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Short Communication

Prevalence of *Anaplasma marginale* in Tropical Area of Khyber Pakhtunkhwa, Pakistan

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SG and KK collected the data and conducted the experiment. TU wrote the paper. SA and SA designed the project and supervised the research. SG analyzed the data. All the authors read and approved the research paper.

Key words
Anaplasmosis, Cattle, ELISA, Major surface protein gene, PCR

ABSTRACT

Bovine anaplasmosis is an infectious tick-borne disease caused by a rickettsial parasite *Anaplasma marginale.* This study investigated the prevalence of *A. marginale* in cattle on different commercial farms in subtropical conditions of Khyber Pakhtunkhwa (KP). A total of 120 cattle from different farms were randomly sampled and tested for the presence of *A. marginale* between May to August 2016. The study revealed that 15.0%, 20.8% and 29.1% of sampled animals were found positive for *A. marginale* using microscopic examination, indirect ELISA and PCR, respectively. Two major surface protein genes (*MSP1a* and *MSP4*) sequence were compared with cattle isolates from different origins. Phylogenetic analysis of local isolates showed a close homology with the isolates from Australia, Brazil, Turkey and Japan. It was found that aged, exotic and crossbreed cattle were more susceptible to *A. marginale* infection in summer season compared to the younger and indigenous cattle breeds, respectively ($P<0.01$). Moreover, the study revealed widespread prevalence of *A. marginale* in the subtropical areas of KP, Pakistan.

Anaplasmosis is one of the most important infectious and hemoparasitic diseases of bovines and other ruminants (Dumlar *et al*., 2001). It is caused by an intraerythrocytic parasite of the genus *Anaplasma* (order: Rickettisale; family: Anaplasmataceae) (Dumlar *et al*., 2001). In the mammalian host, *Anaplasma marginale* produces antigenic variants by altering a surface coat, consisting of many proteins. Six major surface proteins (MSPs) have been identified on *A. marginale* derived from bovine erythrocytes (Kocan *et al*., 2000). The functional pseudogenes that belong to *MSP2* super familyplay a vital role in surface coat antigenic variation (Kocan *et al*., 2000). MSPs involved in host-pathogen interactions evolve more rapidly than other nuclear genes because of selective pressures exerted by the host immune system. Three of these *MSPs* (*MSP1a, MSP4* and *MSP5*) are from single genes and do not vary antigenically during the multiplication of the organism. The other three *MSPs* (*MSP1b, MSP2* and *MSP3)* are from multigene families and may vary antigenically, most notably in persistently infected cattle (Bowie *et al*., 2002). *MSP1a*, encoded by*MSP1a*gene of *A.marginale* play a vital role in providing protective immunity in cattle (Fuente *et al*., 2003).

Ticks are obligate, blood feeding ectoparasites of vertebrates verified to transmit *A. marginale* (Kahn, 2005). Pakistan is located in a subtropical zone (30° N, 70° E) within South Asia and thus offers favorable environmental conditions for ticks to infest a variety of hosts and transmit diseases to livestock, companion animals and humans (Jabbar *et al*., 2015). Due to rapid extension of the dairy industry and an increased import of high milk producing exotic dairy cattle (Holstein Friesian and Jersey), it has become crucial to assess the status of tick born diseases (TBDs) in exotic and indigenous breeds of cattle. Exotic breeds are highly vulnerable to TBDs than indigenous breeds. Therefore, the present study was designed to investigate the prevalence and molecular identification of *A. marginale* in indigenous and exotic cattle breeds of subtropical areas of Khyber Pakhtunkhwa (KP), Pakistan.

Materials and methods

A total of both exotic 120, (Australian Friesian and

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Jersey) and indigenous (Sahiwal and Achai and cross bred cattle) animals, of both sexes were randomly selected from different commercial cattle dairy farms of KP from May-August 2017. The cattle were of different age group, maintained at loose housing system and were fed on the recommended feed. The study was approved by the ethical committee of the University of Agriculture Peshawar. Approximately 6mL of blood was collected from the jugular vein of each animal in two 6mL tubes one without anticoagulant for ELISA and the other with anticoagulant (Improvacuter®, Belgium) for DNA extraction. Serum was isolated after centrifugation and stored at -20°C. Thin blood smears were prepared with fresh blood in EDTA anticoagulant and Giemsa stained using an AMES HemaTek slide stainer (Bayer, Zurich, Switzerland).

Anaplasmosis indirect ELISA was performed using SVANOVIR *A. marginale* Ab (Company Boehringer Ingelheim Svanova, Sweden) following the manufacturer's instruction. The optical density (OD) was measured at 405nm in microplate photometer. The percentage positivity (PP%) for the serum samples was calculated using the following equation:

$$\text{PP \% for serum Sample} = \frac{\text{OD value of serum sample}}{\text{Mean OD value of positive control}} \text{ x } 100$$

Serum sample showing PP% greater than 25% were considered positive for *A. marginale* while those less than 25% were considered negative according to the kit protocol of indirect ELISA kit (SVANOVIR).

For PCR amplification of *MSP1*and *MSP4* genes, DNA was extracted from blood samples (200µL) using commercially available kits (Thermo Scientific; Gene JET Whole Blood Genomic DNA Purification Mini Kit k0781, no k0782) according to the manufacturer's protocol.

The extracted DNA was used for PCR using primers specific for *A. marginale MSP1α* and *MSP4* genes as described by Fuente *et al*. (2005). PCR reaction was performed in a final volume of 20µL containing 2µL of DNA, 6µL distilled water, 10µL PCR master mix (10xTaq buffer, MgCl2, 0.2Mm dNTPs, 1U Taq polymerase, Loading dye). Blood from cattle shown to be free of *A. marginale* was used as a negative control to observe contamination. Amplified PCR products were run on 1% agarose gel containing ethidium bromide and visualized by UV transillumination. A 2300bp and 872bp DNA fragments were identified by comparison with DNA molecular weight marker (100plus bp DNA Ladder, Promega) (Supplementary Fig. 1). For phylogenetic analysis, positive samples confirmed by PCR were loaded on gel. The samples were excised, purified and then sent to Macrogen, Inc. South Korea for nucleotide sequencing.

The *MSP4* amplified region was sequenced completely while *MSP1α* amplified region was partially sequenced using big dye method. The sequences obtained were checked with Chromas v.1.45 and compared to sequence data from GenBank1, using the BLAST 2.2.15. program (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences obtained in this study were aligned by Multiple Sequence Alignment using online software multalin (http://multalin.toulouse.inra.fr/multalin/) and a phylogenetic tree was constructed by maximum likelihood method using Mega 6 software.

The data was analyzed statistically by chi-square test using SPSS version 13.0. A *P* value of less than 0.01 was considered statistically significant. The kappa coefficient was calculated to estimate the degree of agreement between the Indirect ELISA and PCR assay.

Results

Out of 120 blood samples of different breed of cattle, microscopic examination detected 15% samples positive. The proportion of infected erythrocytes in blood smears was low (<4%) (results not presented) and contained 1-3 inclusion bodies in submarginal, marginal, or central position. There were no relevant clinical signs found in majority of the sampled animals. However, some showed nonspecific clinical signs. The prevalence rate was 20.8%, and 29.1% using ELISA and PCR, respectively. Comparing the two techniques in our study, a concordance of 87.6% was found between ELISA and PCR assays. The microscopic examination, although presents a similar result as PCR, yet the chances of sampling error are unavoidable, and thus can provide false negative results.

Table I shows the prevalence of *A. marginale* in animals with different age group, breed, gender and sampling-months using the total number of samples positive for either test. Statistically significant association was found between level of prevalence of *A. marginale* and different age groups, sampling-months and breeds ($P<0.01$). Contrary, no significant difference was observed between the two genders regarding prevalence rate. Animals older than six years were more susceptible to *A. marginale* as compared to young animals (1-3 years). Pure and crossbred Holstein Friesian had a prevalence rate of 34% and 28%, respectively, whereas, the Achai and Sahiwal showed the lowest infestation rate of 8% and 6.2%, respectively (Table I). The *A. marginale* infection was found with an increasing trend from May to August. According to our study, female cattle were found more infected than male cattle with a ratio of 59% and 41%, respectively. The *A. marginale MSP1α* and *MSP4* genes were successfully amplified from bovine samples. Sequence analysis of the PCR products showed that all of them correspond to *A. marginale*, showing 90-95% identity with the reference sequences of *A. marginale* found in GenBank. *A. phagocytophilum* was not noticed in analyzed samples. The phylogenetic analysis in our study

was done using two genes of *A. marginale* i.e. *MSP1α* gene and *MSP4* genes. Similar genes were also used by Fuente *et al*. (2003) for molecular characterization and phylogenetic studies of *A. marginale*. The phylogenetic analysis showed that our local isolates have high homology with the isolates of Australia, Turkey, Thailand and Japan with strong bootstrap value (Figs. 2, 3).

Table I. Prevalence of *Anaplasma marginale* in 120 blood samples of different breeds of cattle maintained at various commercial farms in subtropical area of Khyber Pakhtunkhwa.

Variables	Categories	Prevalence (%)	*P* values
Age	Calf	20	
	Heifer	17	0.002
	Adults	63	
Sex	Male	41	0.06
	Female	59	
Breed	Sahiwal	6.2	
	Achai	8	0.001
	HF	34	
	HFX	28	
	Jersey	23.8	
Summer season	May	15.4	0.002
	June	23.1	
	July	26.9	
	August	34.6	

HF, Holstein Friesian; HFX, Holstein Friesian Cross.

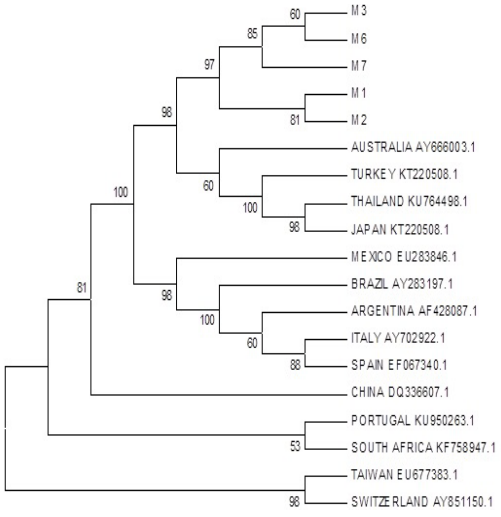

Fig. 1. Nucleotide based tree for *msp1α* gene of *Anaplasma marginale* by Maximum Likelihood Method.

Discussion

Prevalence of *A. marginale* was investigated using microscopic examination, indirect ELISA and further confirmation of antigen through PCR in cattle maintained at commercial dairy farms at KP. According to ELISA, the highest prevalence was recorded in aged animals followed by heifers and calves (Table I). Anaplasmosis is -more prevalent in aged cattle, younger animals might remain liable but show little visible signs (Singh *et al*., 2003). We found inclusion bodies characteristic of *A. marginale* in the RBCs of the majority of the diseased cows. In aged animals, immune system becomes weaker thus are more susceptible to the disease. Calves (3-6 months) on the other hand were less susceptible for anaplasmosis as they receive maternal antibodies and the resistance gradually wanes after one year of age and vulnerability to different infection increases (Kocan *et al*., 2000).

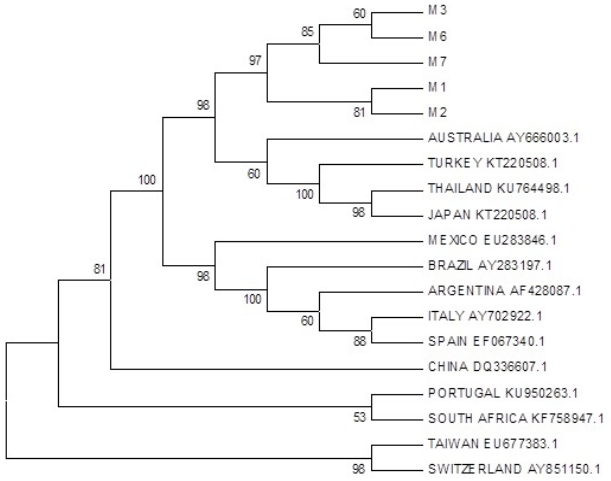

Fig. 2. Nucleotide based tree for *MSP4 gene* of *Anaplasma marginale* by Maximum Likelihood Method.

The highest prevalence (34.66%) of *A. marginale* was found in August. The hot (~ 44˚C) and humid environment of August favors the rapid multiplication of ticks. In closed housing system, higher humidity provides favorable environment for ovipositioning in the crevices and cracks in the floor, walls and roof of house (Muhammad *et al*., 2008). In the present study, highest prevalence of *A. marginale* was seen in exotic and cross bred cattle as compared to indigenous cattle breeds. The natural selection over the millennia made the indigenous cattle more resistant to the prevailing pathogen. This is manifested by lower incidence rate of ticks' infestation in local cattle (Swai *et al*., 2007). Sing *et al*. (2003) found 12.5% and 51% samples positive for *A. marginale* using microscopic examination and nested PCR technique, respectively. His results showed that nested PCR ideally suited for diagnosis of carrier animals. Our results also showed that PCR is more sensitive technique for detecting *A. marginale* in susceptible and carrier animals. In Pakistan, the prevalence of *A. marginale* has been recorded as 7.36-75.71% using microscopic examination of blood smears

(Khan *et al*., 2004; Rajput *et al*., 2005).

Sex influenced the extent and severity of the infection (Khan *et al*., 2004). Our findings showed higher susceptibility of female cattle compared to the male cattle. Higher prevalence of *A. marginale* in female population is due to the higher productivity, climatic and metabolic stress the females are facing which posedthem to a weakened immune system. Findings of the current study are in close agreement to those reported by Rajput *et al*. (2005). In the present study, two major surface protein genes of *A. marginale* i.e. *MSP1α* and *MSP4* were identified as markers for PCR examination.

The sequence alignment and the phylogenetic analysis revealed that the two genes of *A. marginale*, *MSP1α* and *MSP4* can be used as useful markers in evolutionary studies. The *MSP4* and *MSP2* genes were used previously as markers for the genetic characterization of *A. marginale* strains (Fuente *et al*., 2007). The high prevalence rate of *A. marginale* was due to poor management and unhygienic measure found in the present study. These results are in line with those suggested by Swai *et al*. (2005).

Currently, a number of different methods, including chemotherapy as well as prophylaxis, vaccination and chemical tick control are used worldwide to decrease economic damages resulting from TBDs (Muhammad *et al*., 2008). Owing to partial understanding of bovine TBDs, currently, no program for the chemoprophylaxis of these is in place in Pakistan. Grooming is the most commonly used strategy for tick control mostly at small-holding farms. Grooming includes the manual removal of ticks and burning them on the fire made with cattle dung cakes in Pakistan (Mondal *et al*., 2013). The present study showed that the indigenous breeds of cattle are well adapted and are resistant to a higher level towards the TBDs. Thus a wise strategy needs to be developed both at public and private farms for ticks control to avoid the financial losses caused by the TBDs in high yielding exotic dairy cattle especially in the hot and humid months of summer.

Conclusions

The current epidemiological study showed that the prevalence rate of *A. marginale* was approximately 29.1% in dairy cattle at commercial dairy farms maintained under subtropical environment of KP province of Pakistan. The molecular and serological techniques confirmed that exotic and crossbred cattle were highly susceptible to anaplasmosis compare to the indigenous cattle breeds.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20180510110525

Statement of conflict of interest

The authors have declared no conflict of interest.

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