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Short Communication

FIRST MOLECULAR SURVEILLANCE AND ESTIMATION OF RISK FACTORS OF *ANAPLASMA MARGINALE* INFECTION AMONG INDIGENOUS, CROSSBRED AND EXOTIC CATTLE

F. A. Atif[1*], M. A. Zaman[2], K. Hussain[1,4], M. F. Qamar[2], M. S. Sajid[3,4], U. Iqbal[2] and S. Mehnaz[1,4]

[*1]Medicine Section, Department of Clinical Sciences, College of Veterinary and Animal Sciences, Jhang (Sub-campus); University of Veterinary and Animal Sciences, Lahore, Pakistan.
[2]Parasitology Section, Department of Pathobiology, College of Veterinary and Animal Sciences, Jhang (Sub-campus); University of Veterinary and Animal Sciences, Lahore, Pakistan.
[3]One Health Laboratory, Center for Advanced Studies in Agriculture and Food Security, University of Agriculture, Faisalabad, Pakistan.
[4]Department of Parasitology, Faculty of Veterinary Science; University of Agriculture, Faisalabad, Pakistan.
[*]Corresponding author's email: farhan.atif@uvas.edu.pk

ABSTRACT

Anaplasmosis is an important disease of cattle, worldwide including Pakistan. Blood samples (n=384) were collected from asymptomatic dairy cattle located in all tehsils of district Jhang belonging to either age, sex and breed. Samples were analyzed for the detection of *A. marginale* infection using blood smear microscopy and PCR targeting *msp1β* gene. Epidemiological information related to host, area, season and management was collected on a questionnaire. Overall prevalence of anaplasmosis based on blood smear and PCR was 29.43% (113/384) and 37.24% (143/384); respectively. Univariate analysis, indicated age breed, tick infestation, herd size, feeding pattern, interval between acaricidal application and season as significant risk factors ($p \leq 0.20$). Nevertheless, multivariate analysis revealed age of >2-4 years (OR 1.95), exotic cattle (OR 2.23), herd size >50 (OR 2.09), tick infestation (OR 2.56), 30 days interval between acaricidal application (OR 3.21) and summer season (OR 2.56) were significantly at higher risk ($p<0.05$) associated with molecular occurrence of *A. marginale* infection in cattle. Based on the results of first molecular surveillance, it can be concluded that bovine anaplasmosis is endemic in the area and tick infested exotic young cattle kept in larger herds in summer season are at higher risk associated with *A. marginale* as compared to indigenous and crossbred animals. Further studies are required at large scale to investigate genetic diversity for better prevention and control of bovine anaplasmosis in the region.

Keywords: *Anaplasma*, molecular epidemiology, risk factors, cattle, Jhang

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INTRODUCTION

Anaplasmosis is one of the most important disease of cattle and other domestic animals worldwide including Pakistan (Atif, 2016). Disease is mainly caused by *Anaplasma (A.) marginale* characterized by fever, weight loss, abortion, anorexia, emaciation, Jaundice, anemia, decline in milk production and even death of the infected animal (Allan, 2001; Howden *et al*., 2010; Kocan *et al*., 2010). In Pakistan, the prevalence of *Anaplasma* ranges from 4.3 to 60% but most of them were either based on microscopy or serology (Atif *et al*., 2013a; Jabbar *et al*., 2015). Transmission of *Anaplasma* can be accomplished through ticks (biological vectors), mechanically through fomites, biting flies and from dam to calf through placenta (Ewing, 1981; Costa *et al*., 2016). Host, environment, ticks/flies-burden, housing, management system and exposure of animal to fomites are important risk factors from different regions (Kumar *et al*., 2011; Atif *et al*., 2013b; Sajid *et al*., 2014; Zhou *et al*., 2019; Spare *et al*., 2020; Zeb *et al*., 2020; Boucher *et al*., 2020). Blood smear examination is less sensitive and fails to detect persistent infection with low parasitemia. Modern molecular techniques are more sensitive as compared to conventional microscopy and serology. Accurate diagnosis and identification of risk factors are important from disease control standpoint (Rahman *et al*., 2019). As far as we can ascertain, there was no information available regarding molecular prevalence of *A. marginale* in carrier cattle targeting *msp1b* gene and associated risk factor from all tehsils of district Jhang (Pakistan).

MATERIALS AND METHODS

Study Location: The study was performed in Jhang district, Punjab, Pakistan which is at 72° or 72.32 longitudes towards east and 31° or 31.26 latitude towards north and at 512.4 ft elevation. The district Jhang has four tehsils *viz.* Jhang Sadar, Shorkot, Athara Hazari and Ahmadpur Sial. Geologically, district Jhang has additional

importance, as it is located on the embankment of river Chenab and Jhelum. It has been estimated that there are about 1.13 million large animals, 0.69 million small animals and 0.04 million poultry birds in district Jhang. The meteorological circumstances of district Jhang are similar to desert which arrays from 5°C to 40°C temperature with 248mm of annual rain fall (L&DD Punjab, 2019; Pakistan Metrological Department, 2019).

Collection of blood samples: Blood samples (n=384) were randomly collected in sterilized syringe from jugular vein of asymptomatic adult dairy cattle from dairy farms located in all tehsils of district Jhang, Pakistan belonging to either age, sex and breed. The jugular blood was added into vacutainer containing EDTA (Improvacuter®, Improve Laboratory Supply, East-Flanders, Belgium) and transported in ice box to Department of Clinical Sciences, College of Veterinary and Animal Sciences, Jhang for further processing.

Sample size was estimated assuming 50% prevalence using following formula (Thrusfield, 2007):

$$n = 1.96^2 P_{exp} (1 - P_{exp}) / d^2$$

In this formula:

n= Sample size

P_{exp} = Prevalence expected

d= Absolut percision desired

Questionnaire administration for risk factor assessment: A questionnaire was developed having dichotomous/poly-chotomous questions designed for dairy farmers having closed ended questions. Determinants related to host age (<1year, 1-2 years, >2-4 years and >4 Years), breed (indigenous, crossbred and exotic breed), gender (male and female) and presence of ticks (*Rhipicephalus* and/or *Hyalomma)*. Ecological factors including area (Jhang Sadar, Ahmadpur Sial, Shorkot and Athara Hazari), season (Summer, Autumn, Winter and Spring) and managemental factors were included.

Blood smear preparation and examination: Thin blood smears were prepared and fixed with 100% methanol, stained with Giemsa stain diluted at 1:10 ratio for 25-30 minutes. The smears were rinsed for three to four times with tap water to wash extra stain and then air dried. The slides were observed under oil immersion lens of compound microscope (Euromex; Model: BB 4253, Netherland) at 100X magnification. With respect to location of intra-erythrocytic bodies they were declared as *A. marginale* (located on margin) and *A. centrale* (located in the center). The *Anaplasma* was identified as mentioned by OIE Terrestrial Manual, 2012. Microscopic fields (n=20) were observed in search of the selected blood parasites.

Molecular analysis: The DNA from blood was extracted as per directions of manufacturer (Thermofisher Scientific, USA; Catalogue No. K0722 and Catalogue No. K0782). Finally, the elution step was repeated to enhance the quantity and quality of extracted DNA. The extracted DNA was either stored at -20°C or subjected to PCR.

Amplification of *msp1β* gene: The PCR was performed by amplifying *msp1β* gene using 25 µl of PCR reaction mixture comprising 12.5µl of Master mix (PCR) (Thermofisher Scientific, USA; Catalogue No. K1081).The 1µl (b1) of forward primer (MAR1bB2 primer: 5'-GCT CTA GCC GGT TAC GCG TC-3') and 1µl (b2) of reverse primer (MAR1bB2 primer: 5'- CTG CTT CGG AGA ATA CAC CT-3') specific for *msp1β* gene of *A. marginale* was used. The 5.5µl of nuclease free water and 5µl of template DNA was utilized (Bilgiç *et al*., 2013). The PCR mixture of25 µl was placed in thermal cycler T100 (Bio Rad, USA) using protocol as described by Bilgiç *et al*. (2013). The PCR product was run on agarose gel (1.3%) in 1X TAE buffer (Tris-acetate-EDTA; 20 mM acetic acid; 40 mM Trisand 1 mM EDTA) at 90V in gel tank for 30 minutes and visualized on UV illuminator.

Statistical analyses: The potential risk factors based on questionnaire, were estimated initially with univariate model between the presence of disease (outcome variable) and explanatory variables. Those variables that were significantly associated ($p \leq 0.2$) were remained and analyzed further for inter-correlation. Only the significant variables were qualified and assessed from final multivariate logistic regression to determine the association and significance of disease occurrence and variables using Minitab 16 software. The variables with $p<0.05$ were considered significant for multivariate analysis. Those variables that were not kept, later reoffered to the model to evaluate significance and confounding. In this study, positive odds ratio (95% confidence interval) represent that an outcome is more likely associated as compared to negative.

RESULTS AND DISCUSSION

Conventional blood smear microscopy and molecular based surveillance was conducted among indigenous, crossbred and exotic cattle (n=384) in all tehsils of Jhang district. Tehsil-wise prevalence was found highest in Jhang Sadar (48.34%; 73/151) followed by Athara Hazari (44.26%; 27/61), Ahmad pur Sial (30.26%; 23/76) and Shorkot (20.83%; 20/96); tehsils. As far as the spatial factors are concerned, agro-climatic conditions are not much divergent and similar conditions prevail except some parts of desert in district Jhang. Overall prevalence of *A. marginale* in cattle using molecular technique was 37.24% (143/384) including indigenous 28.23% (35/124), crossbred 35.48 (66/186) and exotic cattle 56.76 % (42/74). The 265 base pair PCR product was detected in agarose gel (Plate 1). Whereas, blood smear examination had revealed that 113 samples were positive for anaplasmosis. Among these, 67 and 46 samples were found positive for *A.*

marginale and *A. centrale*, respectively. Based on microscopic scrutiny, the overall prevalence of anaplasmosis was 29.43%. In the present study, molecular prevalence was recorded higher as compared to conventional microscopy. Blood smear examination is simple, convenient, less time consuming, cost effective detection method of hemo-pathogens but it is less sensitive at lower parasitemia because it may show large number of false positive. Additionally, it needs technical expertise and individual variation may encounter upon observation of morphologically similar pathogens.

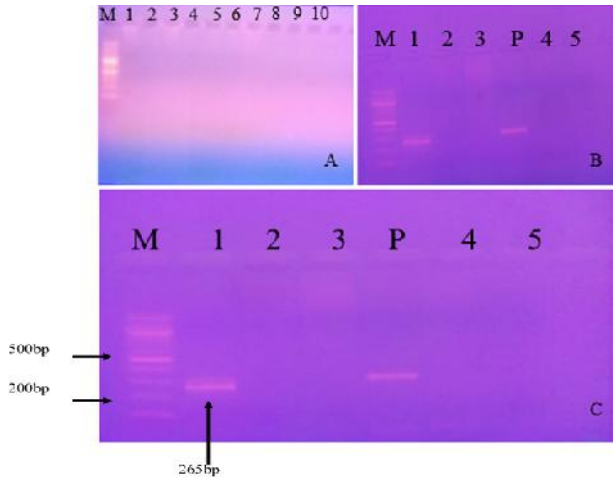

Plate 1. The UV-Illuminated images of *A. marginale* in agarose gel. A. Ladder expression having no amplification of template, B & C. Ladder visualization, with control and positive sample (265 bp).

Female Holstein Friesian breed of >4 years of age was found more positive than male animals of native breed. Some of the researchers have mentioned that prevalence is influenced by gender and climatic conditions. Our findings showed that prevalence was higher in female (45.12%) than male animals (23.19%) with non-significant association (p<0.05). These results are in agreement with Ait Hamou *et al*. (2012); Atif *et al*. (2013 a, b) and Okafor *et al*. (2018). While, incoherent with the findings of Khan *et al*., (2004); Ashraf *et al*. (2013) and Zeb *et al*. (2020). Higher prevalence of infection in female than male animals might be due to immuno-compromised conditions like pregnancy, lactation or using contaminated needles for milk let down. Breed has a significant role in disease outcome. *Bos taurus* is more prone to ticks and tick-borne diseases linked with higher disease severity than *Bos indicus* and their crosses (Atif, 2016).

Prevalence of anaplasmosis was noticed higher during Summer (58.33%) followed by Spring (35.42%), Autumn (31.25%) and Winter (23.96%). Our findings are supported by Atif *et al*. (2013a); Sajid *et al*. (2014), Dharanesha *et al*., 2017 and Okafor *et al*., 2018; they described highest prevalence during late spring and summer. Furthermore, during the months of monsoon higher infection rate has been noticed on account of higher humidity and tick burden (Roy *et al*., 2018). Occurrence of disease during other seasons might be due to its mechanical routes of transmission (Atif, 2015).

For estimation of risk factors initially univariate model and then significant factors (p<0.20) were retained for final multivariate analysis. During univariate analysis age, breed, herd size, feeding pattern, tick-infestation, interval between acaricidal application and season were identified as important risk factors (p<0.20).Whereas, final multivariate model revealed age of >2-4 years (OR 1.95), exotic cattle (OR 2.23), herd size > 50 (OR 2.09), tick infestation (OR 2.56), 30 days interval between acaricidal application (OR 3.21) and summer season (OR 2.56) were significantly at higher risk ($p<0.05$) associated with molecular prevalence of *A. marginale* in cattle (Table-1). Nonetheless, Ait Hamou *et al*. (2012) and Zhou *et al*. (2019) suggested that there is no significant effect of age, gender and breed on prevalence of *A. marginale*. Most of our risk factors finding regarding breed, tick infestation and acaricidal treatment are in agreement from various studies/countries such as USA (Kansas), Puerto Rico, The Union of The Comoros and earlier studies from Pakistan (Punjab and KPK provinces) (Urdaz-Rodriguez *et al*., 2009; Atif *et al*., 2013b; Farooqi *et al*., 2018; Zeb *et al*., 2020; Spare *et al*., 2020; Junsari *et al*., 2020; Boucher *et al*., 2020). Prevalence and risk factors vary from region to region depending on age, gender, breed of animals, diagnostic approach, tick burden, season, geographical region, agro-ecological conditions and husbandry practices (Farooqi *et al*., 2018; Boucher *et al*., 2020; Zeb *et al*., 2020; Spare *et al*., 2020). Based on the results of first molecular surveillance, it can be concluded that bovine anaplasmosis is endemic in the region. Tick infested exotic young cattle kept in large herd size in summer season are at higher risk of *A. marginale* infection. Further studies, on genetic diversity of these isolates is required for devising better prevention and control strategies.

Table 1. Multivariate analyses of risk factors associated with molecular prevalence of *A. marginale.*

					95% C.I	
Variables	**Levels**	**β0**	**P-value**	**Odds ratio**	**Lower CI**	**Upper CI**
Animal variables						
Age[1,3],[5]	< 1 year[a]	1.23	0.033	0.78	0.55	0.95
	1-2 years	1.09	0.038	0.89	1.32	2.78

	>2-4 years	2.01	0.022	1.95	1.11	2.32
	>4 years	1.57	0.028	0.98	0.53	1.21
Breed[2,3],[5, 6]	Exotic	1.56	0.025	2.23	1.98	2.45
	Crossbreed	2.11	0.042	1.98	1.98	2.87
	Indigenous[a]	1.49	0.056	1.11	0.98	1.28
Herd size[2,3, 4, 6]	≤ 10 animals[a]	2.81	0.003	1.21	1.01	1.35
	> 10 animals ≤ 50 animals	0.74	0.043	1.98	1.56	2.32
	> 50 animals	0.97	0.402	2.09	1.89	2.21
Feeding[5,6]	Grazing[a]	1.21	0.048	1.48	1.23	1.76
	Stall feeding	1.60	0.072	2.76	2.12	3.11
Tick	High	0.52	0.053	2.56	2.13	2.87
infestation[12,3]	Low	0.42	0.045	1.87	1.53	2.21
	No[a]	0.44	0.153	1.21	1.12	1.53
Interval b/w	30 days	1.53	0.048	3.21	3.10	3.32
acaricidal	60 days	-0.64	0.037	2.87	2.76	2.98
application[5]	90 days	1.03	0.220	2.34	2.10	2.83
	120 days	2.45	0.046	1.95	1.83	2.25
	180 days[a]	1.44	0.027	1.78	1.45	1.89
	More than 180 days	0.33	0.012	1.34	1.03	1.67
Season[1]	Summer	1.55	0.014	2.56	2.34	2.76
	Spring	1.40	0.058	1.80	1.75	1.90
	Autumn	1.20	0.033	1.31	1.21	1.58
	Winter[a]	1.21	0.022	1.01	0.92	1.13

[a] Reference category; [1]Simuunza *et al*., 2011;[2]Ola-Fadunsin *et al*., 2018; [3]Farooqi *et al*., 2018; [4]Boucher *et al*., 2020; [5]Zeb *et al*., 2020; [6]Spare *et al*., 2020.

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