

## Molecular and pathological investigation of spirochaetosis in a commercial layer flock - case report

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### ABSTRACT

The present investigation was carried out of a natural outbreak of spirochaetosis in 46 weeks old layer birds. The most striking post-mortem lesion was an enlarged mottled spleen. Necrotic foci with thickening of the capillary walls in the spleen, severe fatty changes in the liver, and degenerative tubular epithelial cells with casts and lymphocytic infiltration in the kidney were evident histopathologically. PCR amplification of the extracted DNA from spleen tissue indicated 725 and 750 bp amplicon specific for the flagellin gene (*flaB*) of *Borrelia anserina* by respective primers. The strain causing the disease was phylogenetically closely related to the Iranian and Brazilian strains of *B. anserina*. This report describes the genetic characterization and phylogenetic analysis of *B. anserina* from a field outbreak of spirochaetosis in layer chickens from India.

**Key words:** *Argas persicus*; *Borrelia anserina*; India; layer; phylogenetic study; spirochaetosis

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### Introduction

Fowl spirochaetosis is an acute, septicaemic tick-transmitted disease of avian species caused by *Borrelia anserina* (ABDUL-AZIZ and BARNES, 2013). The soft bodied Argasid ticks serve as reservoirs and important biological vectors for the transmission of *B. anserina*. In most countries, due to improved sanitation, the possibility of the persistence of the Argus tick population has diminished on intensively managed farms (ANONYMOUS, 2002) In spite of this, several

outbreaks have been reported in many different countries, including India (ASLAM et al., 2017; MALLESH et al., 2018). The disease occurred throughout the year and seasonality was not observed, but transmission was related directly to the vector activation (COOPER and BICKFORD, 1993). Due to the complex bacterial structure of *B. anserina*, it acquires poor staining, and is usually diagnosed by a dark field microscope and/or phase contrast microscope (ATALIBA et al., 2007). After

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infection, antibodies against the flagellin appear early compared to other antigens. As Flagellin is one of the immunodominant antigens of *Borrelia*, it can be explored as a diagnostic tool and used as a phylogenetic marker (ROSA et al., 1991). This report describes the molecular characterization and phylogenetic analysis of *B. anserina* from a field outbreak of spirochaetosis in layer chickens on the Indian subcontinent.

**Materials and methods**

*Collection of samples.* Layer birds (Lohman type) of 46 weeks of age were received from a private poultry farm of 7489 birds due to mortality of about 10-15 birds daily for the previous 4-5 days. The clinical material for necropsy was collected from seven birds at the Department of Pathology, Nagpur Veterinary College, Nagpur, India. A detailed post-mortem was conducted and spleen samples were stored at -20°C until further use. Tissue samples of the lungs, liver, kidneys,

heart and spleen were collected in 10% buffered formalin for histopathological study. The formalin-fixed tissues were subjected to histopathological processing as per the standard procedures (LUNA, 1968). Ticks were collected from crevices and cracks on the farms and also from the wing and abdominal area of the birds with the help of forceps, placed in screw-capped plastic tubes and kept at 5°C until further use (GUNER et al., 2003).

*Detection of B. anserina by Polymerase Chain Reaction (PCR).* Genomic DNA was extracted from the spleen samples collected from the necropsied birds using the HiPurA™ Multisample DNA Purification Kit (Himedia, India) following the manufacturer’s protocol. Detection of *B. anserina* was carried out by PCR using the GoTaq®Green Master Mix 2X (Promega, USA) targeting flagellin gene (*flaB*). A fragment of the *flaB* gene of *B. anserina* was amplified using two different primers (Table 1) and PCR reactions were carried out in a thermocycler (BioRad) with the cycling conditions depicted in Table 2.

Table 1. Details of primers used in the present study

Gene	Primer I and II	Product	References
<i>flab</i>	F - 5'CTC AAA TTA GAG GAT TAT CTC AAG C 3' R - 5' TGC TAC AAT TTC ATC TGT CAT TG 3'	725 bp	(CHEGENI et al., 2017)
	F - 5' ACA TAT TCA GAT GCA GAC AGA GGT 3' R - 5' GCA ATC ATA GCC ATT GCA GAT TGT 3'	750 bp	(ASLAM et al., 2013)

Table 2. The cycling conditions in the Master gradient cycler for the *flab* gene

		Primer set I	Primer set II	
Step I	Initial denaturation	94°C for 5 min	95°C for 1 min	1 cycle
Step II	Denaturation	94°C for 30 sec	95°C for 1 min	} 30 cycles
	Annealing	56°C for 1 min	57°C for 1 min	
	Extension	72°C for 1 min	72°C for 50 sec	
Step III	Final extension	72°C for 10 min	72°C for 5 min	1 cycle
Step IV	Hold	4°C	4°C	Optional

PCR for each 25 µl final volume reaction was done using 12.5 µl GreenMaster Mix® 2X, 1 µl each from forward and reverse primers (10 pM), 3 µl gDNA template (100 ng/µl), and 7.5 µl deionized water. The amplified products were visualized in 1.2% agarose gel (Gel Doc, Syngene). PCR products were submitted for sequencing to Eurofins Genomics, Bangalore (India).

*Sequence and phylogenetic Analysis.* The sequences were blasted using a blast search of NCBI for their identity. Both sequences were aligned and the consensus sequence was prepared with Bioedit (www.mbio.ncsu.edu/BioEdit/bioedit.html). The alignment was carried out with Clustal W of MEGA 7.0 and the phylogenetic tree was constructed on the basis of partial sequences of the *flaB* gene by the neighbour-joining method in MEGA 7.0 (KUMAR et al., 2016). The evolutionary distance was calculated and shown as the number of base differences per site. The sequences were submitted to GenBank under accession numbers MK128989 and MK128990.

## Results

*Pathology.* The affected flock demonstrated depression, pale combs and wattles, reduced egg production by up to 40%, and mortality of 0.2%. Large numbers of ticks were seen aggregated on the inner surface of the wings and abdominal area of the birds. The morphological characteristics of *Argas sp.* were studied initially by visual examination and then by light microscopy (Fig. 1). The ticks were identified as *Argas persicus* on the basis of their morphological features as they were brown in colour, oval in shape, narrower anteriorly than posteriorly, with sharp edges on the body and mouth parts on the ventral side (MALLESH et al., 2018; MULLEEN and DURDEN, 2013).

The post-mortem examination revealed congestion and oedema of the lungs. The liver was swollen and fragile, with mild congestion with focal areas of necrosis. Pale kidneys and misshapen ova were also evident. The characteristic pathological lesion observed was an enlarged mottled spleen (Fig. 2). Histopathologically, the spleen showed necrotic

foci with thickening of the capillary endothelial cell proliferation (Fig. 3). Lesions in the liver included severe fatty changes, along with lymphocytic infiltration (Fig. 4). The kidneys revealed severe degenerative tubular epithelial cells, with tubular casts and lymphocytic infiltration (Fig. 5). The lungs showed congestion with inflammatory cells, oedema and haemosiderosis due to phagocytosis of the red blood cells.

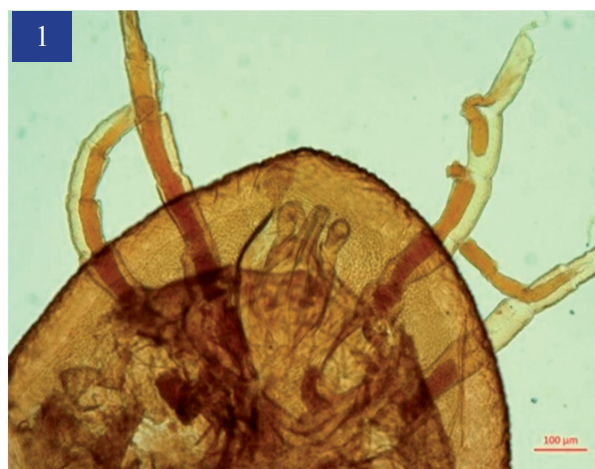


Fig. 1. An *Argas persicus* tick showing that it is brown in colour, oval in shape, narrower anteriorly than posteriorly, with sharp edges of the body and mouth parts on the ventral side



Fig. 2. Enlarged mottled spleen of a hen that has succumbed to infection



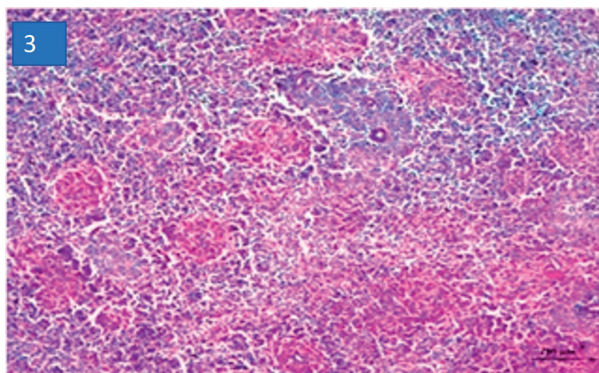


Fig. 3. Spleen showing necrotic foci with thickening of the capillary endothelial cell proliferations H&E, 100X

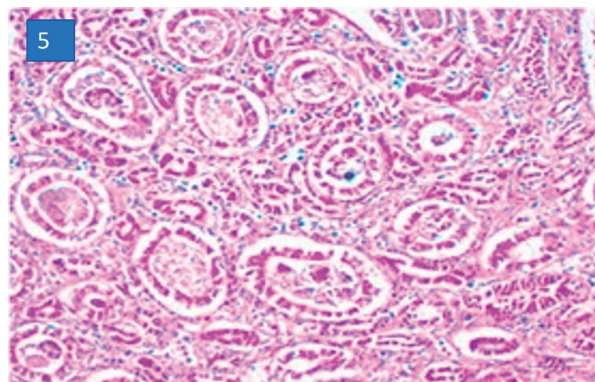


Fig. 5. Kidney showing severe degenerative tubular epithelial cells with tubular casts and lymphocytic infiltration H&E, 200X

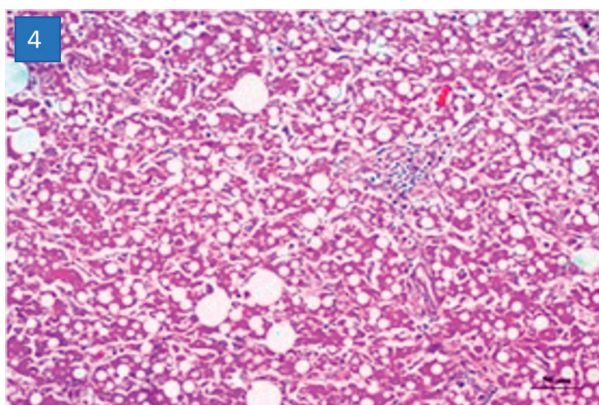


Fig. 4. Liver showing severe fatty changes along with lymphocytic infiltration H&E, 200X

**PCR Amplification.** DNA bands of expected sizes of 725 and 750 bp by respective primers were visualized (Fig. 6).

**Nucleotide Sequence Analysis.** The phylogenetic tree revealed that *B. anserina*/India/Nagpur/2018 was closely related to *B. anserina* from Iran and Brazil (Table 3 and Fig. 7). Comparison of the partial *flaB* gene sequence of *B. anserina* revealed that *B. anserina*/India/Nagpur/2018 was 99% identical to the sequences of KY438930 *B. anserina* isolate (Iran) and DQ849626 *B. anserina* strain PL flagellin *fla* gene (Brazil) (ATALIBA et al., 2007; CHEGENI et al., 2017).

Table 3. Comparison of nucleotide sequence homology of the present strain with other isolates

S. No	Accession number	Gene	Nucleotide homology (%)
1	KY438930	Flagellin ( <i>flaB</i> ) gene (Iran)	99
2	DQ849626	Flagellin ( <i>flaB</i> ) gene (Brazil)	99
3	JF693808	Flagellin ( <i>flaB</i> ) gene (Pakistan)	97
4	CP005830	Flagellin ( <i>flaB</i> ) gene (USA)	98
5	X75201	Flagellin ( <i>flaB</i> ) gene (Sweden)	98
6	CP005829	Flagellin ( <i>flaB</i> ) gene (USA)	98
7	CP013704	Flagellin ( <i>flaB</i> ) gene (USA)	98

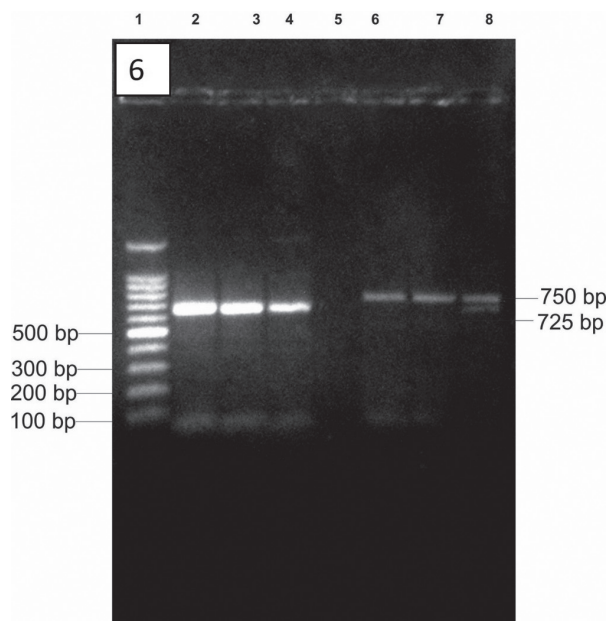


Fig. 6. Detection of *Borrelia* DNA in spleen by PCR targeting the flagellin gene  
Lane 1 – 100bp DNA ladder, Lane 2 – Positive control, Lane 3 and 4 – Spleen, Lane 5 – Negative control, Lane 6 – Positive control, Lane 7 and 8 – Spleen

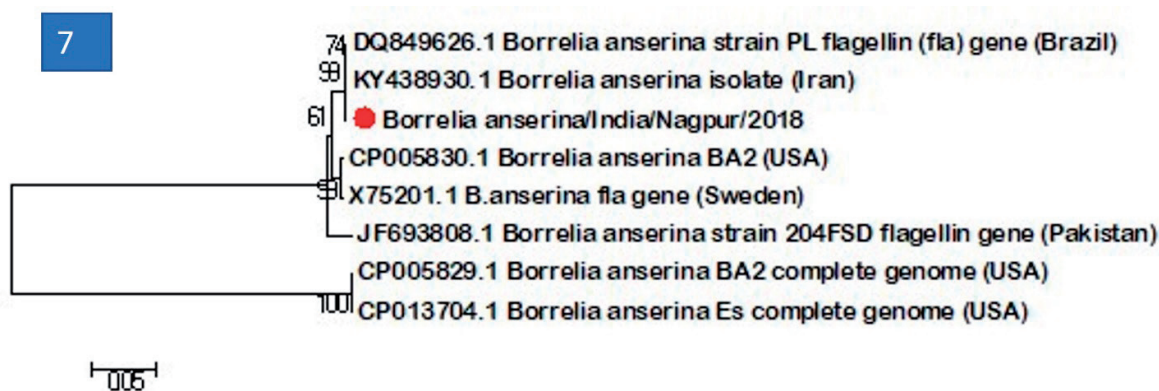


Fig. 7. Phylogenetic tree of the nucleotide sequence based on partial *flaB* gene sequences of *Borrelia anserina*. *B. anserina*/India/Nagpur//2018 is indicated by the red filled circle. Numbers at nodes indicate support by bootstrap resampling of 100 replicates.

## Discussion

Spirochaetosis among chicken in India has been reported since the early 1970s and regularly afterwards in different breeds (CHAWLA and SINGH, 1969).

However, the molecular characterization from these outbreaks has not been studied. In the present study, the outbreak was reported during the month of July, when the temperature was relatively warm

and the humidity high. Outbreaks of spirochetosis are common during the warm humid seasons of the year and are related directly to increased tick activity. Ticks collected from the farm were identified as *Argas persicus* which is known to be the vector transmitting *B. anserina*. The occurrence of *Argas persicus* infestation on poultry farms in India during recent years has already been reported (MALLESH

et al., 2018). This indicates that the agent of fowl spirochaetosis is still prevalent worldwide and its re-emergence as an important veterinary pathogen could occur in the near future (ATALIBA et al., 2007).

The gross lesions observed in this study, such as mottled spleen, are characteristic of *B. anserina* infection. Enlargement and mottling of the spleen were previously described by many researchers and correlated with extensive phagocytosis of the red blood cells in the spleen and haemosiderosis (SHOMMEIN and KHOGALI, 1974). As observed in the present study, sub capsular haemorrhage and fatty degeneration in the liver was noticed in birds infected with virulent strains of *Borrelia anserina* (BANDOPADHYAY and VEGAD, 1983). In an experimental infection of chickens, lesions were observed such as mononuclear cell infiltrations in the spleen and liver, and necrosis and thickening of the capillary walls in the spleen (EL-NASRI et al., 2010).

Due to the highly conserved nature of the flagellin gene among *Borrelia* species, the flagellin gene is usually targeted for PCR analysis. It can be used to distinguish borrelial infections irrespective of differences in the causative species (ASSOUS et al., 1994). The flagellin gene encodes the endoflagellar protein specific to spirochetes and, due to its diversity, it has been used extensively for identification of the *Borrelia* species. The reported disease outbreak in layer birds was diagnosed as fowl spirochaetosis caused by *B. anserina*, which was confirmed by molecular methods. The strain causing the disease was phylogenetically closely related to the Iranian and Brazilian strains of *Borrelia anserina*. The disease is brought to farms by flying wild birds and other international traveller birds which shed *Argas persicus* and *B. anserina*. This report describes the sequence and phylogenetic analysis of *B. anserina* in India, which is the causative agent of fowl spirochaetosis. More data are required to know the molecular epidemiology of the *Borrelia anserina* in India. Understanding the genetic background of circulating *B. anserina* in India will help to formulate control strategies using vaccines and/or the use of therapeutics.

## Conclusions

In the present study, *B. anserina* was detected and genotyped from disease outbreaks in central India, and compared with other previously isolated strains from around the world. The present study describes the molecular characterization of *B. anserina* from India in layer birds. The predominant gross lesions of the spleen, the presence of *Argas persicus* and phylogenetic analysis of the *flaB* gene from the Indian isolates confirmed *B. anserina*.

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**HEDAU, M., M. KAORE, S. W. KOLTE, N. V. KURKURE: Istraživanje molekularnih i patoloških svojstava spirohetoze u komercijalnom jatu nesilica – prikaz slučaja. Vet. arhiv 94, 165-172, 2024.**

### SAŽETAK

Istraživanje je provedeno na slučaju prirodnog izbijanja spirohetoze u nesilica dobi od 46 tjedana. Najupečatljivija je postmortalna lezija bila povećana i prošarana slezena. Histopatološki su uočena nekrotična žarišta sa zadebljanjem kapilarnih stijenki slezene, opsežne masne promjene u jetri te degenerativne tubularne epitelne stanice s prozirnim cilindrima i limfocitna infiltracija u bubrezima. Uporaba odgovarajućih početnica i PCR umnažanje ekstrahirane DNA iz tkiva slezene uputilo je na 725 i 750 bp amplikona specifičnih za gen flagelina (*flaB*) *Borrelia anserina*. Soj koji je uzrokovao bolest bio je filogenetski usko povezan s iranskim i brazilskim sojevima *B. anserina*. Ovaj prikaz slučaja daje genetsku karakterizaciju i filogenetsku analizu spirohete *B. anserina* izdvojene u slučaju izbijanja spirohetoze nesilica u Indiji.

**Ključne riječi:** *Argas persicus*; *Borrelia anserina*; Indija; nesilice; filogenetsko istraživanje; spirohetoza

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