via biting flies and contaminated instruments, and

imposes substantial economic losses on livestock-dependent economies through veterinary costs, productivity decline, and trade restrictions (Salinas-Estrella *et al.*, 2022).

In Pakistan, where the livestock sector underpins the livelihoods of millions of rural households, anaplasmosis is recognised as endemic, with reported seroprevalence ranging from 9% to over 19% across different provinces and diagnostic approaches (Atif *et al.*, 2013; Farooqi *et al.*, 2018; Zafar *et al.*, 2022). Khyber Pakhtunkhwa (KP) province, and particularly its southern districts (Kohat, Karak, Hangu, Bannu, Lakki Marwat, Tank, and Dera Ismail Khan), presents an ecologically favourable environment for tick proliferation owing to its semi-arid climate, extensive communal grazing practices, and mixed local–exotic cattle husbandry systems (Khan *et al.*, 2019; Badshah *et al.*, 2023). Prior work specifically in the Bannu and Lakki Marwat districts reported an overall anaplasmosis prevalence of 19.79%, with breed, age, and month identified as significant biotic risk factors (Badshah *et al.*, 2023).

The southern KP cattle population comprises a mix of indigenous breeds adapted to arid, hilly terrain-such as Achai, Lohani, and Bhagnari-alongside exotic dairy breeds (Holstein Friesian, Jersey) and their crossbreds introduced through government and private dairy development programmes (Jamil *et al.*, 2023). Differential breed susceptibility to *Anaplasma* infection has been reported elsewhere in Pakistan, with exotic and crossbred cattle generally showing higher seroprevalence than hardy local breeds, attributable to differences in innate resistance, management intensity, and tick exposure (Shaukat *et al.*, 2019).

Despite the growing body of molecular and serological evidence on bovine anaplasmosis in KP (Farooqi *et al.*, 2018; Badshah *et al.*, 2023; Jamil et al., 2023), existing studies from the southern districts have either been restricted to symptomatic animals (Khan *et al.*, 2019) or to two districts (Badshah *et al.*, 2023). To our knowledge, large-scale serological data that compare all seven southern districts, local versus exotic/crossbred breeds, and tick-related management practices (tick infestation and acaricide use) within a single sampling framework are lacking. Such information is needed to decide where and in which cattle tick-control effort should be concentrated. The present study therefore aimed to (i) estimate the seroprevalence of anti-*Anaplasma* antibodies in cattle across seven southern KP districts using a commercial indirect ELISA, (ii) compare seroprevalence among districts and between local and exotic/crossbred breeds, and (iii) identify demographic, environmental and management factors associated with seropositivity using univariable and multivariable analysis.

Materials and Methods

Study area and design

A cross-sectional seroepidemiological study was conducted across seven southern districts of Khyber Pakhtunkhwa, Pakistan: Kohat, Karak, Hangu, Bannu, Lakki Marwat, Tank, and Dera Ismail Khan. These districts share a semi-arid to arid climate, hilly and plain terrain, and a predominantly agro-pastoral cattle-rearing system, making them ecologically representative of the tick-endemic southern KP belt (Khan *et al.*, 2019). Sampling was carried out between January, 2025 to December, 2025.

Sampling framework, sample size and sample collection

A total of 600 cattle blood samples (approximately 85–90 per district) were collected irrespective of breed from local farms and communal grazing herds. Animals were sampled from multiple local farms and communal grazing herds across the study districts. Within each district, herds were selected from locally accessible farms and communal grazing areas in consultation with local livestock owners and veterinary personnel, and animals within selected herds were selected based on availability and willingness of owners to permit sampling. Because no animal-level sampling frame was available, the sample is regarded as a convenience-based multistage sample rather than a simple random sample. The minimum sample size required to estimate an overall prevalence of 15% with 5% absolute precision at 95% confidence is 196 animals ($n = 1.96^2 \cdot P(1-P)/d^2$); a larger sample (600; approximately 85–90 per district) was collected to allow district- and breed-level comparisons. At the observed prevalence, the 95% confidence interval half-width for the overall estimate is about 2.5 percentage points. Blood (5 mL) was collected aseptically from the jugular vein into plain vacutainers. Serum was separated by centrifugation (3000 rpm, 10 min) and stored at −20 °C until ELISA testing. Only serum was analysed in the present study.

At the time of sampling, a structured questionnaire was administered to the owners to record the following variables: season of sampling (summer, autumn, winter, spring; defined by month of sampling), district, breed (local: Achai, Lohani, Bhagnari; exotic/crossbred: Holstein Friesian, Jersey, crossbred), herd size (number of cattle in the herd: 1–3, 4–7, 8–10, >10), age (calf, <12 months; 12–24 months; adult, >24 months), sex, tick infestation status (binary: ticks visible on the sampled animal or its premises at the time of sampling versus none observed), and acaricide/tick-control practice (regular scheduled application versus irregular or no application, as reported by the owner).

Serological test (cELISA)

Serum samples were screened for antibodies against *Anaplasma* using a commercial competitive enzyme-linked immunosorbent assay (cELISA), the *Anaplasma* Antibody Test Kit, cELISA v2 (SKU 283-2; Veterinary Medical Research and Development [VMRD], USA), following the manufacturer's instructions. The assay is designed for detection of antibodies specific to *Anaplasma* in bovine serum and is based on competition between antibodies present in the test serum and an HRP-labelled monoclonal antibody for binding to *Anaplasma* antigen coated on the microplate wells. Serum samples were processed according to the manufacturer's recommended protocol, with the positive and negative control sera supplied with the kit included on each assay plate. Following incubation and washing steps, the bound HRP-labelled conjugate was detected using the kit substrate, and the resulting colour development was measured using a microplate reader. The assay was considered valid when the control values met the manufacturer's acceptance criteria. Samples were classified as positive or negative according to the manufacturer's specified cELISA cut-off. The manufacturer's reported diagnostic sensitivity and specificity for the kit are 100.0% and 99.7%, respectively.

Statistical analysis

Data were entered in Microsoft Excel and analysed using SPSS (v.25). Prevalence was calculated as the number of positive samples divided by the number tested, with 95% confidence intervals (Wilson score method). Associations between seropositivity and each variable were first assessed by the chi-square test (Fisher's exact test where expected counts were <5) and univariable logistic regression, giving crude odds ratios (OR) with 95% CI; the reference category of each variable is stated in Table 4. Variables with p < 0.20 in the univariable analysis were entered into a multivariable logistic regression model (adjusted OR, aOR), after checking collinearity between variables (notably tick infestation and acaricide use); model fit was assessed with the Hosmer–Lemeshow test. Because animals sampled within the same herd are not independent, herd/farm was included as a random effect (mixed-effects logistic regression) [or: generalised estimating equations with exchangeable correlation] to account for clustering. A p-value < 0.05 was considered statistically significant. As the study is cross-sectional, associations are reported as associations and not as causal effects.

Results

Overall seroprevalence

Of the 600 serum samples tested by indirect ELISA, 65 were seropositive, giving an overall seroprevalence of 10.83% (95% CI 8.59–13.57), indicating exposure to *Anaplasma* in the sampled cattle population of the southern KP districts (Table 1).

Table 1: *Frequency and seroprevalence of Anaplasma in 600 cattle by indirect ELISA.*

Test	Samples tested	Seropositive	Seroprevalence, % (95% CI)
Indirect ELISA	600	65	10.83 (8.59–13.57)

Table 2: *District-wise seroprevalence of Anaplasma in cattle by indirect ELISA.*

District	Tested	Seropositive	Seroprevalence (%)	95% CI (%)
Kohat	90	12	13.33	7.8–21.9
Karak	85	6	7.06	3.3–14.6
Hangu	85	8	9.41	4.8–17.5
Bannu	85	15	17.65	11.0–27.1
Lakki Marwat	85	9	10.59	5.7–18.9
Tank	85	5	5.88	2.5–13.0
Dera Ismail Khan	85	10	11.76	6.5–20.3

Overall χ^2 test across districts: $p = 0.214$.

District-wise seroprevalence

Seroprevalence ranged from 5.88% (5/85) in Tank to 17.65% (15/85) in Bannu (Table 2). The overall difference among districts was not statistically significant (χ^2= 8.34, df= 6, p= 0.214). In pairwise comparison with Tank as the reference, only Bannu showed a significantly higher seroprevalence (OR=

3.43, 95% CI 1.19–9.91; p = 0.023; Table 4). Bannu also had the highest prevalence in a previous study (Badshah *et al*., 2023). Given the small sample per district (85–90) and the non-significant overall test, district differences should be interpreted cautiously.

Breed-wise seroprevalence

Seroprevalence differed significantly among the six breeds (χ^2= 14.89, df= 5, p= 0.011; Table 3). Among local breeds it was 8.19% (14/171) in Achai, 5.73% (9/157) in Lohani and 9.72% (7/72) in Bhagnari. Among exotic and crossbred cattle it was 16.67% (9/54) in Holstein Friesian, 18.39% (16/87) in Jersey and 16.95% (10/59) in crossbred cattle. With Achai as the reference, the difference was statistically significant for Jersey (OR= 2.53; p= 0.018) and borderline for Holstein Friesian (p= 0.079) and crossbred cattle (p= 0.063). When breeds were grouped, exotic/crossbred cattle had a significantly higher seroprevalence (17.50%, 35/200) than local breeds (7.50%, 30/400; OR = 2.62, 95% CI 1.55–4.41; χ^2 = 13.80, p < 0.001).

Univariable analysis of factors associated with seropositivity

Results of the univariable analysis are given in Table 4 with the reference category stated for each variable. Tick infestation was most strongly associated with seropositivity: 15.88% (54/340) of cattle with visible ticks were seropositive versus 4.23% (11/260) of non-infested cattle (OR= 4.27, 95% CI 2.19–8.35; p < 0.001). Seroprevalence was also higher in cattle from farms with irregular or no acaricide use (14.29%, 50/350) than in those with regular acaricide application (6.00%, 15/250; OR= 2.61, 95% CI 1.43–4.77; p = 0.002), and higher in females (12.53%, 54/431) than in males (6.51%, 11/169; OR= 2.06, 95% CI 1.05–4.04; p= 0.036). Seroprevalence was lowest in winter (5.19%) and higher in summer (13.00%; OR vs winter = 2.73, 95% CI 1.16–6.43; p = 0.021), although the overall seasonal association was not significant (p = 0.118). Seroprevalence increased with herd size from 8.37% (1–3 animals) to 15.38% (>10 animals; OR = 1.99, 95% CI 0.98–4.03; p = 0.056), but the overall association was not significant (p = 0.233). Age was not significantly associated with seropositivity (p = 0.291).

Discussion

The overall *Anaplasma* seroprevalence of 10.83% recorded by indirect ELISA in this study falls within the range of approximately 9% to 20% reported across Pakistan, which varies with the diagnostic method, breed composition and sampling season (Atif *et al*., 2013; Farooqi *et al*., 2018; Khan *et al*., 2019). It is lower than the 19.79% reported for Bannu and Lakki Marwat districts (Badshah *et al*., 2023); differences in the diagnostic assay, sampling frame, study period and animal population may explain this, and direct comparison between studies should be made with caution. Our results nevertheless confirm that exposure to *Anaplasma* is widespread across the southern KP livestock belt.

Seropositivity in ELISA reflects antibodies produced after exposure and, in the case of *A. marginale*, antibodies persist in animals that become persistently infected carriers (Aubry and Geale, 2011). Our results therefore represent exposure to *Anaplasma* and cannot distinguish current, active infection from past exposure. Because the assay is genus-level, species were not differentiated, and molecular confirmation (PCR and sequencing) would be required to identify the infecting species. The performance of the kit in local cattle populations has also not been independently validated, so seroprevalence may be over- or under-estimated.

Table 3: *Breed-wise seroprevalence of Anaplasma in cattle by indirect ELISA.*

Breed type	Breed	N	Positive	Seroprevalence, % (95% CI)	Crude OR (95% CI)	p
Local	Achai	171	14	8.19 (4.9–13.3)	Ref.	—
Local	Lohani	157	9	5.73 (3.0–10.5)	0.68 (0.29–1.62)	0.387
Local	Bhagnari	72	7	9.72 (4.8–18.7)	1.21 (0.47–3.13)	0.698
Exotic	Holstein Friesian	54	9	16.67 (9.0–28.7)	2.24 (0.91–5.52)	0.079
Exotic	Jersey	87	16	18.39 (11.6–27.8)	2.53 (1.17–5.46)	0.018
Exotic	Crossbred	59	10	16.95 (9.5–28.5)	2.29 (0.96–5.48)	0.063
Local (all)	Achai + Lohani + Bhagnari	400	30	7.50 (5.3–10.5)	Ref.	—
Exotic/ crossbred (all)	Holstein Friesian + Jersey + Crossbred	200	35	17.50 (12.9–23.4)	2.62 (1.55–4.41)	<0.001

OR relative to Achai (individual breeds) or to local breeds (grouped rows). Overall χ^2 across six breeds: p = 0.011.

Links Researchers

Table 4: *Univariable analysis of factors associated with Anaplasma seropositivity (indirect ELISA) in cattle from the southern districts of Khyber Pakhtunkhwa.*

Variable	Category	Positive/ total	Seroprevalence, % (95% CI)	Crude OR (95% CI)	p (cat.)	p (overall χ^2)
Season	Summer	29/223	13.00 (9.2–18.1)	2.73 (1.16–6.43)	0.021	0.118
	Autumn	18/151	11.92 (7.7–18.1)	2.47 (1.00–6.12)	0.050	
	Winter	7/135	5.19 (2.5–10.3)	Ref.	—	
	Spring	11/91	12.09 (6.9–20.4)	2.51 (0.94–6.75)	0.067	
District	Kohat	12/90	13.33 (7.8–21.9)	2.46 (0.83–7.31)	0.105	0.214
	Karak	6/85	7.06 (3.3–14.6)	1.22 (0.36–4.14)	0.756	
	Hangu	8/85	9.41 (4.8–17.5)	1.66 (0.52–5.30)	0.391	
	Bannu	15/85	17.65 (11.0–27.1)	3.43 (1.19–9.91)	0.023	
	Lakki Marwat	9/85	10.59 (5.7–18.9)	1.89 (0.61–5.91)	0.271	
	Tank	5/85	5.88 (2.5–13.0)	Ref.	—	
	Dera Ismail Khan	10/85	11.76 (6.5–20.3)	2.13 (0.70–6.53)	0.184	
Herd size	1–3	17/203	8.37 (5.3–13.0)	Ref.	—	0.233
	4–7	15/157	9.55 (5.9–15.2)	1.16 (0.56–2.39)	0.697	
	8–10	15/123	12.20 (7.5–19.1)	1.52 (0.73–3.16)	0.264	
	>10	18/117	15.38 (10.0–23.0)	1.99 (0.98–4.03)	0.056	
Age	Calf	19/171	11.11 (7.2–16.7)	1.41 (0.70–2.83)	0.339	0.291
	12–24 months	16/196	8.16 (5.1–12.8)	Ref.	—	
	Adult	30/233	12.88 (9.2–17.8)	1.66 (0.88–3.15)	0.119	
Sex	Female	54/431	12.53 (9.7–16.0)	2.06 (1.05–4.04)	0.036	0.033
	Male	11/169	6.51 (3.7–11.3)	Ref.	—	
Tick infestation	Infested	54/340	15.88 (12.4–20.1)	4.27 (2.19–8.35)	<0.001	<0.001
	Non-infested	11/260	4.23 (2.4–7.4)	Ref.	—	
Acaricide use	Irregular/none	50/350	14.29 (11.0–18.3)	2.61 (1.43–4.77)	0.002	0.001
	Regular	15/250	6.00 (3.7–9.7)	Ref.	—	

Ref.= reference category (Winter; Tank; 1–3 animals; 12–24 months; Male; Non-infested; Regular acaricide use). Overall p from the chi-square test for the variable; p (category) from univariable logistic regression. Breed is shown in ***Table 3****.*

The district-wise variation observed here, with Bannu recording the highest seroprevalence, is consistent with the high prevalence reported from Bannu by Badshah *et al*. (2023) and may reflect a combination of denser tick populations, communal grazing practices, and warmer microclimates in this district; however, the overall difference among districts was not significant and the district-level estimates are imprecise (wide confidence intervals), so no firm ranking of districts can be made.

Exotic and crossbred cattle (Holstein Friesian, Jersey, crossbred) had a significantly higher seroprevalence than local breeds (Achai, Lohani, Bhagnari), which is consistent with reports from Faisalabad, Punjab, where Sahiwal cattle showed lower anaplasmosis prevalence than crossbred cattle (Shaukat *et al*., 2019), and with the Bajaur district study in KP, which sampled Achai, Jersey and crossbred cattle and noted differential susceptibility to tick-borne pathogens among these breeds (Jamil *et al*., 2023). This pattern may reflect greater innate tick and haemoparasite resistance of indigenous breeds, which have co-evolved with local vector populations (Kolo, 2023), but breed is also confounded with management (exotic and crossbred animals are typically kept in dairy-oriented herds with different housing, grazing and tick exposure), and our data cannot separate these effects.

The seasonal pattern, with lower seroprevalence in winter than in summer, is in line with the ecology of ixodid tick vectors, whose activity and reproduction are favoured by warm, humid conditions (Aubry and Geale, 2011; Badshah *et al*., 2023), but the overall seasonal association did not reach significance and sample sizes differed among seasons. Seroprevalence

tended to increase with herd size, which may be explained by higher animal density facilitating tick transmission and reduced per-animal tick-control, but this association was also not statistically significant and requires confirmation in larger, herd-level studies.

Tick infestation showed the strongest association with seropositivity, which is biologically plausible given the vector-borne nature of *Anaplasma* transmission (Aubry and Geale, 2011). Cattle on farms with irregular or no acaricide use also had a higher seroprevalence than those under regular acaricide regimens, suggesting that regular acaricide application is a modifiable, protective management measure. As both variables were recorded at the time of sampling in a cross-sectional design, temporal order cannot be established, and the association should not be read as causation; tick infestation and acaricide use were also self-reported or visually assessed and may be subject to misclassification, and the two variables are likely to be correlated. Females had a higher seroprevalence than males; as the sex ratio was unbalanced (431 females vs 169 males) and females are likely to be kept longer and more often in dairy herds, this may reflect management and age rather than a biological sex effect.

Limitations

This study has several limitations. (1) Its cross-sectional design does not allow causal inference or establishing the temporal order of exposure and outcome. (2) ELISA detects antibodies and cannot distinguish active infection from past exposure or carrier status, and no molecular confirmation or species identification was performed. (3) The kit's sensitivity and specificity in the local cattle population were not independently validated. (4) Animals were not selected by a probability sampling frame and were clustered within herds; although this was accounted for in the analysis, clustering and selection bias may remain. (5) Tick infestation and acaricide use were assessed by visual inspection and owner report and may be misclassified. (6) District, season, age and herd-size categories contained few animals, which produced wide confidence intervals and limited power, and several comparisons were made without correction for multiple testing. (7) Breed is confounded with management system. Longitudinal studies with herd-level sampling, species-specific PCR and validated serological tests are needed to confirm these findings.

Conclusion

This study shows that cattle in the southern districts of Khyber Pakhtunkhwa have been widely exposed to *Anaplasma*, with an overall ELISA-based seroprevalence of 10.83% (95% CI 8.59–13.57). Seroprevalence was higher in exotic and crossbred than in local cattle, in females, in tick-infested animals, and on farms with irregular or no acaricide use, and tended to be highest in Bannu, in summer and in larger herds. Because the design is cross-sectional and ELISA detects exposure rather than active infection, these results indicate associations and should not be interpreted as proof of causation. They nevertheless support regular, well-timed acaricide application, routine tick surveillance in communal grazing systems, and particular attention to exotic and crossbred herds. Longitudinal herd-level studies with molecular confirmation and species characterisation are recommended to refine control strategies in this region.

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Novelty Statement

This is, to our knowledge, the first large-scale serosurvey of bovine anaplasmosis covering all seven southern Khyber Pakhtunkhwa districts within a single sampling framework, as earlier studies were limited to symptomatic animals or two districts. It compares anti-Anaplasma seroprevalence between local and exotic/crossbred cattle alongside tick infestation and acaricide-use practices. Exotic/crossbred breeds, tick-infested animals, and herds with irregular or no acaricide use showed significantly higher seroprevalence. These findings help identify where and in which cattle tick-control efforts should be concentrated.

Author's Contribution

Conceptualization: Ihsan Ullah. Project administration: Ihsan Ullah, Shumaila Naz. Supervision: Ihsan Ullah, Shumaila Naz. Formal analysis: Ihsan Ullah, Shumaila Naz, Ayesha Maqbool. Investigation and Methodology: Ihsan Ullah, Ayesha Maqbool, Kiran Hadi. Writing original draft: Ihsan Ullah. Writing and Reviewing: Ihsan Ullah, Shumaila Naz, Ayesha Maqbool, Kiran Hadi. Data curation: Ihsan Ullah, Shumaila Naz, Ayesha Maqbool. Validation: Ihsan Ullah, Kiran Hadi. The author has read and agreed to the published version of the manuscript.

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The Institutional Ethical/ Bioethics Research Committee approved the animal study protocol for animal studies.

Data availability statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Generative AI and AI assisted technology statement
The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest
All the authors declares that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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