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Molecular detection of *Anaplasma* in apparently healthy Cholistan breed of cattle from the Bahawalpur district, Pakistan

Hussain, M.F.[1], Qamar, M.[1], Malik, M.I.[1], Hussain, M.[1,2], Saeed, Z.[1,2], Shaikh, R.S.[1*] and Iqbal, F.[3*]
[1]Institute of Molecular Biology and Biotechnology. Bahauddin Zakariya University Multan 60800, Pakistan
[2]Livestock and Dairy Development Department, Punjab, Pakistan
[3]Institute of Pure and Applied Biology, Zoology Division, Bahauddin Zakariya University Multan 60800, Pakistan
*Corresponding author e-mail: furhan.iqbal@bzu.edu.pk
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Abstract. The present study was designed to report the prevalence of *Anaplasma* sp. in blood samples of Cholistan breed of cattle from Bahawalpur District and to determine the risk factors associated with the prevalence of this parasite. A total of 148 blood samples were randomly collected from apparently healthy cattle. On the sampling sites, data on the characteristics of the animals (species, gender, age) were collected through questionnaires. 47 blood samples (31.8% of total) produced the 577 base pairs DNA fragment specific for *16S rRNA* gene of *Anaplasma* sp. by PCR amplification. Out of 47 *Anaplasma* sp. positive PCR products, 9 were found to be *Anaplasma marginale* by restriction with *BssNa1* and 9 were confirmed to be *Anaplasma phagocytophilum* (*A. phagocytophilum*) as they amplified 550 bp fragment from the amplified *MSP 2* gene of this species. Risk factor analysis indicated that the presence of parasite was not limited to a particular sex or age group of the infected animals. Comparison of hematological profile revealed that *Anaplasma* sp. positive cattle had significantly reduced levels of mean corpuscular volume (P=0.02) and eosinophils (P=0.02) than in parasite negative animals. While studied serum biochemical profile remain unaffected when compared between the two groups.

INTRODUCTION

Pakistan has been blessed with 15 indigenous breeds of cattle belonging to Zebu breed, *Bos taurus indicus*. Cattle constitute 43% of the total cattle population in the country (Ijaz *et al*., 2008a). Heavy work has been documented on many aspects of Sahiwal (Ijaz *et al*., 2008b) and Red Sindhi (Mustafa *et al*., 2003) breeds of cattle in their respective local climates that have rightly earned them an international fame as being the vital tropical milk breeds of Pakistan (Ahmad *et al*., 2005). However, Cholistan breed of cattle is still unexplored for many aspects of their biology including the parasite prevalence in this breed (Farooq *et al*., 2010).

Tick borne diseases (TBDs) are among the major constraints to the livestock industry in developing countries adversely affecting economic performance, mainly by the spread of severe pathogens, provoking paralysis or toxicosis and causing physical damage to livestock (Rajput *et al*., 2005; Durrani *et al*., 2008; Zulfiqar *et al*., 2012). One of the TBDs is anaplasmosis which is an infectious but non-contagious disease caused by various *Anaplasma* species (Razzaq *et al*., 2015). They are obligate intracellular Gram-negative bacteria placed in order Rickettsiales, which infect domestic and wild ruminants. They are transmitted biologically by tick species (*Ixodes* sp., *Dermacentor* sp., *Rhipicephalus* sp., and *Amblyomma* sp.) (Dumler, 2010; Ashraf *et al*., 2013) and mechanically from infected to susceptible cattle by biting flies or by blood-contaminated fomites including needles,

ear tagging, and dehorning and castration equipment (Skotarczak *et al.*, 2003; Lew and Jorgensen, 2005; Rymaszewska and Grenda, 2008). Various forms of *Anaplasma* infection may reduce the body weight of infected animals, causes abortion, reduce milk production and frequently lead to death causing economic losses to livestock owners (Sainz *et al.*, 1999; Melendez, 2000; Stuen *et al.*, 2002).

As limited information is available regarding the prevalence of *Anaplasma* sp. infection in ruminants from Pakistan, the present study was designed to report the prevalence of *Anaplasma* sp. in blood samples of Cholistan breed of cattle from Bahawalpur district. Furthermore, the present study will provide a baseline data regarding risk factors associated with *Anaplasma* sp. infection and the potential affect of the parasite on the hematology and selected parameters of serum biochemical profile in Cholistan cattle.

MATERIALS AND METHODS

Sample and Data Collection

Blood samples of 148 apparently healthy cattle of Cholistan breed were randomly collected from Bahawalpur district (29° 23. 44" N, 71 41' 1" E). Bahawalpur has a desert climate, with hot, arid summers and virtually no rainfall throughout the year (Saeed *et al.*, 2016). Blood (5 ml) was collected from the jugular vein of the animals and preserved in micro centrifuge tubes by adding 0.5 M EDTA. A questionnaire was filled at the spot in order to gather data of risk factors associated with bovine anaplasmosis. All the animal handling procedures and lab. protocols were approved by the ethical committee of Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University Multan, Pakistan (Permission number IMBB/ETH/ 2014-2212).

DNA Extraction

Inorganic method of DNA extraction was used following Ashraf *et al.* (2013). The purity and integrity of DNA samples were assessed by measuring the optical density at 260/280 nm and conducting submerged gel electrophoresis. The quantity of DNA was measured by formula given as:

DNA ng/µl = Dilution factor x A260 x 50 (standard conc. of DNA i.e. 25ng/µl)
Where dilution factor = Total water / total stock DNA

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 µl. 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* positive sample, isolated during previous study, and 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 electrophorsed on a 1.8% agarose gel and visualized under a UV Trans illuminator (Biostep, Germany).

Digestion of the *Anaplasma* sp. positive PCR products 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 Tris-HCl (pH 8.5 at 30ºC), 10 mM $MgCl_2$, 100 mM KCl, and 100 µg/ml BSA], 0.4 µl *BssNA1* Enzyme (Vivantis, UK).

The *Anaplasma* sp. positive 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') that can amplify 550-base pair amplicon of *MSP2* gene of *A. phagocytophilum* following 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 p mol), *Taq* DNA polymerase (Vivantis, UK) (0.625 U) and 1X reaction buffer (50 mM KCl; 10 mM Tris HCl of 8.3pH). Thermal profile used for amplification was the same as formerly applied by Ashraf *et al.* (2013). Initial denaturation was brought about at 95°C for 5 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 90 s and final extension was carried out at 72°C for 5 min.

Hematological and Serological Analysis Various hematological and serum biochemistry parameters in blood samples from Cholistan cattle i.e. packed cell volume, total red and white blood cell count, mean corpuscular volume, total lymphocytes count, granulocytes count, hemoglobin (Hb), Aspartate aminotransferase (AST), Triglycerides (TG), Gamma-glutamyl transferase (GGT), Cholesterol (CHO) and Albumin (ALB) were determined in *Anaplasma* sp. positive and negative blood samples of Cholistan breed cattle by using Metertek SP-8SO spectrophotometer (Korea) and diagnostic kits manufactured by BioMed (Germany) following Hussain *et al.* (2016).

Statistical Analysis

Statistical package Minitab (Minitab @ 17 USA) was used for statistical analysis. Cattle were categorized into two age groups, less than 1 year and more than 1 year old. Association between the presence of *Anaplasma* sp. and various studied epidemiological parameters, i.e. sex, age was analyzed by contingency table analysis using the Fisher's exact test (for 2 x 2 tables. Two sample T-test was applied to compare the hemato-biochemical profile between parasite positive and negative animals.

RESULTS

Prevalence of *Anaplasma* sp.

Anaplasma sp. was detected in 47 out of 148 collected blood samples (31.8%) from Cholistan breed cattle during present study as they amplified a 577 base pair product of *16S rRNA* gene specific for *Anaplasma* sp. (Fig. 1).

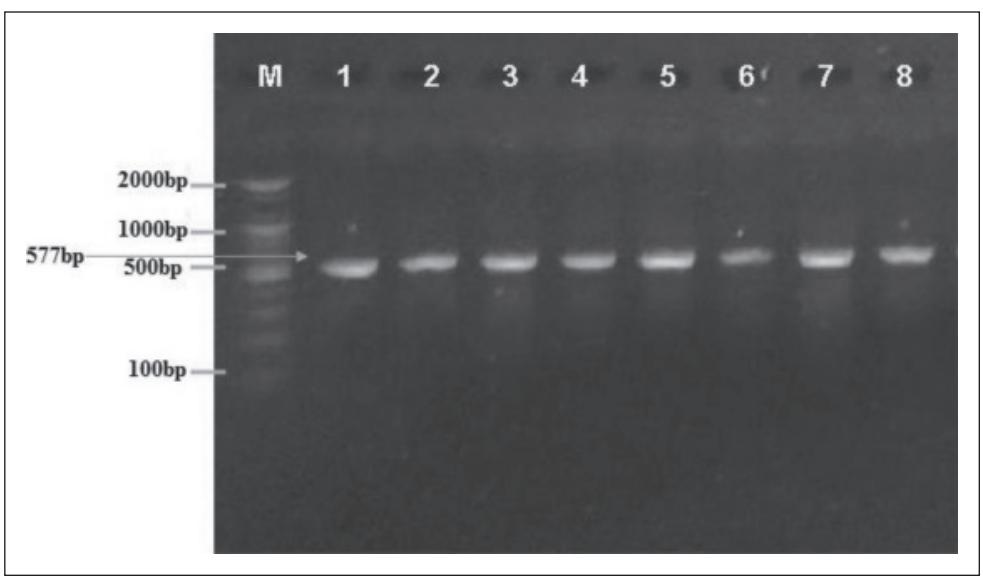

Figure 1. PCR amplification of 16S rRNA gene in *Anaplasma* sp. Lanes: M, 100 bp DNA ladder; 1-3 *Anaplasma* negative, 4 control negative 5 *Anaplasma* positive.

Prevalence of *Anaplasma marginale*

In restriction analysis all the *Anaplasma* sp. positive PCR products with *BssNA1* resulted into confirmation of 9/47 (15.2%) samples positive for *Anaplasma marginale* (restriction activity cleaved the 577 base pair product into two fragments of 509 and 68 bp) (Fig. 2).

Prevalence of *Anaplasma phagocytophilum*

By using *Anaplasma phagocytophilum* specific oligonucleotide primers resulted in amplification of 550 base pair fragment from *MSP 2* gene of this parasite in 9 of the total 47 (15.2%) *Anaplasma* sp. positive samples (Fig. 3).

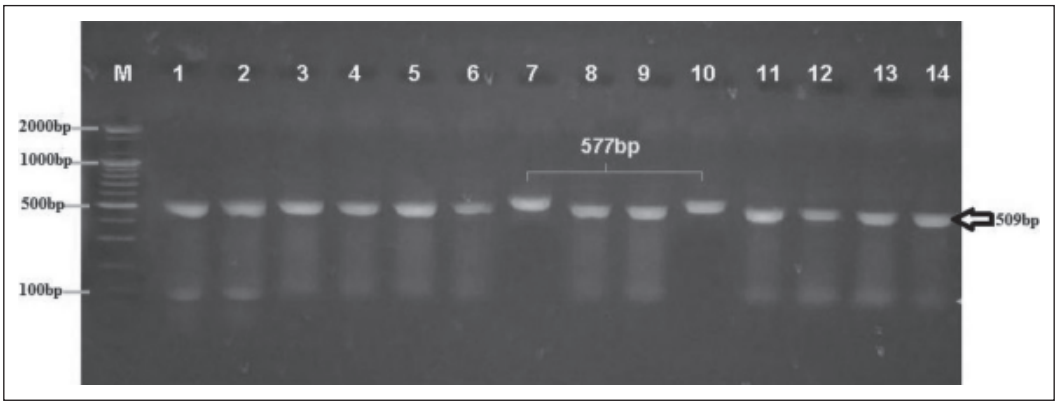

Figure 2. *Anaplasma* sp. positive PCR products were restricted by restriction enzyme BssNA1 producing 509 bp fragments. M 100 bp DNA ladder; Lanes 8-9 and 11-14 *Anaplasma marginale*; Lanes 1-7 and lane 10 undigested *Anaplasma* sp.

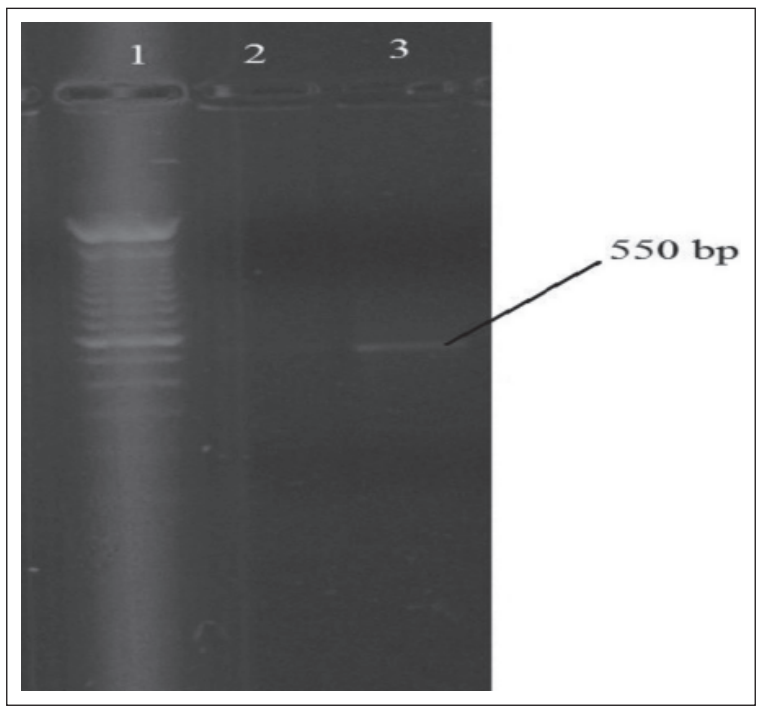

Figure 3. PCR amplification of *MSP 2* gene of *Anaplasma phagocytophilum*; Lane: 1, 100 bp DNA marker; Lane: 1-2 *A. phagocytophilum* positive.

Risk factor associated with *Anaplasma* sp.

Analysis of studies risk factors revealed that prevalence of *Anaplasma* sp. was not restricted to a particular gender or age group of Cholistan cattle under investigation as both these parameters were non significantly ($P > 0.05$) associated with the presence of the parasite (Table 1).

Analysis of hematobiochemical profile

Comparison of hematological profile revealed that *Anaplasma* sp. positive cattle had significantly reduced levels of mean corpuscular volume (P=0.02) and eosinophils (P=0.02) than in parasite negative animals. All other studied parameters varied non significantly ($P > 0.05$) between the two groups. Serum biochemical profile remain unaffected when compared between the *Anaplasma* sp. positive and negative animals (Table 2).

DISCUSSION

Animal diseases are perhaps the most important limitation to animal production in developing countries. According to Food and Agriculture Organization (FAO) estimates, there are 50% production losses because of their fatality and adverse effects on animal productivity in these countries due to various tick borne diseases like East coast fever, anaplasmosis and piroplasmosis (Nasir, 2000; Akhter *et al.*, 2010).

As limited information is available in literature regarding *Anaplsma* sp. incidence from Pakistan, present research was designed to report the prevalence of genera *Anaplasma* sp. in blood samples of Cholistan breed cattle from Bahawalpur and to detect the presence of *Anaplasma marginale* and *Anaplasma phagocytophilum* among the positive samples.

The primers 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 the primer sequence had a high homology to the corresponding sequences in *A. centrale* (Accession Nos. AF414868 and AF414869) and *A. ovis* (Accession Nos. AF414870 and AF309865) and it was difficult to differentiate these 3 species from each other, the 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 products of *A. marginale* and cut it in the position 68, whereas the used restriction enzyme cannot cut the corresponding PCR product of *A. ovis* (GTACGC) or *A. centrale* (GTACGC). In the present study, the prevalence of *Anaplasma* sp., *Anaplasma marginale* and *Anaplasma phagocytophilum* was 47/148(31.8%), 9/148 (6.08%) and 9/148(6.08%) respectively in blood samples of Cholistan cattle collected from Bahawalpur district. In a recent study from Pakistan, Ashraf *et al.* (2013) has reported 41% prevalence of *Anaplasma* sp. in buffalo samples collected from 7 cities on 2 provinces. While 7.1% of the total 281 analysed samples were found positive for

Table 1. Association between prevalence of *Anaplasma* sp. in blood samples of Cholistan breed cattle collected from Bahawalpur and animal characteristics studied during present study. P values represent the output of Fisher's exact test

Subject	Parameters		Sample number (N)	*Anaplasma* sp. +ve	*Anaplasma* sp. -ve	P-value
Cholistan breed cattle	Sex	Male	59	19	40	0.92
		Female	89	28	61	
		All	148	47	101	
	Age	> 1 Year	116	36	80	0.72
		< 1 Year	32	11	21	
		All	148	47	101	

Table 2. Comparison of various studied parameters of hematobiochemical profile between *Anaplasma* sp. positive and negative Cholistan breed cattle samples from Bahawalpur. P-value represents the result of 2 sample t-test calculated for each parameter

	Parameters	*Anaplasma* +ve	*Anaplasma* -ve	P- value
Hematological Profile	WBC (10^3/µ1)	9.35 ± 0.04	9.59 ± 0.03	0.41
	LYM (10^3/µ1)	73.65 ± 0.07	73.21 ± 0.50	0.94
	NEUT (10^3/µ1)	21.68 ± 0.07	22.49 ± 0.05	0.48
	MONO (10^3/µ1)	2.17 ± 0.1	2.36 ± 0.06	0.78
	EOS (10^3/µ1)	2.44 ± 0.05	1.89 ± 0.02	0.02*
	RBCs (10^6 /µ1)	7.32 ± 0.03	7.34 ± 0.02	0.93
	Hb (g/dl)	11.13 ± 0.1	11.27 ± 0.02	0.48
	HCT/PCV	35.32 ± 0.03	34.89 ± 0.88	0.16
	MCV (f/l)	19.78 ± 0.06	48.19 ± 0.25	0.02*
Serum Biochemical Profile	AST (U/L)	98.80 ± 0.07	56.52 ± 0.05	0.15
	TG (mg/dl)	23.4 ± 0.04	24.45 ± 0.03	0.89
	GGT (U/L)	15.95 ± 0.06	18.22 ± 0.05	0.52
	CHO (U/L)	156.76 ± 0.07	151.73 ± 0.04	0.24
	ALB (U/L)	3.41 ± 0.03	3.39 ± 0.02	0.92

$P < 0.05$ = significant.

WBCs = White blood cells, LYM = Lymphocyte, NEUT = Neutrophils, MONO = Monocytes, EOS = Eosinophils, RBCs = Red blood cells, Hb = Hemoglobin, HCT / PCV = Hematocrit / Packed cell volume, MCV = Mean corpuscular volume, AST = Aspartate aminotransferase,TG = Triglycerides, GGT = Gamma-glutamyl transferase CHO = Cholesterol, ALB = Albumin.

Anaplasma marginale. In an earlier report, Haider and Bilqees (1988) had reported 61% *Anaplasma* sp. prevalence in Karachi and adjoining areas. All these reports are confirming that *Anaplasma* sp. infection is endemic and the differences observed regarding *Anaplasma* sp. prevalence in various regions of the same country are due to different elements associated with the occurrence of tick borne ailments including gender, age, animal type, ticks load, seasonal variation, climate and geographical distribution of the area (Magona *et al.*, 2011).

Analysis of studied risk factors revealed that prevalence of *Anaplasma* sp. was not restricted to a particular gender or age group of Cholistan cattle under investigation (Table 1). Our results are in agreement to Ashraf *et al.* (2013).

In present study, various hematological parameters were compared between *Anaplasma* sp. positive (N=47) and negative (N=101) blood samples and Mean corpuscular volume (P=0.02) and eosinophil count (P=0.02) was significantly lower in cattle infected with the parasite (Table 2). Haemogram of the infected animals revealed microcytic anaemia as indicated by significant decrease ($P<0.05$) in MCV in the infected groups positive by PCR as compared to the health control group. Haemopiotic system was activated in response to erythrophagocytosis, Our results are in agreements to Sharma *et al.* 2013 as they found same results studied in dairy animals in Punjab (India).

Concerning the effect of *Anaplasma* infection on activity of liver enzymes, the obtained results of the study revealed no significant increase in serum AST in infected cattle when compared with healthy cattle. These results were in agreement with other studies reported by Allen *et al.* (1981). For the studied serum biochemical parameters [AST (Aspartate aminotransferase), TG (Triglycerides), GGT (Gamma-glutamyl transferase) CHO (Cholesterol) and ALB (Albumin)], we did not observed any significant difference ($P > 0.05$) between *Anaplasma* sp. positive and negative

animals (Table 2). Our results are in agreement with the findings of Jassem and Agaar 2015 as they found none of the studied sero-parameter varying significantly between *Anaplasma* sp. positive and negative animals.

In summary, we have used PCR-RFLP protocols for the molecular detection of *Anaplasma* sp., *Anaplasma marginale* and *Anaplasma phagocytophilum* in the blood of apparently healthy cows. As anaplasmosis is endemic in Pakistan with very high prevalence, we recommend this technique to livestock owners for the screening of their herds that will enable them to detect *Anaplasma* sp. even when the parasitemia is extremely low and prophylactic treatment their animals will result in better output and hence improved economy of livestock owners and Pakistan.

Conflict of interest

Authors declare no conflict of interest of any sort with anyone.

Author's contributions

RSS designed the study, ZS, MH, MQ and MIM collected the samples, MFH conducted the lab experiments, FI analyzed the data and prepared the manuscript.

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