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Prevalence of *Borrelia anserina* in *Argas* Ticks

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Abstract.- *Borrelia anserina* is a pathogen of high importance in poultry industry which causes fowl spirocheatosis. This study was designed to determine the prevalence of *Borrelia anserina* in poultry soft ticks, *Argas persicus* collected from birds and poultry farms. A total of 1500 ticks were collected from poultry farms located in Faisalabad and Kamalia, Pakistan. *B. anserina* was isolated using BSK-H medium and confirmed by dark field microscopy and indirect immunoflourescence. In addition, *B. anserina* was characterized using polymerase chain reaction by employing specific primer set of *fla* B gene. Of 750 tick samples collected from poultry birds, 144 (19.2%) were positive for *B. anserina*; whereas 131 (17.4%) were positive collected from poultry farms. The data indicated that the *Argas* ticks had the significant prevalence of *B. anserina*. Furthermore, the data may warrant future studies towards the vector control and/or immunoprophylaxsis against *B. anserina* which might indirectly be helpful for the eradication of this threatening disease.

Key words: *Borrelia anserina, Argas persicus,* poultry industry.

INTRODUCTION

Poultry products are a good source of protein; as eggs contain superior quality protein relative to that in mutton and beef (Shah *et al*., 2004). However, this productivity is directly affected by tick infestation. Ectoparasites such as ticks and mites transmit different pathogens which lead to a number of threatening diseases. Genus *Argas* belongs to argasid ticks and has a great veterinary importance especially in poultry throughout the world (Jongejan and Uilenberg, 2004). The argasid ticks usually live close to their host and feed for a short period of time and then return to their hiding places. In contrast, the larvae of *Argas persicus* attach and feed for few days on domestic poultry. Among *the* genus, *Argas persicus* is very important tick for poultry because it transmits an important poultry pathogen *B. anserina* (Aslam *et al*., 2013).

Spirocheatosis caused by *B. anserina* is generally characterized by fever, generalized weakness, ruffled feathers, loss of appetite, increased thirst and greenish diarrhea (Ataliba *et al*., 2007). Morphologically, *B. anserina* bacterium is 0.2-0.5 µm in diameter and 10-50 µm in length. It has a very complex morphological structure, so it gains poor staining and only observed by dark field or phase contrast microscope (Barbour, 1984). It contains two membranes; an outer membrane encloses the protoplasmic cylinder while all inner cellular components are enclosed by an inner membrane. *B. anserina* moves with the help of sub-terminally attached 7-11 flagella (Goldstein *et al*., 1994). Flagellum is very critical for the survival of *Borrelia*, whereas, a mutant deficient of flagellum turns into a rod shaped structure (Motaleb, 2000).

A lot of work has been conducted on the epidemiological aspects of tick infestation in poultry throughout Pakistan (Shah *et al*., 2006). We know very little about the pathogens transmitted by ticks. Since these pathogens cause fatal diseases in poultry, serious efforts to understand their prevalence, isolation and characterization are required to formulate better good control strategies against the diseases.

In this study we have focused on one of the aforementioned aspects regarding this pathogen and reported the temporal prevalence of tick born *B. anserina* from poultry birds and farm premises using cell culture, immunoflourescence and molecular methods

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MATERIALS AND METHODS

Collection of Argas *ticks*

Argas persicus (*n*=1500) were collected in two summer seasons (May 2008 to August 2009) from poultry birds and farms located in Faisalabad and Kamalia, Punjab, Pakistan (Table I). A complete history of fowl spirocheatosis was recorded alongwith the sample collection. Ticks were collected with the help of forceps and placed into screw-capped plastic tubes having small cotton swabs soaked in 1% mycostatin solution, in order to avoid the dryness of the ticks. The collected ticks were stored at refrigerating temperature until further processing (Guner *et al*., 2003).

Table 1.- Distribution of tick samples collected from various farms.

Farm location	Farm name	Source of sample A=birds & B=farms	Total No. of ticks collected A+B
Faisalabad	Anwar poultry farm	A= 135 B= 133	268
Faisalabad	204 poultry farm	A= 155 B= 160	315
Kamalia	Arif poultry farms	A= 151 B= 145	296
Kamalia	Arif poultry farm 2	A= 142 B= 140	282
Kamalia	Kamalia poultry farm	A= 167 B=172	339

Isolation of B. anserina

For the isolation of *B. anserina*, a total of 1500 dissected *Argas* ticks were inoculated discretely into 5ml of BSK-complete medium (Sigma-Product No. B8291, USA) enriched with sodium bicarbonate and 6% rabbit serum and then, incubated at 37°C (Barbour, 1984) with microaerophilic conditions. The presence of spirochaetes was confirmed by dark field and phase contrast microscopy (Nikon). The confirmation of the isolates was accomplished by observation of motility of *B. anserina* with indirect immunoflourescence assay using *IgG* polyclonal antibodies (Bactrace^{TM,} Sweden) additionally.

Indirect Immuno-flouresencent assay

Indirect Immuno-flouresencent assay was performed as described by (Horta *et al*., 2004). Briefly, spirochaetal culture was centrifuged at 12,000 × *g* for 10 minutes, followed by a washing of pellet in 0.1M phosphate-buffered saline (PBS). After a brief centrifugation, pellet was resuspended in PBS containing 1% bovine calf serum. A 10 µl of sample was added into all the wells of microscopic slide chamber excluding the control well. The slide chamber was air-dried and fixed in acetone for 10 minutes at room temperature. A 10 µl of specific *B. anserina* IgG polyclonal antibodies (Bactrace^{TM}) was dispensed to each well of the chamber. The slides were incubated at 37°C for 30 minutes and then washed twice for 10 minutes in PBS. The slides were incubated again with fluorescein-labeled anti *B. anserina IgG* and washed as described earlier. The slides were mounted with gel mount under cover slips. Finally, slides were visualized under standard immunoflourescence microscope (Nikon) using suitable filters.

Molecular characterization of B. anserina

DNA extraction of spirochaetal culture was done by using the DNeasy Tissue kit (Qiagen) in the BioRobot EZ1. In brief, 5 ml of culture was centrifuged at 5000 rpm and the pellet was resuspended into 200µl of G2 buffer (Qiagen). A 20µl lysozyme was added into the tube having sample and incubated at 37°C for 30 minutes. EZ1 BioRobot procedure was followed for the DNA extraction. Isolates were subjected to PCR for their molecular identification using the specific primers for flagellin gene (*fla* B) with following sequences, FP: 5-ACA TAT TCA GAT GCA GAC AGA GGT-3´, RP: 5´-GCA ATC ATA GCC ATT GCA GAT TGT-3´ (Barbour *et al*., 1996). An amount of 3µl DNA was used as template for the flagellin gene amplification in 25µl of final reaction volume. Specifically, final composition of PCR master mix was 2.4µl of 25 mM of $MgCl_2$, 0.6µl of 10nM of dNTPs, 0.6µl of each 10µM forward and reverse primers and 1 U of platinum Taq DNA polymerase (Invitrogen®). A 30 repeated cycles reaction was performed with the prescribed profile: 94°C for 30°C sec, 52°C for 30 sec and 72°C for 1 min, final extension at 72°C for 10 min and finally reaction was terminated at 4°C. A 0.8% agarose gel electrophoresis with positive control was performed

to visualize the PCR product under Gel documentation system (Dolphin-Doc, Wealtec, USA). Furthermore, phylogentic analysis of the isolates was done to observe the homology with already available sequences in NCBI (Bukhari *et al.*, 2013).

Statistical analysis

Percentage prevalence was estimated and results were statistically analyzed with 95 % confidence level. Epidemiological software called Winpepi is used for the statistical analysis.

RESULTS

Isolation of B. anserina

It was observed that BSK-H complete medium having tick samples become turbid on the 7th day of post inoculation into the medium. Best growth of *Borrelia* was observed at 37°C after 7 days of inoculation. Samples showed granular growth earlier possibly were due to the existence of the infection inside the tick sample. Initial 2-3 passages took 18-20 days to show the proper growth of *Borrelia* in some ticks, later the passage time was declined almost to 5-7 days. Numerous viable spirochaetes were observed under the dark field microscope (Fig. 1).

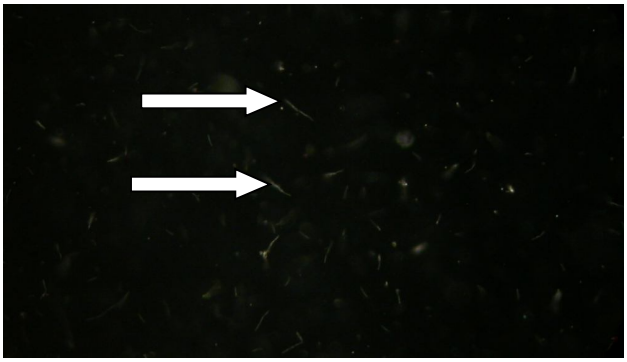

Fig 1. Arrows indicate the dark field microscopic (Nikon) 100X image showing the thread like structures of *B. anserina* isolated form *Argas* ticks by using the BSK-H complete medium (Sigma).

Confirmation of spirochaetes

All the samples which exhibited granular growth were processed for indirect immuno-flourescence assay. In spite of using the polyclonal IgG *Borrelia* antibodies all the samples (100%) showed clear fluorescence. All positive samples were further run in PCR for molecular identification. DNA was extracted from the spirochaetal culture and subjected to the PCR for the amplification of the flagellin gene, positive samples amplified a product of 750bp. The PCR products were sequenced; their BLAST analyses indicated that the isolates had highest percentage similarity to the *B. anserina* sequences present in the GenBank. Flagellin gene (*fla* B) sequence of the isolate of present study was submitted to NCBI database (accession number JF693808). Phylogenetically, isolate of the current study showed strong homology with already presented isolated of *B. anserina*.

Prevalence of B. anserina *in* Argas *ticks collected from poultry birds*

Out of 750 ticks collected from poultry birds during summer 2008-09, 96 (19.2%) showed presence of *B. anserina* in BSK-H complete medium (Table II). Immunifluorescent assay (IFA) showed 100% results as all the culture positive samples gave clear fluorescence. Whereas, out of 144 positive samples, 135 (94%) showed positive results with PCR. On the basis of *B. anserina* isolation a statistically significant prevalence was recorded ($P < 0.05$) among *Argas* ticks collected form poultry birds. However, the difference between culture positive and PCR positive results was statistically non significant ($P > 0.05$).

Prevalence of B. anserina *in* Argas *ticks collected from poultry farms*

Out of 750 ticks collected from poultry farms, 131 (17.4%) showed culture positive results (Table III). As mentioned earlier, IFA showed 100 % results. Whereas, 118 (90%) out of 131 positive samples amplified a product of 750pb when they were subjected to PCR. A statistically significant prevalence was recorded ($P<0.05$) among *Argas* ticks collected form poultry farms whereas difference between culture positive and PCR positive was again non significant ($P > 0.05$).

Table II.- Isolation of spirochaetes (*B. anserina*) from the *A. persicus* adult ticks collected from poultry birds (on host) during summer 2008-09 with their respective 95 % C. I. Table shows the percentage of positive sample of *B. anserina* isolated from different tick sample of *A. persicus* collected form poultry farms of Faisalabad and Kamalia. 144 (19%) sample out of 750 samples showed the culture positive results, 144 (100%) IFA results out of culture positive and only 135 (94%) were found PCR positive out of culture positive.

Months	Ticks collected from poultry birds	Culture positive samples (%) With 95% C.I	IFA positive samples (%) out of culture positive With 95% C. I	PCR positive samples (%) out of culture positive With 95% C. I
2008 May	75	12(16) 8.9 to 25.6	12(100) 77.9 to 100.0	12(100) 77.9 to 100.0
June	75	21(28) 18.7 to 38.9	21(100) 86.7 to 100.0	21(100) 86.7 to 100.0
July	75	12(16) 8.9 to 25.6	12(100) 77.9 to 100.0	11(92) 65.2 to 99.5
August	75	9(12) 6.0 to 20.8	9(100) 71.6 to 100.0	8(89) 56.1 to 99.4
September	75	9(12) 6.0 to 20.8	9(100) 71.6 to 100.0	8(89) 56.1 to 99.4
Total	375	63 (17) 13.5 to 21.1	63 (100) 95.3 to 100.0	60 (95) 87.5 to 98.7
Summer 2009 May	75	9(12) 6.0 to 20.8	9(100) 71.6 to 100.0	8(89) 56.1 to 99.4
June	75	24(32) 22.2 to 43.1	24(100) 88.2 to 100.0	22(92) 75.1 to 98.5
July	75	18(24) 15.3 to 34.6	18(100) 84.6 to 100.0	17(94) 75.5 to 99.7
August	75	18(24) 15.3 to 34.6	18(100) 84.6 to 100.0	17(94) 75.5 to 99.7
September	75	12(16) 8.9 to 25.6	12(100) 77.9 to 100.0	11(92) 65.2 to 99.5
Total	375	81(22) 18.1 to 26.5	81(100) 96.3 to 100.0	75(93) 85.2 to 96.9
Total 08-09	750	144 (19%) 16.3 to 22.0	144(100%) 97.9 to 100.0	135 (94 %) 88.8 to 96.9

DISCUSSION

The purpose of prevalence of *B. anserina* was to reveal the fact that it is a primary factor for the distribution of disease in the area and it could be helpful for the eradication of the disease. Role of ticks as important ectoparasites in poultry especially layers has been established globally because they are responsible for the transmission of different infectious diseases (Shah *et al*., 2006). Among different ticks Argas is most prevalent tick in Pakistan (Shah *et al*., 2004; Aslam *et al*., 2013). To formulate any control strategies against different ominous diseases caused by infectious pathogen transmitted by tick vector, the epidemiological assessment of pathogens could provide one sort of foundation.

In this study *Argas* ticks were collected form poultry and different layer farm of Faisalabad District during summer 2008-09. Parasitizing role of genus *Argas* has been reported in layer chicken among different regions of Pakistan (Khan, 2001). It has been established that incidence of fowl spirochaetosis remains higher during the months of May to August due to presence of active vector (Aslam *et al*., 2013).

In the present study, tick samples were collected from old layer farms because of deprived housing conditions. With high temperature and humidity these ‘’kacha houses’’ (unpaved houses) may play a vital role for *Argas persicus* breeding. Overall health status of the poultry birds was very poor which further aggravated the situation. In Pakistan, the vector role of *A. persicus* in the

Table III.- Isolation of spirochaetes (*B. anserina*) from the *A. persicus* adult ticks collected from poultry farms (off host) during summer 2008-09 with their respective 95 % C.I. Table shows the percentage of positive sample of *B. anserina* isolated form different tick sample of A. persicus collected form poultry farms of Faisalabad and Kmalia, 131 (17%) sample out of 750 samples showed the culture positive results, 131(100%) IFA results out of culture positive and 118 (90%) PCR positive out of culture positive.

Months	Ticks collected from poultry farms	Culture positive samples (%)With 95% C. I	IFA positive samples (%) out of culture positive With 95% C. I	PCR positive samples (%) out of culture positive With 95% C. I
Summer 2008 May	75	11(15) 7.9 to 24.0	11(100) 76.1 to 100.0	11(100) 76.1 to 100.0
June	75	19(25) 16.4 to 36.0	19(100) 85.4 to 100.0	19(100) 85.4 to 100.0
July	75	8(11) 5.0 to 19.2	8(100) 68.7 to 100.0	6(75)
August	75	8(11) 5.0 to 19.2	8(100) 68.7 to 100.0	8(100) 68.7 to 100.0
September	75	12(16) 8.9 to 25.6	12(100) 77.9 to 100.0	11(92) 65.2 to 99.5
Total	375	58 (15) 11.5 to 18.8	58 (100) 94.9 to 100.0	55 (95) 86.5 to 98.6
Summer 2009 May	75	11(15) 7.9 to 24.0	11(100) 76.1 to 100.0	9(82) 51.7 to 96.8
June	75	22(29) 19.8 to 40.3	22(100) 87.2 to 100.0	19(86) 67.2 to 96.4
July	75	17(23) 14.2 to 33.1	17(100) 83.8 to 100.0	15(88) 66.2 to 97.9
August	75	14(19) 11.0 to 28.6	14(100) 80.7 to 100.0	12(86) 60.2 to 97.5
September	75	9(12) 6.0 to 20.8	9(100) 71.6 to 100.0	8(89) 56.1 to 99.4
Total	375	73 (19) 15.2 to 23.1	73 (100) 95.9 to 100.0	63 (86) 76.9 to 92.8
Total 08-09	750	131(17 %) 14.5 to 19.8	131 (100%) 97.7 to 100.0	118 (90 %) 84.0 to 94.3

transmission of spirochaetosis in layer chicken has been established (Shah *et al*., 2004).

In the present study, for the isolation of the *B. anserina* from different tick samples, Barbour Stoenner Kelly (BSK)-H complete medium (Sigma USA) was used which has frequently been used for the cultivation of the spirocheates (Ataliba *et al*., 2007). It contains N-acetyleglosamine, yeast extract, amino acids, vitamins and serum which facilitate the growth of the *Borrelia*. Additionally, BSK-H complete medium is supplemented with 6 % rabbit serum and sodium bicarbonate (Sigma, USA: Catalogue No. 8291).

Micro-aerophillic conditions at 37^{o}C for the isolation of *Borrelia* were found the best for spirochaetal growth has already been established (Barbour, 1984). Nalidixic acid (Negrom®) was used with a dose rate of 100µg/ml to avoid the culture contamination; since Borrelia is resistant to nalidixic acid (Barbour, 1984). *Borrelia* gets stain very poorly because of complex structural composition (Ataliba *et al*., 2007). *Borrelia* was confirmed in the isolates by observing under the dark field and phase contrast microscopes. The isolates were further confirmed by IFA; an established confirmatory and sensitive test for *Borrelia* (Oslen *et al*., 1993). The molecular identification of the *Borrelia* isolates was done by performing PCR by using the specifically designed primers of Flagellin gene (*fla* B). Flagellin gene has already been used extensively for molecular identification of *Borrelia* (Ataliba *et al*., 2007; Barbour *et al*., 1996; Picken, 1992; Fukanaga *et al*., 1996). Some culture positive samples for *B.*

anserina showed negative results in PCR due to due to the insufficient DNA in the reaction or due to some unknown reasons. Similar results have been reported by Livesely *et al*. (1994) where they isolated *Borrelia* from the ticks collected form UK and confirmed with PCR.

In our study, 19.2% *Argas* ticks collected form poultry birds showed the presence of *B. anserina*. Ninety-four percent (94 %) of culturally positive Borrelia samples were also positive in PCR test. *A* significant prevalence of *B. anserina* was recorded among *Argas* ticks collected from poultry birds. The difference between culture positive and PCR positive results was not significant statistically. While (17.4%) isolates were culture positive collected from poultry farms. However, no significant difference was found between the samples collected from poultry birds and poultry farms throughout the summer season. In results of the present study, we found comparatively more good number of culture positive samples (19.2%) than those already reported (9%) by Davidson *et al*. (1999).

In conclusion, our temporal prevalence based studies have revealed that a significant number of poultry birds and premises are affected by the tick parasites harboring *B. anserina* and thus ultimately causing fowl spirocheatosis. Serious efforts are emergently required to carry out the isolation and molecular characterization of *B. anserina* for future studies. Further, a vaccine prepared against the prevalent strains of *B. anserina* will certainly lead to the better control measures of this malady and thereby boosting the poultry industry in Pakistan.

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