International Journal of Engineering Research and General Science Volume 2, Issue 3, April-May 2014
ISSN 2091-2730

First Record on Serological Study of *Anaplasma marginale* Infection in *Ovis aries* by ELISA, in District Peshawar, Khyber Pakhtunkhwa, Pakistan

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ABSTRACT – Geographical sero-prevalence of *Anaplasma marginale* (T) in sheep, *Ovis aries* (L) was done from January-May, 2012 in district Peshawar which is a crowded area of Pakistan. In this area sheep's infection with *A. marginale* is not reported before. For this purpose, 376 serum samples were obtained conveniently from 4 different breeds of sheep, from different geographical areas of Peshawar. An indirect ELISA using recombinant MSP-5 as antigen of *A. marginale*, was performed. Totally, 92/376 (24.47%) of the overall sheep sera were positive. In 6 areas of Peshawar, Peshthakhara and Mashokhel area were found highly infected i.e. 32.00% and 32.00% respectively, while Ghazi baba area was less infected comparatively. While age wise, adults were highly infected specially turkai ones. This is the first record of *A. marginale* showing high rate infection in sheep in Peshawar, Pakistan, This research should be useful in epidemiological applications.

Keywords: Sheep; epidemiology; *A. marginale*; MSP-5; indirect ELISA; Peshawar.

Introduction:

Peshawar, the capital city of Khyber Pakhtunkhwa is the administrative center and central economic hub for the Federal Administrated Tribal Areas (FATA) of Pakistan. It is situated in a large valley near the eastern end of the Khyber Pass, between the eastern edge of the Iranian Plateau and the Indus valley strategically it has an imoptant location on the crossroads of central Asia and South Asia. Peshawar under Koppen's climate classification features has a semi-arid climate with very hot summers and mild winters. It is located at 34°01′N and 71°35′E, area with on of 1,257 km2 and population of 3,625,000 [9] (Figure 1). Sheep, *Ovis aries* (L) is one of the initial animals, domesticated for agricultural purposes; it is raised for meat, (hogget or mutton, lamb) milk and fleece production. These quadru-pedal ruminant mammals are members of the order *Artiodactyla*, the even-toed ungulates typically kept as livestock. It has great economic potential because of their early maturity and high fertility as well as their adaptability to moist environment [7]. However, the benefits derived are too low from the expected due chiefly to low productivity. Numerous factors are involved in this low productivity, in which the major one is disease [2].

Diseases caused by heamoparasites are most apparent. These heamoparasites are parasites found in the blood of mammals in which *A. marginale* is also include. Ticks are biological vectors of *Anaplasma* sp.; tick, mammalian or bird hosts with persistent *Anaplasma* sp. infection can serve as reservoir of infection naturally. *Anaplasma* sp. is intracellular, gram-negative bacteria and representatives of the

order *Rickettsiales* classified into *Rickettsiaceae* and *Anaplasmataceae* families [5]. The tick vector distribution is the factor influencing the transmission of tick-borne diseases [3]. However, for *A. marginale*, mechanical transmission through contaminated hypodermic needles and biting flies plays an important role [9].

Erythrocytes are phagocyted by reticulo-endothelial cells during infection. Animals may die older than 2 years due to the infection [7]. Nevertheless, concerning ovine anaplasmosis, little information is available, in despite of the expressive number of sheep, goat and expansion of small ruminant herds in this country. Diagnosis of anaplasmosis in small ruminants mainly based in the identification of the rickettsia in stained blood smears. However, below 0.1% rickettsemias in chronic carriers are not detected by this method [9]. Serological assays, based on Major Surface Protein 5 (MSP-5) of *A. marginale* have been successfully used, for the detection of antibodies against *Anaplasma* sp. [11]. In this study, we observed for the first time sero-prevalence of *Anaplasma* sp., in different breeds of sheep using an indirect ELISA based on MSP5 recombinant of *A. marginale,* in Peshawar, Pakistan. This research should be particularly useful for epidemiological applications such as prevalence studies, awareness, education, research and control programs in this region.

Materials and Methods:

Samples collection: Conveniently, 376 blood sampling was collected from the overall sheep population of different areas of Peshawar from January to May 2012. About 5 ml blood samples were collected from the jugular vein of each sheep with a sterile hypodermic syringe into an evacuated tube containing gel and clot activator. Some information like breed, age, and sex were noted. The blood sample was then centrifuge for 5 minutes at 12000 rpm to separate serum and stored at −35°C until further use [6]. The SVANOVIR® *A. marginale*-Ab ELISA kit (Svanova Biotech AB, Uppsala, Sweden) was used for the diagnosis of specific antibodies against *A. marginale* in bovine serum samples. The kit procedure was based on the Indirect Enzyme Linked Immunosorbent Assay (Indirect ELISA). The whole procedure was done according to the protocol given with the kit.

Protocol for Indirect Enzyme Linked Immunosorbent Assay (iELISA):

All reagents were equilibrated to room temperature 18 to 25 °C before use. Pre-dilution of control and samples 1/40 in PBS-tween buffer (e.g., 10 µl sample in 390 µl of PBS-tween buffer). Hundred micro liter of pre-diluted serum sample was added to selected wells. The plate was then seal and incubate at 37 °C for 30 minutes. The plate was rinse 4 times with PBS-tween buffer. Hundred micro liter of conjugate dilution was added to each well and then sealed the plate and incubate on 37 °C for 30 minutes. Again, the plate was rinse 4 times with PBS-tween buffer. Hundred micro liter substrate solution was added to each well and then incubated for 30 minute at room temperature (18 to 25 °C). Hundred micro liter of stop solution was added to each well and mixed thoroughly. The optical density (OD) of the controls and sample was measured at 405 nm in a micro-plate photometer (BIOTEK Instruments Inc., Winooski, Vermont, U.S.A.). Mean OD values were calculated for each of the control and samples.

Data analysis:

The following formula was used for the percent positivity (PP):

PP= [(Sample OD ×100)/Mean positive control OD]

Interpretation of the results:

The calculated percent positivity (PP) if less than 25%, the sample was consider as negative and if PP was equal or more than 25%, then the sample was consider as positive.

Results:

There were overall 92 (24.47%) positive samples for *A. marginale* of *O. aries*. In Ghazi Baba 19 (19.00%) positive cases were detected in which 6 (13.33%) were Balkhai, 4 (16.00%) Watanai, 1 (16.67%) Punjabai and 8 (30.77%) Turkai. In Warsak road 17 (22.66%) positive cases were detected in which 3 (12.00%) were Balkhai, 7 (31.82%) Watanai, 3 (18.75%) Punjabai and 4 (33.33%) Turkai. In Badabher 19 (25.33%) positive cases were detected in which 5 (20.83%) were Balkhai, 6 (50.00%) Watanai, 4 (16.67%) Punjabai and 4 (26.67%) Turkai. In Peshtakhara 16 (32.00%) positive cases were detected in which 4 (40.00%) were Balkhai, 1 (8.33%) Watanai, 3 (23.10%) Punjabai and 8 (53.33%) Turkai. In Mashokhel 16 (32.00%) positive cases were detected in which 4 (33.33%) were Balkhai, 4 (25.00%) Watanai, 3 (33.33%) Punjabai and 5 (38.46%) Turkai. In Barha 8 (32.00%) positive cases were detected in which 2 (28.57%) were Balkhai, 3 (27.027%) Watanai, 3 (37.5%) Punjabai and 0 (0.00%) Turkai (Table 1).

The infection was high in Peshtakhara, Mashokhel and Barha, while lower in Ghazi baba as compare to other areas. In total 17 (18.28%) positive Balkhai males, 9 (16.07%) were adult and 8 (21.62%) were young, in 21 (29.57%) positive Watanai males, 16 (40.00%) were adult and 5 (16.13%) were young, in total 12 (20.68%) positive Punjabai males, 7 (22.58%) were adult and 5 (18.52%) were young and in total 22 (33.85%) positive Turkai males, 6 (14.28%) were adult and 14 (60.87%) were young (Table 2).

In total 6 (19.35%) positive Balkhai females 5 (41.67%) were adults and 1 (5.26%) was young, in 5 (19.23%) positive Watanai females 2 (14.28%) were adults and 3 (25.00%) were young, in 4 (22.22%) positive Punjabai females 1 (12.50%) was adults and 3 (30.00%) were young and in 7 (50.00%) positive Turkai females 4 (50.00%) were adults and 3 (50.00%) were young (Table 3).

Discussion:

The research on sheep anaplasmosis (*A. marginale*) is rare and little literature is available. The frequency of sero-positivity of sheep anaplasmosis in this research were (24.47%) which is very low as compared to the prevalence of sero-positive sheep found by Hornok et al. [6] (99.4%) in Hungry and high as compared to the prevalence of sero-positive sheep found by Cabral et al. [4] (8.92%). Sero-prevalence were found by Ramos et al. [10] (16.17%) in Ibimirim county, semi-arid region of Pernambuco State, Brazil using monoclonal antibody ANAF16C1 and De La Fuente et al. [5] (75.0%), in sicily, Italy, using competitive ELISA, based on recombinant

MSP-5 of *A. marginale*. The low sero-prevalence rate in this research work can be the cause of low tick vector population in Peshawar area. However, some ticks were also observed in sheep during blood samples collection. This result represents the first description of antibodies for Anaplasma sp. in sheep from Peshawar, Pakistan. Further studies are require to know the epidemiology of Anaplasma sp. infection in sheep, in Pakistan, particularly to define which species is involved, possible impacts and vectors in animal production and in public health.

Figure 1. Map of Distract Peshawar, Pakistan (Google, 2012)

Table 1. Area wise collected and positive blood samples for *A. marginale* by indirect Enzyme Linked Immunosorbent Assay (iELISA) in sheep during January -May, 2012 in Peshawar, Pakistan.

S No.	Area	Total sample	Positive (%)	Balkhai		Watanai		Punjabai		Turkai	
				n^1	P (%)	n^2	P (%)	n^3	P (%)	n^4	P (%)
1	Ghazi Baba, Ring road	100	19 (19.00)	45	6 (13.33)	25	4 (16.00)	6	1 (16.67)	26	8 (30.77)
2	Warsak Road	75	17 (22.66)	25	3 (12.00)	22	7 (31.82)	16	3 (18.75)	12	4 (33.33)
3	Badabher	75	19 (25.33)	24	5 (20.83)	12	6 (50.00)	24	4 (16.67)	15	4 (26.67)
4	Peshtakhara	50	16 (32.00)	10	4 (40.00)	12	1 (8.33)	13	3 (23.10)	15	8 (53.33)
5	Mashokhel	50	16 (32.00)	12	4 (33.33)	16	4 (25.00)	9	3 (33.33)	13	5 (38.46)
6	Barha	26	8 (32.00)	7	2 (28.57)	11	3 (27.27)	8	3 (37.5)	0	0 (0.00)

n^1, n^2, n^3 and n^4: Shows the total number of collected samples of Balkhai, Watanai, Punjabai and Turkai breed respectively.
P: Indicate the positive samples for *A. marginale*.

Table 2. Male age wise collected and positive blood samples for *A. marginale* by indirect Enzyme Linked Immunosorbent Assay (iELISA) in sheep during January -May, 2012 in Peshawar, Pakistan.

S No.	Breeds	Total samples	Male samples	Male +v (%)	Male			
					Total *adult	Adult +v (%)	Total **young	Young +v (%)
1	Balkhai	124	93	17(18.28)	56	9 (16.07)	37	8 (21.62)
2	Watanai	97	71	21(29.57)	40	16 (40.00)	31	5 (16.13)
3	Punjabai	76	58	12(20.68)	31	7 (22.58)	27	5 (18.52)
4	Turkai	81	65	22(33.85)	42	6 (14.28)	23	14(60.87)

*****More than one year
******Less than one year

Table 3. Female age wise collected and positive blood samples for *A. marginale* by indirect Enzyme Linked Immunosorbent Assay (iELISA) in sheep during January -May, 2012 in Peshawar, Pakistan.

S No.	Breeds	Total samples	Female samples	Females +v (%)	Female			
					Total *adult	Adult +v (%)	Total **young	Young +v (%)
1	Balkhai	124	31	6 (19.35)	12	5 (41.67)	19	1 (5.26)
2	Watanai	97	26	5 (19.23)	14	2 (14.28)	12	3 (25.00)
3	Punjabai	76	18	4 (22.22)	8	1 (12.50)	10	3 (30.00)
4	Turkai	81	14	7 (50.00)	8	4 (50.00)	6	3 (50.00)

*****More than one year
******Less than one year

Acknowledgments:

We are grateful to Dr. Ghufran Ullah, Dr. Ikhwan Khan and Dr. Ijaz Khan, Senior Researchers, Veterinary Research Institute (VRI), Peshawar for their full support and cooperation at every step in current research work. The experiments comply with the current laws of the country in which they were performed.

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