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SERO-EPIDEMIOLOGICAL STUDY OF *ANAPLASMA MARGINALE* AMONG CATTLE

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ABSTRACT

The study was designed to determine the risk factors associated with seroprevalence of *Anaplasma marginale* among cattle in Sargodha, Khushab and Rawalpindi districts of the Punjab, Pakistan during September, 2009 to August, 2010. A total of 1050 serum samples were collected from selected small holders and private livestock farms using multistage cluster random sampling technique. The epidemiological data and relevant information regarding management, host and area determinants were collected on a questionnaire through interview. Antibodies against *Anaplasma marginale* were detected by MSP-5 competitive enzyme linked immunosorbent assay. Multivariate analysis of risk factors revealed that cattle of more than 4 year of age (OR=5.42), heavy tick infested (OR=2.10), crossbred (OR=1.59) cattle were significantly at higher risk for *Anaplasma marginale*. Use of Ivermectin (OR=16.50), moderate interval of acaricide frequency (OR=16.50), stall feeding (OR=8.30) and use of unhygienic needles (OR=24.00) at farms were significantly associated with seroprevalence to *Anaplasma marginale* in cattle (*P<0.05*). The Sargodha district was at higher risk (OR=1.81) for *Anaplasma marginale* infection as compared to Khushab and Rawalpindi districts. The study would help in the prevention and control of bovine anaplasmosis in Northern Punjab, Pakistan.

Key words: *Anaplasma marginale,* seroprevalence, risk factors, cattle.

INTRODUCTION

Anaplasmosis is one of the most prevalent haemo-rickettsial diseases of cattle especially in tropical and subtropical regions including Pakistan (Dumlar *et al*., 2001; Khan *et al*., 2004). Bovine anaplasmosis is usually caused by *Anaplasma (A.) marginale* characterized by weight loss, severe anaemia, jaundice, weakness, fever, brownish urine, pale mucous membranes, decreased milk production, abortion, hyper-excitability and death in severe cases (Richey and Palmer, 1990).

The potential tick vectors of bovine anaplasmosis are not known in Pakistan. Biting flies, blood-contaminated needles, nose tongs, dehorning instruments, ear tagging devices and castration instruments or other contaminated fomites contribute in mechanical transmission (Kahn, 2005; Aubry and Geale, 2011).

Adequate knowledge regarding disease determinants is necessary for the effective prevention and control of anaplasmosis in the region. Knowledge about the risk factors of *A. marginale* is lacking in study areas (Sargodha, Khushab and Rawalpindi districts) of the Punjab, Pakistan. Therefore, the study was designed to estimate the risk factors associated with seroprevalence of *Anaplasma marginale* among cattle in Punjab, Pakistan.

MATERIALS AND METHODS

Study area: Epidemiological studies were conducted in Sargodha, Khushab and Rawalpindi districts of the Punjab, Pakistan. Each district has a distinct agro-ecological zone based on temperature, precipitation, topography, irrigation and soil type (PARC, 2011).

Sargodha district has flat flooded plain with an average temperature ranging from 25-49^{o}C in summer and 5-23^{o}C in winter and annual rainfall of 526 millimeter. The district has loamy soil with canal water and tube well irrigation **(**GOP, 2011**).** Agro-ecologically the district is included in central mix cropping zone (PARC, 2011). Khushab is the driest and hottest district with diverse topography having arid hills of salt range with bushy vegetation in its north (Soon Sakesar Valley) and central part have irrigated lowland plains while southern part has hot dry Thal sandy desert with scarce vegetation. The temperature ranges from 25-48^{o}C in summer and 19-29^{o}C in winter with average annual precipitation of 521 millimeter (GOP, 2011). Rawalpindi district is categorized as arid high rainfall zone (PARC, 2011). It has hilly to sub-hilly topography with humid subtropical climate. The northern part of the district has high hills with annual average rainfall of 1364 millimeter and an average temperature range from 14.1-28.4^{o}C (RRCAP, 2011).

Sampling strategy: A total of 1050 serum samples were collected from randomly selected small holders *(n=90)*

and private livestock farms *(n=12)* using multistage cluster random sampling technique (Thrusfield, 2005). All the union councils in each district were included in the sampling frame. A total of 30 union councils, 34 cattle farms (30 small holders and 4 livestock farms) and 350 animals were selected as primary, secondary and tertiary sampling units from each district. Sampling unit was indigenous and crossbred cattle of both the sexes. The criteria for the selection of small holders and private livestock farms were: a) small holder having 1-10 cattle; (b) Livestock farm having ≥50 cattle; (c) Distance between small holder farms ≥5 kilometer; (d) Distance between livestock farms ≥10 kilometer.

Serological screening: Antibodies against *A. marginale* were detected by MSP-5 competitive enzyme linked immunosorbant assay (cELISA) using commercially available Anaplasma Antibody Test Kit, cELISA (VMRD, Pullman, WA, USA). The test was performed according to the manufacturer's instruction. An ELISA reader (Statfax® 2100 Microplate Reader, Awareness Technologies, Inc.) was used to measure the optical density at 620 nm wavelength. Serum samples with >30% inhibition were considered positive and samples with <30% inhibition were considered negative. The percent inhibition was calculated using formula:

$$\text{Inhibition percentage} = \frac{100 - \text{Sample optical density}}{\text{Mean negative control optical density}} \times 100$$

Questionnaire administration: The epidemiological data and related information regarding area, host and management were collected on a questionnaire through interview from livestock owners, attendants or managers of cattle farms. The informants were requested to provide information regarding farm category, farm type, herd size, type of housing, feeding, animal species at farm, type of acaricide and their frequency and use of unhygienic needles. The selected farms were visited each month in each district for the collection of relevant epidemiological data.

Statistical analysis: A multivariate logistic regression was applied for analyzing risk factors by using Statistical Package for Social Services (SPSS) version 13.0. A *p*-value <0.05 was considered statistically significant.

RESULTS

Multivariate analysis of risk factors revealed that cattle of more than 4 year of age were significantly (P<0.001; OR=5.42) at higher risk as compared to >2-4, 1-2 and <1 year age cohorts. Heavy tick infested (P<0.001; OR=2.10), crossbred (P<0.01; OR=1.59) cattle was significantly at higher risk as compared to low tick infested indigenous cattle. On the contrary, sex and the health status of the animals had non significant association (P>0.05; Table 1).

Farm management factors, significantly associated (P<0.05) with seroprevalence of *A. marginale* include stall feeding (P<0.05; OR=8.30), moderate acaricide frequency (P<0.05; OR=16.50), use of ivermectin (P<0.05; OR=3.97) and unhygienic needles (P<0.001; OR=24.00). Avermectin (ivermectin) was found at higher risk compared to pyrethroid and organophosphate class of acaricide. Among Avermectin, ivermectin was the only product used by the farmers. Farm type (beef, dairy, mix), herd size (1-4, 5-10, 11-20, 21-50, >50), livestock species at farm (cattle, buffalo, mix livestock), sources of animals (brought in vs. home bred) and farm instruments (nose punch, ear pliers, dehorner, docking instruments) were not significantly involved (P>0.05). Significant association was found among different districts (P<0.01). The Sargodha district (OR=1.81) was at higher risk for seropositivity whereas, Khushab and Rawalpindi districts were at lower risk for *A. marginale* (Fig. 1; Table 1).

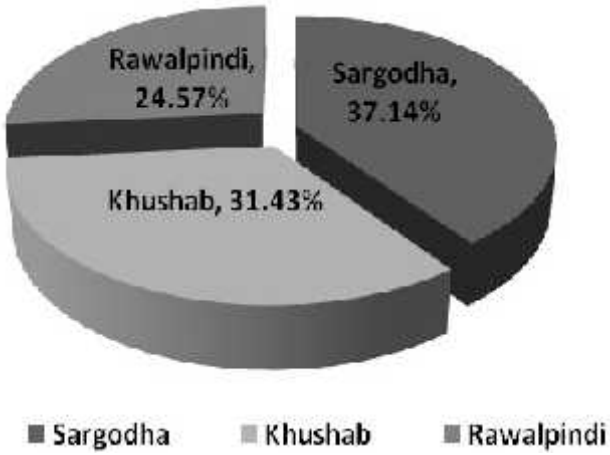

Fig 1. District-wise seroprevalence of *Anaplasma marginale* in cattle.

Table 1. Multivariate analysis of risk factors associated with seroprevalence for *Anaplasma marginale* in Punjab, Pakistan from September, 2009 to August, 2010.

					95% CI	
Variables	**Levels**	***b*[a]**	***p*-value**	**Odds ratio**	**Lower CI**	**Upper CI**
ANIMAL VARIABLES						
Age	<1 yr	-1.751	0.000	-	-	-
	1-2 yrs	0.439	0.061	1.55	0.98	2.46
	>2-4 yrs	1.266	0.000	3.55	2.34	5.38
	>4 yrs	1.690	0.000	5.42	3.71	7.92
Sex	Male	-0.926	0.000	-	-	-
	Female	0.171	0.271	1.19	0.87	1.61
Breed	Indigenous	-1.041	0.000	-	-	-
	Crossbred [b]	0.465	0.001	1.59	1.22	2.07
Level of tick infestation	Low[c]	-1.179	0.000	-	-	-
	Moderate[d]	0.147	0.441	1.16	0.80	1.69
	Heavy[e]	0.742	0.000	2.10	1.48	2.98
Health status	Fat	-0.899	0.000	-	-	-
	Fair	0.151	0.412	1.16	0.81	1.67
	Weak	0.095	0.613	1.10	0.76	1.59
FARM VARIABLES						
Farm type	Beef	1.609	0.142	-	-	-
	Dairy	0.875	0.483	2.40	0.21	27.78
	Mix	1.280	0.304	3.60	0.31	41.37
Presence of *R. microplus*	No	1.673	0.008	-	-	-
	Yes	1.309	0.107	3.70	0.75	18.17
Herd size	1-4	1.770	0.098	5.88	0.72	47.90
	5-10	1.791	0.165	6.00	0.48	75.34
	11-20	1.011	0.440	2.75	0.21	35.84
	21-50	0.223	0.869	1.25	0.09	17.65
	> 50	1.386	0.080	-	-	-
Livestock species	Cattle	2.803	0.000	-	-	-
	Cattle & buffalo	-0.931	0.303	0.39	0.07	2.32
	Mix livestock	0.780	0.532	2.18	0.19	25.20
Sources of animals	Homebred	2.382	0.000	-	-	-
	Brought in	1.018	0.356	2.77	0.32	24.03
Feeding	Grazing	1.481	0.003	-	-	-
	Stall feeding	2.115	0.015	8.30	1.50	45.76
Acaricide frequency	Intensive[f]	0.693	0.327	-	-	-
	Moderate [g]	2.803	0.023	16.50	1.46	186.40
	Rare[h]	2.233	0.015	9.33	1.53	56.60
Type of acaricide	Pyrethroid	0.693	0.327	-	-	-
	Avermectin	2.803	0.023	16.50	1.46	186.40
	Organophosphate	2.233	0.015	9.33	1.53	56.93
Use of unhygienic needles	No	0.223	0.739	-	-	-
	Yes	3.178	0.000	24.00	4.18	137.69
Farm instruments	Nose punch	-0.451	0.709	0.64	0.06	6.86
	Ear pliers	0.969	0.506	2.64	0.15	45.91
	Dehorner	2.397	0.022	-	-	-
	Docking inst.	0.435	0.732	1.55	0.13	18.73
AREA VARIABLES						
Districts	Sargodha	0.595	0.000	1.81	1.31	2.51
	Khushab	0.341	0.044	1.41	1.01	1.96
	Rawalpindi	-1.121	0.000	-	-	-

[a] *b*: Estimated effect (coefficient); [b]indigenous x Holstein Friesian; [c]low: 1-25% tick infestation; [d]moderate: >25-50% tick infestation; [e]heavy: >50-100% tick infestation; [f]intensive: acaricide application <60 days interval; [g]moderate: acaricide application with in 60-90 days, [h]rare: acaricide application >90 days interval; C.I: Confidence interval; n= number of animals found positive; N= total number of animals sampled.

DISCUSSION

As far as we can ascertain, the knowledge regarding risk factors of *Anaplasma marginale* among cattle from Punjab, Pakistan is extremely lacking. Probably this is the first study estimated risk factors associated with seroprevalence of *Anaplasma marginale.* The cELISA was used for seroprevalence which is based on major surface protein-5, it has obvious advantage over other serological tests because of higher sensitivity 96% and specificity 95% for the serodiagnosis of anaplasmosis (OIE, 2004).

Cattle of more than 4 year of age were significantly ($P<0.05$) at higher risk as compared to <2-4, 1-2 and <1 year age groups. Our findings are in accord with Urdaz-Rodriguez *et al*. (2009) and Aubry and Geale (2011). They mentioned seroprevalence increased with age.

Rhipicephalus (Boophilus) microplus is the major vector of *Anaplasma marginale* in various countries including India, South Africa and Brazil (Guglielmone, 1995; Potgieter, 1996; Ghosh, 2007). This may serve as efficient vector in Pakistan. The association has been found with the seroprevalence and the presence of *R. (Boophilus) microplus* in the present study. Most of the risk factors identified in the present study were also shared by other researchers in developing countries including Peurto Rico and Kenya (Urdaz-Rodriguez *et al*., 2009; Gachohi *et al*., 2010). There is no known zoonotic potential of *Anaplasma marginale*. However, *Anaplasma phagocytophilum*, an obligate intracellular bacteria closely related member of the family *Anaplasmataceae* is responsible for causing human granulocytic anaplasmosis. This is currently becoming an emerging disease in humans it has the potential to be transmitted by ticks as well as nosocomial infection (Dumlar *et al*., 2005; Walker *et al.*, 2008; Zhang *et al*., 2012). During the present questionnaire survey it was revealed that the farmer's knowledge about bovine and human anaplasmosis was poor.

Use of Pyrethroid and veterinary instruments has been identified as major risk factors in Maxican tropics (Rodriguez-vivas *et al*., 2004). Farm instruments, surgical tools and inanimate objects also play their part in the mechanical transmission to *Anaplasma marginale* (Swai *et al*., 2005). Farm instruments have not been identified as significant risk factor in the present study. Whereas, Avermectin (Ivermectin) has been identified at higher risk in our study. Regarding the efficacy of ivermectin, it was better acaricide earlier but indiscriminate use and over the counter availability are the possible reasons for drug resistance against ticks. The drug resistance of Ivermectin has been reported by Sajid (2007) from Punjab, Pakistan. Selection of inappropriate product, indiscriminate use, inadequate contact time and incomplete application contribute resistance in ticks and tick borne diseases (George, 2000).

In the current study, stall feeding was found at higher risk as compared to grazing. The agreement was not found with Swai *et al*. (2005) and Urdaz-Rodriguez *et al*. (2009) who observed a significant association between pasture grazing and high seropositivity for *Anaplasma marginale.* Rope tied animals with kacha (un cemented) floor are at higher risk of tick infestation (Sajid, 2007) support the higher risk of *Anaplasma marginale* in stall fed cattle. Pasture grazing, Permethrin and lack of fly control programme and presence of monkeys were the major risk factors in Puerto Rico (Urdaz-Rodriguez *et al*., 2009). Tick control frequency of less than five times were the two major risk factors in mixed livestock production system in Mbeere district, Kenya (Gachohi *et al*., 2010).

Moderate climate of Sargodha district favours the growth and multiplication of vector ticks. The higher prevalence of ticks and stall feeding practices have possibly accounted for higher risk. Lowest seroprevalence associated with *Anaplasma marginale* in Rawalpindi district is attributed to the fact that more grazing pattern was observed in Rawalpindi district. Grazing help in lesser tick infestation and maximum tick anorectic effect (Durrani, 2008).

It is concluded that unhygienic needles, stall feeding and crossbred cattle of more than 4 years of age are the major risk factors associated with seroprevalence to *Anaplasma marginale* in cattle in Punjab, Pakistan. Furthermore, there is a need to study the prevalence and isolation of the causative agent of human anaplasmosis.

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