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RESEARCH NOTE

Pathological and molecular studies of the renal trematode *Paratanaisia bragai* in Indian peafowls (*Pavo cristatus*)

M. Asok Kumar[1], Deepak Kumar[2], Munuswamy Palanivelu[1], Latchumikanthan Annamalai[3], Karikalan Mathesh[4], Rajendra Singh[5], Anil Kumar Sharma[5] and Kuldeep Dhama[1]

[1]Avian Diseases Section, Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly – 243122, U.P., India; [2]Genetic Engineering Virus Laboratory, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly – 243122, U.P., India; [3]Veterinary University Training and Research Centre (VUTRC), Villupuram – 605601. Tamil Nadu; [4]Centre for Wildlife, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly – 243122, U.P., India; [5]Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly – 243122, U.P., India

Abstract

Endoparasitic diseases are commonly encountered in free-ranging birds. Although not all endoparasites cause disease, persistent infection with large numbers of parasites almost always affects normal physiological functions, leading to deleterious effects on the host. This paper describes the anatomopathological alterations caused by the renal trematode *Paratanaisia bragai* in Indian peafowl (n = 3) and examines the phylogeny of these and related parasites. Peafowl from forests in and around the Bareilly region, Uttar Pradesh, India, were necropsied, and microscopic and molecular investigations were performed. The peafowl were confirmed to be infected with *P. bragai*. Significant gross pathological lesions suggested nephrosis, and microscopic findings indicated a mild-to-moderate degree of nephrosis caused by the parasites in the tissue. The parasites were identified as *P. bragai* by histomorphological analysis of adult and eggs in the ureters, and the identification was confirmed by PCR and phylogenetic analysis. Nucleotide sequencing of the PCR products from the renal trematodes recovered from Indian peafowl revealed a close association with *P. bragai* from Columbiformes in the United Kingdom and Spain. The pathology and molecular epidemiology of parasitic diseases affecting peafowl is not well understood in India. This is the first report from India and the second report worldwide to document *P. bragai* infection in peafowl.

Keywords

Kidney, *Paratanaisia*, peafowl, phylogenetic analysis, trematode

Introduction

The *Pavo cristatus* peafowl (Galliformes: Phasianidae: Phasianinae), commonly referred to as a peacock, is the national bird of India. Endoparasitic infections are one cause of mortality in peafowl (Freitas *et al*. 2002). The pathology and molecular epidemiology of parasitic diseases affecting peafowl is not well understood in India compared to other areas. Among endoparasitic diseases, coccidiosis results in poor growth and diarrhea that can lead to death (Jaiswal *et al*. 2012). Renal coccidiosis caused by *Eimeria* spp. are the most important cause of parasitic nephritis (Pollock, 2006). Kidney trematodes are seldom reported in peafowl in India. *Paratanaisia bragai* parasitizes the kidneys and ureters of several avian species (Brandolini *et al*. 1997; Kanev *et al.* 2002). The parasite *P. bragai*, a member of the family Eucotylidae, has a two-host life cycle. The metacercariae develop as sporocysts in fresh water as well as in land snails, and the infected snails are ingested by birds. The ingested metacercariae excyst and develop in the kidneys of the definitive host to become mature parasites (Unwin *et al.* 2013). *P. bragai* has been identified and reported in various host species viz., *Gallus gallus domesticus* (chicken), *Columba livia domestica* (pigeon), *Phasianus colchicus* (pheasant), *Numida meleagris* (helmeted guineafowl), *M. gallopavo* (turkey), *Melopsittacus undulatus* (white-eared parakeet), *Rhynchotus rufescens* (red-winged

*Corresponding author: drasokvet@gmail.com

timamou), *Primolius maracana* (blue-winged macaw), *Pavo cristatus* (peafowls) and *Columbina talpacoti* (ruddy ground dove) from various parts of the world including Central America, South America, Asia, islands of the tropical Pacific Ocean and India (Keller and Araujo, 1992; Menezes *et al.* 2001; Mapeli *et al.* 2003; Pinto *et al.* 2004; Gomes *et al.* 2005; Luppi *et al.* 2007; Unwin *et al.* 2013; Costa *et al.* 2015; Malik *et al.* 2016). In India, *P. bragai* has been reported only in pigeons (Borah *et al*. 2009; Malik *et al*. 2016). The infection is typically subclinical in certain species and fatal in others. Mortality is common in Psittaciformes infected with *P. bragai* (Luppi *et al.* 2007; Unwin *et al.* 2013). Microscopically, adults and eggs are frequently found in the renal parenchyma, with pathological changes associated with dilation of the collecting ducts (Pinto *et al.* 2004; Gomes *et al.* 2005). The occurrence and pathology of renal trematodiasis caused by *P. bragai* infection in avian species is not well defined in India. A literature survey revealed that there are only two reports from India on *P. bragai* infection in pigeons (Borah *et al*. 2009; Malik *et al*. 2016), but whether it occurs in other avian species in this country is unknown. This paper describes *P. bragai* infection in free-roaming Indian peafowl, with an emphasis on gross and histopathological tissue alterations in the renal parenchyma and ureters. Understanding the occurrence and pathology of renal trematodiasis in other aquatic, captive, and free-ranging avian species is crucial to ascertaining the host range of *P. bragai*.

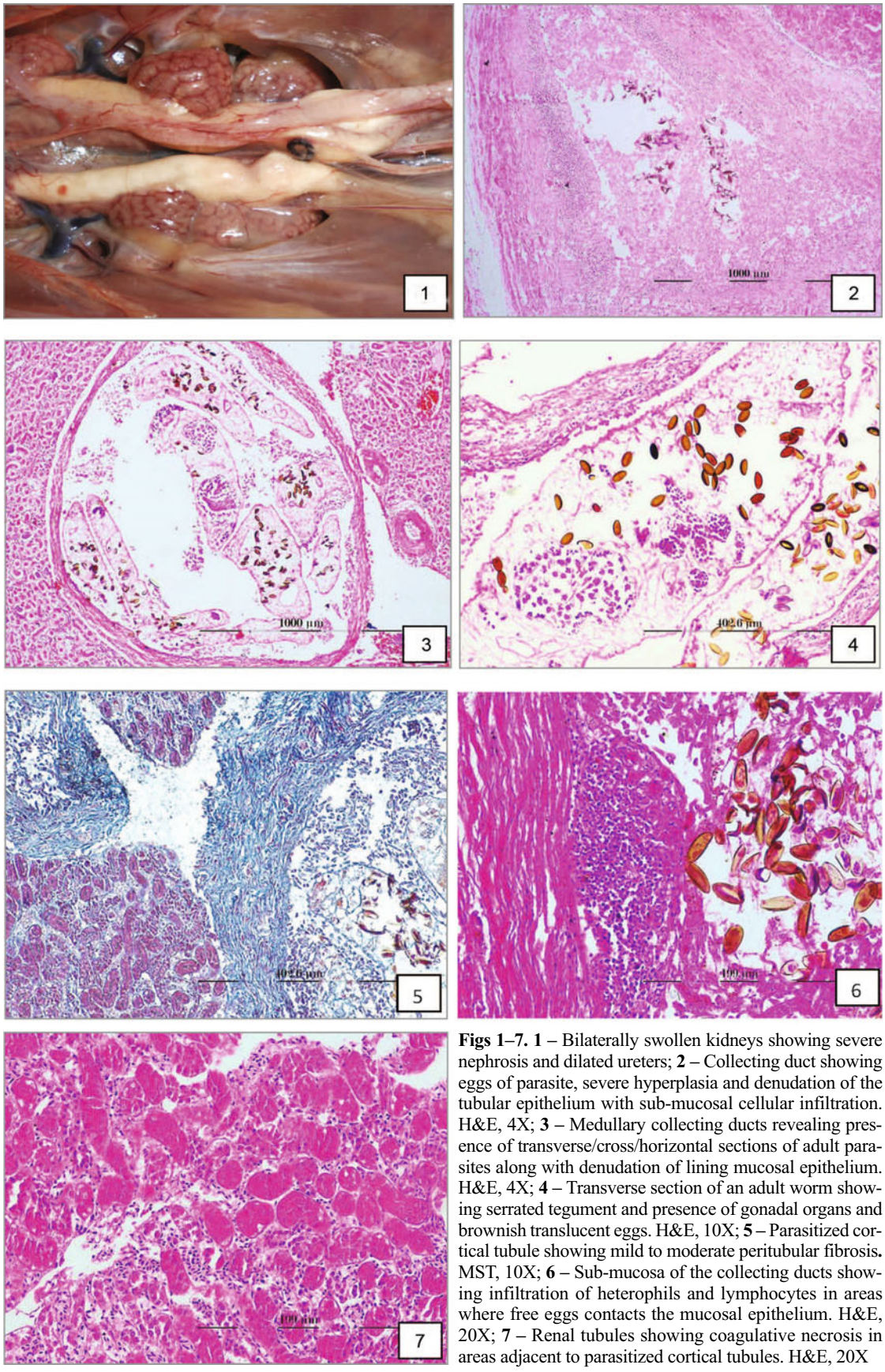
Case report

Three carcasses of peafowl from a forest range in and around Bareilly region, Uttar Pradesh, India, were presented to the Avian Diseases Section, Division of Pathology, for necropsy. Detailed necropsies of the carcasses were performed, and gross lesions were recorded. At necropsy, all the three peafowl showed visible gross pathology of the kidneys and ureters. The kidneys were swollen and edematous with surfaces revealing prominent tubular patterns suggestive of nephrosis. Ureters were dilated and contained a whitish gelatinous fluid admixed with urate crystals (Fig. 1).

Representative tissue samples were preserved in 10% neutral buffered formalin (NBF) for histological examination, and kidney tissues were preserved by freezing at −80°C for molecular study. The tissue (kidney and ureter) samples fixed in 10% NBF were processed by routine paraffin embedding, and 4–5-µm thick sections were stained with hematoxylin and eosin (H&E) for histopathological analysis and Masson's trichrome (MST) stain to determine the degree of fibrosis (Luna, 1968). Histologically, the lesions observed could be broadly categorized into circulatory disturbances, degenerative changes, growth disturbances, and inflammation due to the presence of parasites. Circulatory disturbances included focal areas of sub-capsular hemorrhage, generalized congestion of interstitial blood vessels, and multifocal areas of hemorrhage in the parenchyma. Degenerative changes recorded were generalized cloudy swelling of the tubular epithelium, denudation of the tubular epithelium in its lumen, and focal areas of coagulative necrosis. Growth disturbances were observed in focal areas of the glomeruli that exhibited hypoplastic changes with widened peri-glomerular spaces, flattening of the tubular epithelium in the collecting tubules in contact with parasites, and severe hyperplasia of the tubular epithelium with denudation in the lumen (Fig. 2). Lesions were very prominent in the medullary collecting ducts, revealing dilatation and the presence of numerous transverse/cross-sectional sections of adult parasites (Fig. 3), along with congested blood vessels. The transverse sections of adult worms showed serrated cuticle/tegument on their surfaces and the presence of gonadal organs, as well as brownish translucent eggs within their body cavities (Fig. 4). A few cortical tubules containing parasites exhibited mild-to-moderate peritubular fibrosis (Fig. 5). Dilatation of the medullary collecting ducts and flattening of the epithelial lining were observed in each case, and a few calcified parasitic eggs were found. Severe epithelial hyperplasia and denudation were evident in places where there were free eggs, along with mild-to-moderate infiltration of inflammatory cells, which were predominantly heterophils and lymphocytes (Fig. 6). The renal parenchyma adjacent to the parasitized collecting ducts showed focal areas of degeneration and coagulative necrosis (Fig. 7).

DNA was extracted from kidney tissues collected and stored at −80°C using the commercial DNeasy Blood & Tissue Kit (Qiagen, Germany). The primers sequences used were forward Para 28S_F (5′-AAGCCTGTGTCCACTTGGTC-3′) and reverse Para 28S_R (5′-CGTGCTGTTTACC-CTCTCTTC-3′), which amplified a partial-length *lsrDNA* gene segment of 310 bp (Unwin *et al.* 2013). The 50-µl reaction mixture contained 10 µl of Phusion HF Buffer (5×), 1 µl of dNTP (10 mM), 10 pM each of forward and reverse primers, 1.0 U of Pfu polymerase, and 5.0 µl of template DNA (5 ng). The PCR was performed in a thermal cycler (QB96 cycler, Quanta biotech) with an initial denaturation at 94 r extension at 72°C for 45 s, and final product extension at 72°C for 10 min. The amplified PCR products were then purified using a GeneJET PCR Purification Kit (Thermo Scientific, USA). PCR testing of the DNA extracted from kidney tissues yielded partial-length *lsrDNA* with a single specific amplicon of 310 bp (Fig. 8).

Purified PCR products were sequenced by a commercial sequencing service provider (Eurofins, India). The sequence chromatogram was annotated using DNA Star software. The annotated sequences were identified by the closest match in a BLASTN search of sequences submitted in the non-redundant NCBI GenBank nucleotide database (http://www.ncbi.nlm.nih.gov) (Altschul *et al*. 1997). The sequence analyzed in this study was submitted to GenBank (accession numbers KX431562 and KX420705). Full/partial length sequences of the 28 ribosomal RNA (rRNA) in trematodes from various geographical locations were retrieved from GenBank and then assembled and aligned using the ClustalW program in MEGA 7.0



Figs 1–7. 1 – Bilaterally swollen kidneys showing severe nephrosis and dilated ureters; **2** – Collecting duct showing eggs of parasite, severe hyperplasia and denudation of the tubular epithelium with sub-mucosal cellular infiltration. H&E, 4X; **3** – Medullary collecting ducts revealing presence of transverse/cross/horizontal sections of adult parasites along with denudation of lining mucosal epithelium. H&E, 4X; **4** – Transverse section of an adult worm showing serrated tegument and presence of gonadal organs and brownish translucent eggs. H&E, 10X; **5** – Parasitized cortical tubule showing mild to moderate peritubular fibrosis**.** MST, 10X; **6** – Sub-mucosa of the collecting ducts showing infiltration of heterophils and lymphocytes in areas where free eggs contacts the mucosal epithelium. H&E, 20X; **7** – Renal tubules showing coagulative necrosis in areas adjacent to parasitized cortical tubules. H&E, 20X

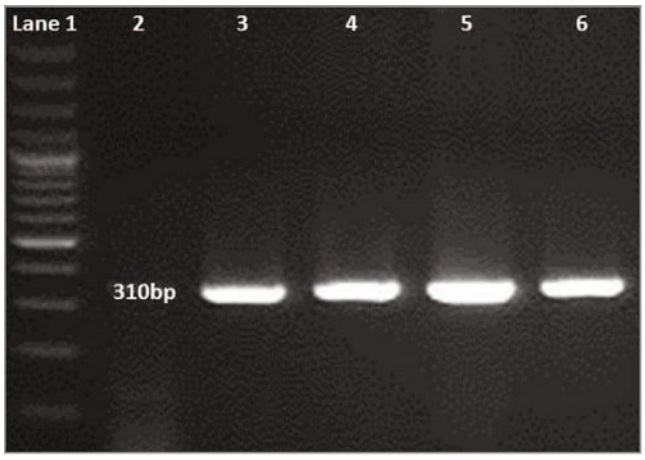


Fig 8. Agarose gel electrophoresis: Lane 1: 100bp plus molecular DNA marker; Lane 2: Template control; Lane 3–6 test samples showing single and specific amplicon of 310 bp

(Kumar *et al*. 2016). The evolutionary history of the trematodes was inferred using the maximum likelihood method based on the Kimura two-parameter model (Kimura, 1980). The tree with the highest log likelihood (−632.1014) is shown. The percentage of trees in which the associated taxa clustered together in a bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with a higher log likelihood value. A discrete gamma distribution was used to model differences in the evolutionary rates among sites (five categories [+*G*, parameter = 0.6505]). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 42.2490% sites). The tree is drawn to scale, with branch lengths representing the number of substitutions per site. Eight nucleotide sequences were analyzed. All positions with less than 95% site coverage were eliminated. Thus, fewer than 5% alignment gaps, missing data, or ambiguous bases were allowed at any position. There was a total of 302 positions in the final dataset. All evolutionary analyses were conducted in MEGA 7.0 (Kumar *et al.* 2016). Phylogenetic analysis by pairwise alignment between KX420705 and KX431562 revealed a 2.4% difference between the 28S RNA gene sequences (Fig. 9).

Discussion

The peafowl is the national bird of India. Recently, there have been a few reports on the deaths of these birds due to various diseases and malicious killing (Shivaprasad and Galey, 2001; Aulakh *et al.* 2005; Shetty *et al.* 2011; Desingu *et al.* 2016). Newcastle disease is an infectious disease whose epidemiology has been widely studied in peafowl (Kumar *et al*. 2013; Desingu *et al.* 2016). Among endoparasites affecting peafowl, intestinal coccidiosis is frequently reported to cause mortality. There are reports documenting *P. bragai* infection in a wide range of avian species, including peacocks in other countries. *P. bragai* was first reported in India in doves by Borah *et al.* (2009). Renal trematodiasis caused by *P. bragai* has been reported in peafowl in Brazil (Costa *et al.* 2015). The present report details the pathology, diagnosis, and molecular characterization of *P. bragai* in peafowl, which is the first study of its kind in India. Grossly, the nephrosis and dilatation of ureters observed was in accordance with the results from previous studies (Unwin *et al.* 2013; Malik *et al.* 2016*;* Silva *et al.* 2016). The gross lesions associated with *P. bragai* infection vary among species, ranging from no visible lesions to severe nephritis. Disease susceptibility of various birds is well documented, and Galliformes have been found highly susceptible to *P. bragai* infection (Unwin *et al.* 2013). In Galliformes, death resulted mainly from renal failure because of mechanical and immunological responses to the parasite.

Microscopically, the parasites were located in the medullary collecting tubules as reported previously, and there was severe dilatation of the tubules due to a compensatory mechanism to pass urine (Pinto *et al.* 2004). There are reports that the extent of microscopic lesions induced by the parasites is mainly attributable to the size of the parasites, site of infec-

Fig 9. Evolutionary relationships of *P. bragai* isolated from Indian peafowl (*Pavo cristatus*) with other Eucotylidae members

tion, parasite strain, and hosts affected (Gomes *et al.* 2005). In this study, severe hyperplasia of the tubular epithelium was noted; a possible reason for this might be constant mechanical irritation by the parasites and eggs over the mucosal surface. Inflammation around the parasites has not been reported in all species studied (Unwin *et al.* 2013), but in the present study, there was inflammation in the submucosa of the collecting ducts that were occluded by parasites. The presence of tegumentary spines on the surface of the parasite (Costa *et al.* 2015) and free eggs (Abdo *et al*. 2013) may have triggered the inflammatory reaction. Calcification of old lesions, previously reported by Costa *et al.* (2015), was observed in the present study as bluish material in the renal parenchyma. Based on the extent of tissue reaction to the parasites, *P. bragai* in peafowl might be moderately pathogenic, which contrasts with the results of an earlier study, in which the authors found very mild inflammation in peafowl (Costa *et al.* 2015). This difference in inflammatory reactions may be attributable to the strains of parasite and host. A point of interest is that birds exhibiting mild-to-moderate inflammation might act as reservoirs for dissemination of the pathogen to other avian hosts.

Unwin *et al.* (2013) reported that *P. bragai* infection can be confirmed using established PCR methods, and the PCR results obtained in the present study corroborated their findings. The PCR products were sequenced, and a phylogenetic tree was reconstructed using the maximum likelihood method based on a Kimura 2-parameter model analysis of the 28S rRNA. A bootstrap analysis (1000 replicates) was performed to evaluate the clustering of taxa. Alignments with other trematodes confirmed the close association between *P. bragai* isolates from a Socorro dove (*Zenaida graysoni*) and helmeted guineafowl (*Numida meleagris*) and their position within the system Digenea: Eucotylidae, grouping with *Tanaisia fedtschenkoi* isolated from mallard ducks (*Anas platyrhynchus*); *Tamerlania* spp. and *Nephromonorcha varitestis* isolated from American white pelicans (*Pelecanus erythrorhynchos*); and *Renicola* spp. isolated from Eurasian curlew *(Numenius arquatus)*.

To conclude, endoparasites infecting organs outside of the gastrointestinal tract in wild birds are underreported. *P. bragai* infection in peafowl can cause mild nephrosis to severe nephritis, mild-to-moderate fibrosis, and renal gout, and the outcome of this infection in different birds varies, as evidenced in the present and previous investigations. Since the parasite is transmitted by ingestion of freshwater or land snails, it is important to analyze snails in their wild habitats to understand the transmission and epidemiology of *P. bragai* and to control the infection in wild avian species. In India, *P. bragai* infection has been reported in pigeons, and now we report it in peafowl; however, other free-ranging aquatic and wild birds should be analyzed for infection. Careful histological screening of nephrotic and nephritic kidneys from free-ranging aquatic and wild avian species will provide information on the occurrence of this parasite in different avian populations. Based on this study, it is very clear that *P. bragai* exists in wild birds of India, suggesting the possibility that these parasites may infect other wild avian species.

Disclosure statement. The authors declare that they have no competing interests.

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