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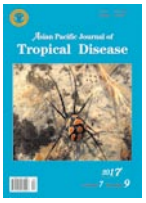

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# First detection on prevalence of *Anaplasma marginale* in sheep and goat in Karak District, Pakistan

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ABSTRACT

**Objective:** To evaluate prevalence of *Anaplasma marginale* (*A. marginale*) and associated risk factors in sheep and goat.
**Methods:** A total of 500 blood samples (250 from sheep and 250 from goat) were collected from three different tehsils of Karak District and analyzed for presence of *A. marginale* using microscopy, indirect ELISA and real-time PCR.
**Results:** The overall prevalence rate was 33.87%. The infection rate was significantly higher ($P < 0.05$) in Karak and Banda Daud Shah compared to Takht-e-Nasrati. It was observed that prevalence rate was higher by real-time PCR compared to indirect ELISA and microscopy. In tehsil Karak, the infection rate by microscopy, indirect ELISA and real-time PCR was 24.38%, 40.62% and 56.25%, respectively. Similar findings were observed in other two tehsils that real-time PCR results were more reliable.
**Conclusions:** The overall prevalence rate was higher in sheep (47.25%) compared to goat (34.85%). Furthermore, among all the risk factors associated, presence of tick and unhygienic condition were highly associated with infection as shown by coefficient of correlation. This is the first report regarding *A. marginale* prevalence in sheep and goat in Karak District, Pakistan.

## 1. Introduction

*Anaplasma marginale* (*A. marginale*) is host-specific rickettsial intraerythrocytic pathogen. The main hosts to infect are ruminants and primarily cattle[1,2]. The parasite is biologically or mechanically transmitted by biting flies and most tick species. The disease caused by *A. marginale* is characterized by fever and general depression, followed by weight loss, progressive anemia, and icterus[3]. It is the most pathogenic species that causes outbreak worldwide compared to other species of anaplasmosis which is one of the most common tick-born, haemo-rickettsial diseases[4,5]. The causative agent, *A. marginale*, an intraerythrocytic parasite, is regarded as the most pathogenic species causing mild infection to clinical outbreak in small ruminants[6].

It is an important issue for animal breeders because of the economic losses associated with it and its threat to human health[7-9]. Pakistan is an agricultural country and livestock is an important sector with 11.9% contribution in national GDP and 56.2% share in agricultural economy[10]. Throughout the developing countries, small ruminants make a very valuable contribution, especially to the poor people in the rural areas. These

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contributions range from precious animal proteins (meat and milk) to fiber and skins, draught power in the highlands, food security and stable households.

Geographically Karak is one of the southern district of KPK and is located toward the southern side of provincial capital Peshawar. Total area of the district is 3 371 km$^2$ with 29% cultivated and 71% non-cultivated. Out of 29% cultivated area, only 3% is irrigated, and 97% is non-irrigated area, so livelihood is totally dependent on small animals rearing. Moreover, this district is totally hilly area and rich in sheep/goat (0.034/0.292 million population) because of livelihood of people. It has been reported that anaplasmosis significantly compromises animal production and reproduction resulting in significant losses to owner. Therefore, the objective of the present study was to evaluate the seroprevalence and molecular detection of *A. marginale*, and to further evaluate associated risk factors responsible for disease spread.

## 2. Materials and methods

The study was conducted at Department of Microbiology, Faculty of Biological Sciences, Kohat University of Science and Technology, Pakistan and Veterinary Research Institute (VRI), Peshawar, Pakistan. A questionnaire was developed to gather general information, herd composition, feeding regime, farming conditions, floor type, farming type, vaccination and individual animal physiological parameters and tick presence as per guidelines described[11]. A total of 500 blood samples (250 from sheep and 250 from goat) were collected from jugular vein of clinically suffering animals from various villages from March to August 2015–2016 (Figure 1).

About 3 mL of blood sample was aseptically collected from the jugular vein of selected small ruminants in sterile EDTA-containing (for PCR) and non-EDTA (for ELISA) tubes. The collected samples were transferred to ice-added container and stored at 4 ℃ until used for further diagnosis[12]. Two thin fresh blood smears were prepared for Giemsa staining. Further the detection of anaplasmosis in small ruminants through ELISA was performed to spot specific antibodies. The kit protocol is based on the indirect ELISA. Then 100 μL of diluted serum (1:40) sample was used for each sample well. The microtiter plate was used to test the fresh and refrigerated serum samples. HRP conjugate was added to the tubes. Subsequently, a blue color developed due to the conversion of the substrate by the conjugate. The development of blue color showed the positive result. The reaction was stopped by addition of the stop solution. The result was read by micro-plate reader Clindiag MR-96 Belgium Photometer at 405 nm. The optical density (OD) was measured within 10 to 15 min to avoid fluctuation in results.

Further, DNA was extracted from the blood collected in EDTA tubes from different regions of villages of Karak District. QIAGEN® DNeasy® blood and tissue kit quick start protocol (GmbH) (Hilden, USA) was used[13]. Bio-Rad real-time PCR (CFX-96) was used for amplification of DNA. Forward and reverse primers were designed with following sequences: forward 5′ TTGGCAAGGCAGCAGCTT 3′, reverse 5′ TTCCGCGAGCATGTGCAT 3′. PCR was run by using a protocol that was previously used by Decaro *et al*.[14]. PCR conditions were maintained as: initial denaturation at 95 ℃ for 2 min followed by 35 cycles of denaturation at 95 ℃ for 1 min, annealing at 60 ℃ for 1 min, extension at 72 ℃ for 1 min and final extension at 72 ℃ for

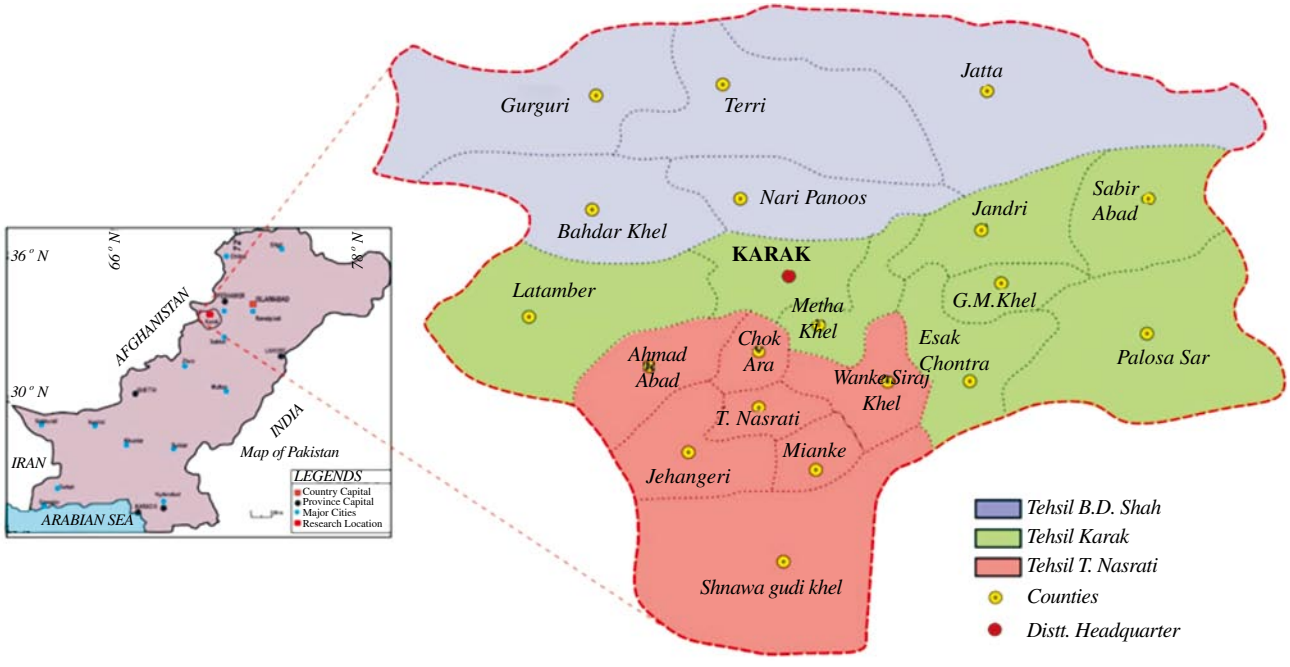


**Figure 1.** Map of Karak District showing transects selected for current research work.

5 min.

Comparison of the prevalence of *A. marginale* in sheep and goats according to risk factors and localities of different villages was performed with statistical software SAS Enterprise Guide (version 4.2; SAS Inst. Inc., Cary NC, USA) using the $\chi^2$ test and Fisher's exact test, and statistical significance was set at $P < 0.05$. Relation of various parameters was determined by Pearson correlations. One animal was used as a unit of analysis[15].

## 3. Results

A total of 500 samples were collected from Karak District. All the samples were processed for determination of seroprevalence of anaplasmosis by microscopy, serological technique (ELISA) and real-time PCR. In small ruminants, the overall prevalence rate was 33.87%. The infection rate was significantly higher ($P < 0.05$) in Karak compared to Takht-e-Nasrati (Table 1), while, the highest prevalence rate was observed in tehsil Banda Daud Shah (Figure 2). The prevalence rate was higher by real-time PCR as compared to indirect ELISA and microscopy (Figure 2). In tehsil Karak, the infection rate by microscopy, indirect ELISA and real-time PCR was 24.38%, 40.62% and 56.25%, respectively. These findings were consistant with those in other two tehsils that the prevalence rates by real-time PCR were higher than other two techniques (Figure 2). Moreover, the overall higher prevalence rate was observed in sheep as compared to goat (47.25% *v.s* 34.85%) (Figures 3 and 4). Furthermore, among all the risk factors associated, tick presence and unhygienic condition were highly associated with infection as shown by coefficient of correlation (Table 2).

**Table 1**

Prevalence rates of *A. marginale* in goats and sheep from different localities.

| Locality | Villages | Percentage of positive animals (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Sheep | | | Goat | | |
| | | Microscopy | ELISA | PCR | Microscopy | ELISA | PCR |
| Karak $n = 80$ (16 per village) | Esak Chontra | 37.50 | 56.25 | 81.25 | 12.50 | 68.75 | 56.25 |
| | Palosa Sar | 31.25 | 18.75 | 68.75 | 25.00 | 12.25 | 87.50 |
| | Latamber | 18.75 | 43.75 | 37.50 | 25.00 | 21.25 | 8.75 |
| | Metha Khel | 18.75 | 25.00 | 68.75 | 6.25 | 50.00 | 43.75 |
| | Sabirabad | 18.75 | 31.25 | 75.00 | 50.00 | 68.75 | 75.00 |
| | Sub-Total | 25.00 | 35.00 | 66.25 | 23.75 | 46.25 | 46.25 |
| Takht-e-Nasrati $n = 80$ (16 per village) | Ahmad Abad | 6.25 | 12.50 | 31.25 | 6.25 | 18.75 | 25.00 |
| | Chokara | 18.75 | 25.00 | 25.00 | 18.75 | 12.50 | 18.75 |
| | Jehangeri | 0.00 | 25.00 | 37.50 | 0.00 | 18.75 | 25.00 |
| | Shnawa Kudi Khel | 25.00 | 31.25 | 25.00 | 25.00 | 25.00 | 37.50 |
| | Wanki Siraj Khel | 12.50 | 25.00 | 18.75 | 12.50 | 12.50 | 12.50 |
| | Sub-Total | 12.50* | 23.75 | 27.50* | 6.25* | 17.50* | 23.75* |
| Banda Daud Shah $n = 90$ (18 per village) | Bahaddar Khel | 27.78 | 44.44 | 77.78 | 44.44 | 50.00 | 55.56 |
| | Jatta | 22.22 | 72.11 | 83.33 | 27.78 | 61.11 | 72.22 |
| | Guguri | 39.00 | 55.56 | 66.67 | 16.67 | 11.11 | 44.44 |
| | Nari panos | 16.67 | 27.78 | 44.44 | 11.11 | 20.22 | 27.78 |
| | Terri | 33.34 | 50.00 | 72.22 | 5.56 | 27.22 | 38.89 |
| | Sub-Total | 27.78 | 50.00* | 68.89 | 21.11 | 34.44 | 47.78 |

*: Significant difference ($P < 0.05$) within column. The prevalence of anaplasmosis was significantly low ($P < 0.05$) in tehsil Takht-e-Nasrati as compared to other two tehsils.

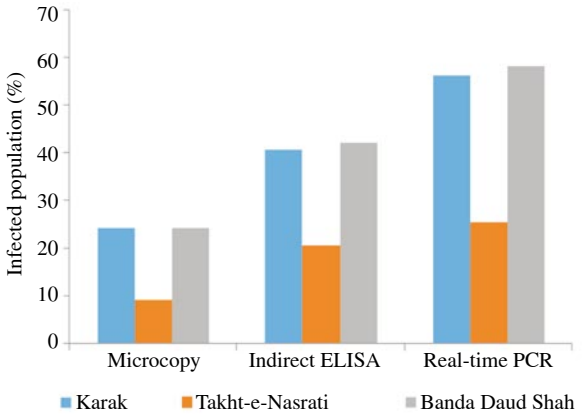


**Figure 2.** Overall prevalence of *A. marginale* in small ruminants in Karak District through different analysis.

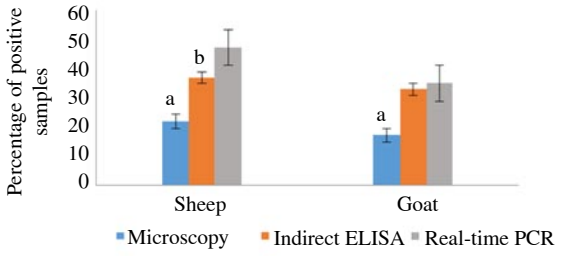


**Figure 3.** Comparison of three different diagnostic techniques for *A. marginale* in sheep and goat ($n = 250$ animals in each technique). Different letters indicate significant difference at $P < 0.05$.

**Table 2**

Risk factors associated with prevalence of *A. marginale* in goats and sheep.

| Sr. No | Risk factors | Infection confirmed by PCR (%) | Pearson correlation coefficient ($r$) |
|---|---|---|---|
| 1 | Tick presence | 95.00 | 0.84 |
| 2 | Unhygienic condition | 90.12 | 0.78 |
| 3 | Muddy floor | 86.65 | 0.73 |
| 4 | Deworming | 75.34 | 0.63 |
| 5 | Open grazing system | 69.14 | 0.60 |
| 6 | Awareness about TBDs | 80.35 | 0.73 |
| 7 | Season of ticks infestation | 78.42 | 0.64 |
| 8 | Long hair sheep/goat | 72.23 | 0.65 |

95% diseased animals have tick infestation and $r = 0.84$ shows correlation of tick infestation with disease. TBD: Tick-borne disease.

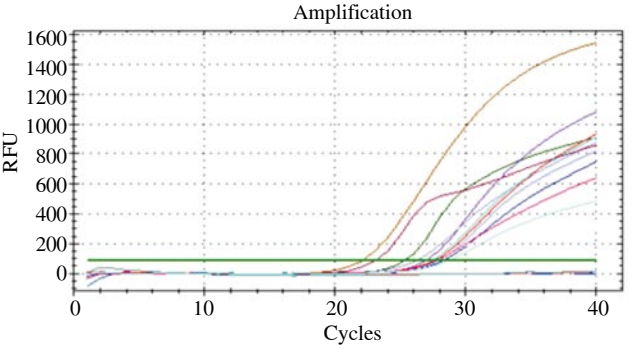


**Figure 4.** Sensitivity of the primer-probe combination specific to anaplosmosis pathogen in real-time PCR with the positive control quantified at 19 cycles.
The earlier the quantification cycle comes, the more the infection occurs. RFU: Relative fluorescence unit, a unit of measurement used in analysis which employs fluorescence detection.

## 4. Discussion

To our knowledge, this is the first report about incidence of *A. marginale* infection and associated risk factors in sheep and goat population ($n$ = 250 each) in Karak District, Pakistan. Our results are consistent with those of other PCR studies showing that domestic ruminants are infected with a range of *Anaplasma*[2,8,16-18]. In small ruminants, the overall prevalence rate was 33.87% with 47.25% in sheep and 34.85% in goat. In the world, *A. marginale* prevalence is different, for example, 99.4% reported in Hungry[19], 81% in China[20], 28% in Egypt[3,21], 27.5% in Iran and 16.17% in Ibimirim County, Brazil[22]. It is worthful to mention that a study conducted in Pakistan reported prevalence of 24.47% for anaplasmosis in sheep using indirect ELISA[23], but in the present study higher prevalence 36.8% was observed. Moreover, that study used only indirect ELISA, but we used indirect ELISA and real-time PCR and prevalence was higher compared to that study. Furthermore, the study area was also different.

The gold standard diagnosis of anaplasmosis relies on microscopic examination of blood smear, so samples were first screened microscopically, and the overall prevalence recorded in sheep and goat was 22% and 17.25% respectively, while indirect ELISA showed prevalence of 36.8% and 32.8% in sheep and goat, respectively. However, real-time PCR detected all the positive samples of microscopy and indirect ELISA and prevalence was 47.25% and 34.85% in sheep and goat, respectively. Although microscopy is gold standard test, prevalence recorded was low as compared to molecular techniques. PCR is more advantageous due to high sensitivity and effectiveness in diagnosing active and carrier state infection[6,17,21]. Similar findings were reported by Salih *et al*.[24] who investigsted the incidence rate of different protozoan species in various livestock through indirect ELISA in Sudan. Moreover, another study[25] proved that real-time PCR is more sensitive for the diagnosis of *A. marginale*. They further explained that real-time PCR is more specific that it produced the same results in multiple runs and never confused with other haemo-parasites which are antigenically similar to *A. marginale*.

In sheep the prevalence rate was higher through all diagnostic techniques *i.e* real-time PCR (47.25%) than that in goats (24.85%) (Figure 3). Similar findings were also reported[26,27] that sheep was more susceptible as compared to goat[20]. This could be due to susceptibility of each animal and differences in risk factors associated with infection, as it has also been reported that prevalence of tick infestation is significantly higher in sheep as compared to goat. Moreover, sheep housing and management is comparatively poor in rural areas, hence sheep is more susceptible to tick infestation due to rough wool. Other studies reported[20,28], however, higher prevalence in goat than sheep. This may be due to difference in the geographical location and farming condition of the study areas.

We came to the conclusion that several risk factors such as housing and management, ticks infestation, deworming and awareness and education of farmers significantly contribute to the disease prevalence. The ticks were observed in 95% of sick animals and these animals were devoid of ticks control. Other factors like housing and management of farms including hygienic conditions ($r$ = 0.78), deworming ($r$ = 0.63) and awareness about tick-borne diseases ($r$ = 0.73) have strong correlation with disease prevalence as shown in Table 2.

It is concluded that *A. marginale* is highly prevalent in Karak District and overall prevalence of about 33.87% was recorded. Higher prevalence was found in sheep as compared to goat. However, the evaluation of associated risk factors revealed that hygienic measures, vector control, scheduled deworming and farmer's awareness about disease and control measures can significantly reduce the risk of *A. marginale* infection.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgments

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