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Prevalence of *Anaplasma phagocytophilum* in horses from Southern Punjab (Pakistan)

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Abstract. The present study was designed to optimize a PCR-RFLP protocol for the molecular detection of *Anaplasma* sp. and to compare its prevalence in blood samples of equines from Southern Punjab (Pakistan) and to find out the risk factors involved in the spread of anaplasmosis. A total of 210 blood samples were collected from equines from 2 sampling sites (Dera Ghazi Khan and Khanewal districts). Data on the animals' characteristics (age, species and gender) were collected through survey. PCR amplified the 577bp product specific for 16S rRNA gene of *Anaplasma* spp. in 9 blood samples (4.3% of total), [Dera Ghazi Khan (N = 3) and Khanewal (N = 6)]. These *Anaplasma* spp. positive blood samples were used for PCR amplification using *A. phagocytophilum* specific primers and parasite was detected in all of them. Also it was revealed that the characteristics of the animals i.e. age, gender, species had no significant association with the presence of *Anaplasma* sp. Hematological parameters remained unaffected while lymphocyte count was significantly lowered in *A. phagocytophilum* positive samples.

INTRODUCTION

In Pakistan, equines are mainly reared by the people for draught and game purposes and are essential part of farming and transport system in rural as well as urban areas (Gebreab, 2004). Working equines are the sources of income for small scale farmers as they allow them to take part in market economy. Horse carts act as strong carriers and are hence capable of transporting many things like people, products, building material, food, water and fuel (Wilson, 2002).

Equine granulocytic anaplasmosis is an acute febrile disease with an incubation period of about 1-2 weeks after which the infected equines may experience clinical signs including pyrexia, depression, anorexia, reluctance to move, limb edema, decrease in body weight, icterus and ataxia. Mild to severe morbidity and even mortality has been seen in certain cases of the disease (Franzen *et al.*, 2005).

Anaplasma phagocytophilum, the etiological agent of equine anaplasmosis is an obligate intracellular gram negative bacterium formerly known as *Ehrlichia equi* (Bermann, 2002). It is a tick transmitted pathogen that mainly infects endothelial and blood cells, principally granulocytes and especially neutrophils of the host and is also of zoonotic importance (McQuiston, 2003). Its incidence is more common from June to August, when there is increased activity of ticks (Rymaszewska & Grenda, 2008). Infected equines should be treated with the antibiotic oxytetracycline which is extremely effective against this rickettsia and should be given for about 7 to 14 days for early recovery (Butler *et al.*, 2008).

The aim of the present study was to establish and optimize a specific, reliable and sensitive molecular tool, the polymerase chain reaction (PCR), for the detection of *Anaplasma* sp. in blood samples of horse from Southern Punjab. Furthermore, the present study provides a baseline data regarding *Anaplasma* sp. prevalence and risk factors involved in the spread of equine anaplasmosis in horses. Comparison of hematological parameters of parasite positive and negative animals was also reported.

MATERIALS AND METHODS

Sample and Data Collection

Blood samples of 210 horses were randomly collected from Dera Ghazi Khan (N = 145) and Khanewal (n = 65) districts of Punjab. Blood was collected from the jugular vein of the animals and immediately preserved in eppendorf tubes by adding a few drops of 0.5 M EDTA. A questionnaire was filled at the spot in order to gather data of risk factors associated with equine anaplasmosis. All the animal handling procedures and lab protocols were approved by the ethical committee of Institute of Pure and Applied Biology, Bahauddin Zakariya University Multan, Pakistan.

DNA Extraction

Inorganic method of DNA extraction was used following Shahnawaz *et al.* (2011). The purity and integrity of DNA samples were assessed by measuring the optical density at 260/280 nm and conducting submerged gel electrophoresis.

PCR-RFLP studies

A set of oligonucleotide primers, Fwd 5'AGAGTTTGATCCTGGCTCAG 3' and Rev 5'GTTAAGCCCTGGTATTTCAC 3', was used to amplify the *16S rRNA* gene sequences of *Anaplasma* sp. as previously described by Ashraf *et al.* (2013). PCR was performed in a final reaction volume of 25 ml. PCR reaction mixture contained 10X buffer with KCL [100 mM Tris HCL (pH 8.8 at 25ºC), 500 mM KCL, 0.8% (v/v) Nonidet P40], 250 ng genomic DNA, 20 pM of each primer, 0.12 mM of dNTPs, 1.5 U *Taq* DNA polymerase (Fermentas, UK) and 1.5 mM Magnesium chloride. *Anaplasma marginale* positive sample, isolated during previous study, and negative control (PCR mixture without DNA) were run during every PCR amplification as positive and negative controls, respectively.

DNA amplification was carried out in a DNA thermal cycler (Gene Amp® PCR system 2700 Applied Biosystems Inc., UK). The thermo-profile used by Noaman *et al.* (2009) was modified for the present study with an initial denaturation at 95ºC for 5 min followed by 35 cycles of denaturation at 94ºC for 45s, annealing at 56ºC for 45s, elongation at 72ºC for 45s and final extension at 72ºC for 10 min. PCR products were stored at 4ºC until electroporesed on a 1.8% agarose gel and visualized under a UV Trans illuminator (Biostep, Germany).

Restriction of the *Anaplasma* sp. positive PCR product was performed in a final reaction volume of 25 µl at 37ºC for 12-16 hours as previously described by Noaman *et al.* (2009). Each restriction reaction mixture consisted of 10 µl amplified PCR product, 2.5 µl of 10X buffer V4 [10 mM Tis-HCl (pH 8.5 at 30ºC), 10 mM MgCl2, 100 mM Kcl, and 100 µg/ml BSA], 0.4 µl *BssNA1* Enzyme (Vivantis, UK).

The *Anaplasma* spp. positive equine samples were examined for the presence of *A. phagocytophilum* by using primers MSP 465F (5'- TGA TGT TGT TAC TGG ACA GA - 3') and MSP 980R (5'- CAC CTA ACC TTC ATA AAG AA - 3') which can amplify 550-base pair product of *msp2* gene of *A. phagocytophilum* (Caspersen *et al.*, 2002).

PCR was performed in a 25 µl reaction mixture comprising of DNA template (50 ng), $MgCl_2$(1.5 mM), [dGTP, dTTP, dATP and dCTP] each 200 uM, primers (each 10 pmol), *Taq* DNA polymerase (Vivantis, UK) (0.625 U) and 1X reaction buffer (50 mMKCl; 10 mMTris HCl of 8.3pH). Thermal profile used for amplification was the same as formerly applied by Caspersen *et al.* (2002). Initial denaturation was brought about at 95ºC for 5 min followed by 40 cycles of denaturation

at 94ºC for 30 s, annealing at 55ºC for 30 s, elongation at 72ºC for 90 s and final extension was carried out at 72ºC for 5 min.

Hematological and Serological Analysis

Various hematological parameters in blood samples from horses i.e. packed cell volume, total RBC and WBC count, mean corpuscular volume, total lymphocytes count, granulocytes count and hemoglobin level were determined in *Anaplasma* sp. positive and negative equine blood samples by using Metertek SP-8SO spectrophotometer (Korea).

Statistical Analysis

Data are expressed as Mean ± Standard deviation. Statistical package Minitab (version 16, USA) was used for the statistical analysis of the results. Animals were divided into two age groups, animals up to 5 years (young) and more than 5 years (mature). Association between the presence of *Anaplasma* sp. and various risk factors, i.e. gender and age of animal was evaluated by contingency table analysis using the Fisher's exact test (for 2 x 2 tables). Two sample t-test was performed to compare various studied hematological parameters between *Anaplasma* sp. positive and negative blood samples.

RESULTS

Analysis of results revealed that 9 out of 210 (4.28%) blood samples were positive for *Anaplasma* sp. (Fig. 1) as they amplified a 577-bp amplicon specific for *16S rRNA* gene of *Anaplasma* genera. It was observed that the blood samples from Khanewal district were more infected with the parasite (6/65, 9.23% prevalence) than those collected from Dera Ghazi Khan (3/145, 2.1% prevalence) but difference in parasite prevalence did not reached the statistical significance (P = 0.06) (Table 1).

Upon restriction with *BssNA1*, all 9 *Anaplasma* sp. positive PCR products remained uncut indicating the absence of *A. marginale* in these blood samples. All these 9 *Anaplasma* sp. positive samples amplified a 550-bp DNA fragment from *msp2* gene specific for *A. phagocytophilum* (Fig. 2).

Analysis of data revealed that characters of animals i.e. age and gender had no relative association with equine anaplasmosis (Table 2). It was observed that all the *A. phagocytophilum* infected animals were mares (P = 0.12). It was also observed that young animals were more prone to *A. phagocytophilum* than adult ones (Table 2),

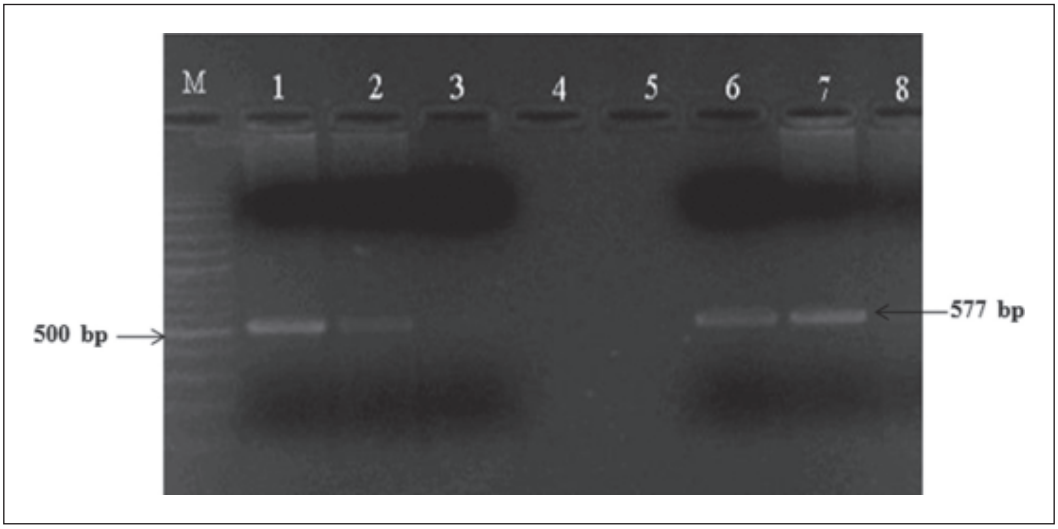

Figure 1. PCR amplification of 16S rRNA gene in *Anaplasma* spp.
Lanes: M, 100 bp DNA marker; 1, positive control (From a clinically confirmed cattle suffering from anaplasmosis); 2, *Anaplasma* sp. +ive blood sample; 3-5, *Anaplasma* sp. –ive blood sample; 6 to 8, *Anaplasma* sp. +ive blood samples.

Table 1. Total number of samples collected (N) from all sampling sites. Prevalence of *Anaplasma* sp. is given in parenthesis. P value indicates the Probability of Fisher's exact test

Sampling site	N	*Anaplasma* spp. +ive	*Anaplasma* spp. –ive	P-value
Dera Ghazi Khan	145	3 (2.1%)	142 (97.9%)	
Khanewal	65	6 (9.2%)	66 (91.8%)	0.06
Total	210	9 (4.3%)	208 (95.7)	

P > 0.05 = Non Significant

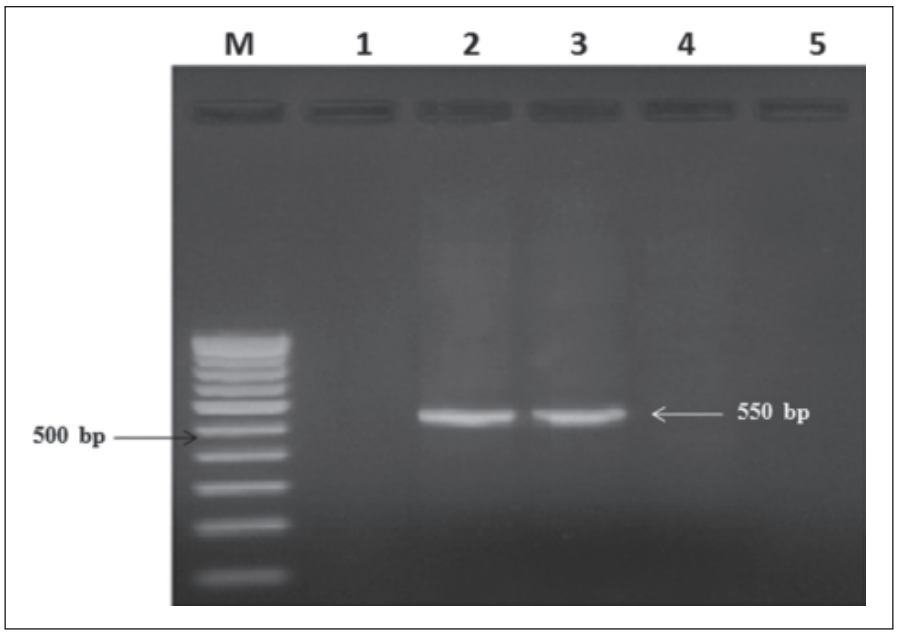

Figure 2. PCR amplification of *Anaplasma phagocytophilum msp2* gene.
Lanes: M, 100 bp DNA marker; 1, Negative control; 2, 3. *A. phagocytophilum* positive horse blood sample; 4 and 5. *A. phagocytophilum* negative blood sample.

Table 2. Association between parasite incidences in blood samples of horses from all the samples gathered from Dera Ghazi Khan and Khanewal districts and the considered parameters describing animal characters. Prevalence of *Anaplasma* sp. is given in parenthesis. Probability of Fisher's exact test is mentioned for each parameter

Parameters	N	*Anaplasma* spp. +ive	*Anaplasma* spp. –ive	P-value
Sex	Male	52	0 (0%)	0.12
	Female	165	9 (5.5%)	
Age	> 5 Year	107	2 (1.87%)	0.17
	≤ 5 Year	110	7 (6.4%)	

P > 0.05 = Non significant

Table 3. Comparison of various hematological parameters between *Anaplasma phagocytophilum* positive and negative horse samples from Dera Ghazi Khan District. Data is expressed as Mean ± Standard Error of Mean. P-Value indicates the probability of 2 sample t-test

Parameters	*Anaplasma phagocytophilum* positive samples N = 3	*Anaplasma phagocytophilum* negative sample N = 142	P value
WBC (x 10^3 μL^{-1})	9.03 ± 1.04	9.35 ± 0.15	0.85
LYM (x 10^3 μL^{-1})	2.8 ± 0.38	24.8 ± 1.7	$P < 0.001^{***}$
NEUT (x 10^3 μL^{-1})	44.2 ± 8.7	49.3 ± 1.6	0.62
RBC (x 10^6 μL^{-1})	7.17 ± 0.38	7.1 ± 0.15	0.88
HGB (gdL^{-1})	10.57 ± 0.47	10.08 ± 0.14	0.43
MCV (f L)	44.83 ± 2.4	40.92 ± 0.67	0.26
PCV (%)	32.13 ± 2.4	28.35 ± 0.52	0.26

P > 0.05 = Non significant; P < 0.001 = Highly significant (***)
WBC = White blood cells, LYM = Lymphocytes, RBC = Red blood cells, HGB = Hemoglobin, MCV = Mean cell volume, PCV = Pack cell volume, PLT = Platelets.

however all these differences did not reached the statistical significance.

Evaluation of hematological parameters between parasite positive (N = 3) and negative blood (N = 142) samples from Dera Ghazi Khan revealed that the studied parameters remained unaffected except lymphocyte count (P < 0.001) which was significantly lowered in *A. phagocytophilum* positive blood samples (Table 3).

DISCUSSION

Animal diseases are important factors affecting the productivity and economy of the developing nations. Parasitic diseases like anaplasmosis and piroplasmosis are the major limitations to the livestock industry as they globally affect human health, trade and economy (Akhter *et al.*, 2010; Zulfiqar *et al.*, 2012). Rickettsia, *A. phagocytophilum*, is responsible for equine anaplasmosis, is mainly found in Europe, United States after Asia causing economic loss in these areas (Rikihisa, 2010). The aim of present study was to analyse the prevalence of *Anaplasma* sp. and *A. phagocytophilum* in blood samples of equines from Khanewal and Dera Ghazi Khan Districts of Punjab.

The primer used in the present study can amplify the highly conserved sequence of *16S rRNA* gene in *A. marginale*, *A. centrale*, *A. bovis*, *A. phagocytophilum* and *A. ovis*. Since reverse primer sequence had high homology to the corresponding sequences in *A. centrale* (Accession no. AF414868 and AF414869) and *A. ovis* (Accession no. AF414870 and AF309865) and it was difficult to differentiate these three species from each other. Therefore PCR-RFLP method was used to determine the specificity of the PCR products for *A. marginale*. The restriction endonuclease *BssNA1* recognizes the sequence (GTATAC) in corresponding PCR product of *A. marginale* and cut it in the position 68, whereas it cannot cut the PCR product of *A. ovis*, *A. phagocytophilum* or *A. centrale* (GTACGC) (Ashraf *et al.*, 2013).

Hansen *et al.* (2010) reported 22.3% seroprevalance of *A. Phagocytophilum* in Danish horses but seroprevalance is generally considered a less reliable tool as compared to PCR (Ashraf *et al.*, 2013). Torina *et al.* (2007) also detected occurrence of tick borne pathogen, *A. Phagocytophilum* in a variety of domestic animals from Sicily (Italy) by comparison of serological testing and PCR amplification. They found high prevalence of *A. Phagocytophilum* in horses (7.8%),

donkeys (8.9%) and cattle (16.7%) by ELISA. However, none of the ELISA positive samples revealed specific PCR product indicating high false positive. Teglas *et al.* (2005) also reported 27.6% prevalence of *A. phagocytophilum* in cattle and horses from Guatemala based on polymerase chain reaction. Very limited information is available regarding the equine piroplasmosis in Pakistan. There are some reports regarding prevalence of *Anaplasma* spp. in cattle (Sajid *et al.*, 2009; Ashraf *et al.*, 2013) but, to our knowledge, this is the first report regarding the prevalence of *A. phagocytophilum* from horses in Southern Punjab. In the present study, the PCR based prevalence of *A. Phagocytophilum* in horses was 4.3% (Table 1).

Various haematological parameters were also studied using *A. Phagocytophilum*. positive (N = 3) and negative (N = 142) blood samples. Results revealed that parasite prevalence had affected the white blood cells. *Anaplasma* spp. positive equines had lower WBC, lymphocytes and neutrophils than parasite negative animals but difference in lymphocyte count reached the statistical significance ($P < 0.001$) (Table 3). These results are in agreement with general observation that *A. phagocytophilum* infect various types of WBC (McQuiston, 2003). Pusterla *et al.* (2002) had observed a significant decrease in WBC in horses experimentally infected with anaplasmosis by using infected *Ixodes scapularis* ticks.

In conclusion, we have validated a procedure for the detection of *Anaplasma* spp. at molecular level by performing PCR amplification of 16S rRNA gene from the blood of equines and found PCR to be extremely specific and sensitive. As anaplasmosis is common in Pakistan, this technique is recommended to the livestock owners to detect *Anaplasma* sp. and treat their animals to get better productivity.

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