Scientific Report

The first record of *Heterakis gallinarum* as a cause of fatal nodular typhlitis in golden pheasants (*Chrysolophus pictus*) in India

Sharma, M.[1]; Asok Kumar, M.[1*]; Karikalan, M.[2]; Faslu Rahman, A. T.[1]; Vivek Srinivas, M.[3]; Ram, H.[4]; Pawde, A. M.[2]; Shukla, U.[5]; Yadav, B. M.[5]; Dhama, K.[1] and Saikumar, G.[1]

[1]Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly-243122, Uttar Pradesh, India; [2]Centre for Wildlife, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly-243122, Uttar Pradesh, India; [3]Department of Veterinary Microbiology, Rajiv Gandhi Institute of Veterinary Education and Research, Puducherry, 605 009, India; [4]Division of Parasitology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly-243122, Uttar Pradesh, India; [5]BVSc, Nawab Wajid Ali Shah Zoological Garden, Hazratganj, Lucknow, Uttar Pradesh, 226 001, India

[*]***Correspondence:*** *M. Asok Kumar, Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly-243122, Uttar Pradesh, India. E-mail: drasokvet@gmail.com*

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Abstract

Background: Heterakidosis is one of the most prevalent parasitic diseases in birds, the caecae of a variety of wild and domestic birds are infected with these nematodes. In pheasants, nodular typhlitis is a lethal disease caused mainly by infection with *Heterakis isoloncheh* alone or in conjunction with *Heterakis gallinarum*. *H. gallinarum* has long been recognized to infect birds with low pathogenicity, with only a few fatal cases previously reported. **Case description:** This paper describes a case of fatal nodular typhlitis due to *H. gallinarum* in a male and female pair of adult golden pheasants (*Chrysolophus pictus*) from a zoological garden in Uttar Pradesh, India. **Findings/treatment and outcome:** The caecum had multiple serosal and mucosal nodules, the majority of which were found to contain various stages of parasites embedded in the center along with the free forms in the caecal contents. Histopathologically, these nodules were generally represented by granulomas centered on necrotic parasite debris, with the occasional reactive fibrous hyperplastic tissue reaction. Based on the morphology and nematode-specific internal transcribed spacer (ITS) ITS1-5.8 rRNA-ITS2 region-based PCR, the nematode was identified as *H. gallinarum*. The presence of *H. gallinarum* was further confirmed by sequencing the ITS region followed by phylogenetic analysis. According to the author's best knowledge, this is the first instance of *H. gallinarum* being linked to nodular typhlitis in pheasants in India. **Conclusion:** Our findings confirm that *H. gallinarum*, other than *H. isolonche*, can induce severe nodular typhlitis with a fatal outcome in pheasants.

Key words: *Heterakis gallinarum*, *Heterakis isolonche*, India, Nodular typhlitis, Pheasants

Introduction

Heterakidosis is one of the most prevalent parasitic diseases in birds, rarely in rodents, and is caused by several *Heterakis* species. The Heterakis genus belongs to the family Heterakidea, the superfamily Heterakoidea, the order Ascaridida, and the phylum Nematoda. Various species include *Heterakis gallinarum*, *H. isolonche*, *H. dispar*, *H. beramporia*, *H. indica*, *H. spumosa*, *H. dahomensis*, and *H. papillosa* (Schoch *et al*., 2020). Among all the species, *Heterakis gallinarum*, *H. isolonche*, and *H. dispar* often lead to pathological lesions in the caeca of a variety of wild and domestic birds (Amundson *et al*., 2016). These three species of Heterakis are differentiated mostly by the morphological characteristics of male parasites. *H. gallinarum* is probably the most common species frequently infecting birds maintained on soil or litter. *H. isolonche* invasions are mainly responsible for nodular lesions in pheasants, resulting in high mortality (Halajian *et al*., 2013). There are reports of *H. gallinarum* infection in domestic chickens from different parts of India (Ara *et al*., 2021; Das *et al*., 2022). By far, the importance of *H. gallinarum* is mainly due to its role in the transport of *Histomonas meleagridis*, which causes blackhead disease/Histomoniasis (Tyzzer, 1926). Chicken, turkey, pheasant, grouse, guinea fowl, partridge, duck, goose, and quail are known hosts of *H. gallinarum* (Wang *et al*., 2012). The life cycle of this worm is direct, without the involvement of any intermediate hosts. Earthworms may serve as paratenic hosts, allowing the larval stages to develop before being consumed by birds.

In pheasants, nodular typhlitis is a lethal disease caused mainly by infection with *H. isolonche* (Rao,

1994) alone or in conjunction with *H. gallinarum*. The defining feature of this condition is the presence of nodules in the caecum. The histology of nodules can range from granulomatous to neoplastic (Mendonca, 1953). Nodular typhlitis due to *H. isolonche* is well documented in the literature (Griner *et al*., 1977; Callinan, 1987; Balaguer, 1992; Himmel and Cianciolo, 2017), but reports due to *H. gallinarum* are scarce (Menezes, 2003). There are very few reports available globally in which infection with *H. gallinarum* alone was sufficient to cause granulomatous or neoplastic caecal nodules in chicken, guinea fowl, and pheasants (Meads and Taylor, 1963; Kaushik and Deorani, 1969; Riddel and Gajadhar, 1988; Khan *et al*., 1994; Menezes, 2003).

H. gallinarum has long been recognized to infect birds with low pathogenicity, with only a few fatal cases previously reported. This paper describes pathological features of two cases of *H. gallinarum* infections inducing severe nodular typhlitis in a male and female co-inhabited pair of golden pheasants from Uttar Pradesh (U.P.) that is the first report in India, to the best of our knowledge. Molecular identification and phylogenetic analysis of the nematode were also performed.

Case description

A male and female pair of adult golden pheasants were found dead in their enclosure in the Nawab Wazid Ali Shah Zoological Garden, Lucknow, U.P., India, and were necropsied in the Avian Section of the Department of Pathology, Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, U.P., India. The birds have been kept and reared on a semi-intensive soil system co-housed with silver pheasants. Housing details include a confined enclosure of 20.7 sq.m. along with an open area floored with soil and sand. The feeding schedule of pheasants includes unpeeled rice, green gram, millet, and wheat grains. As per the history provided by zoo keepers, six pheasants (3 golden pheasants and 3 silver pheasants) showed clinical signs like dullness, depression, anorexia, and loose droppings in the last five days, of which two golden pheasants died and presented for necropsy on the same day. The salient gross lesions were noted from both carcasses, and ileal feces samples were collected for direct microscopic examination. Helminths extracted from the caeca of both birds were collected in 10% formalin and ice for the morphological and molecular studies, respectively. Representative samples were taken from all the visceral organs for histopathological examination.

Necropsy findings

External examination revealed pale conjunctival mucous membranes, fair body condition, and soiled peri cloacal area with greenish faecal contents. The most striking finding was the bilateral symmetrical enlargement of the caeca with multiple, poorly demarcated, variable sized nodules on the serosal wall of both the caeca, which on dissection revealed similar nodules of mahogany brown to reddish color, about 1-3 mm in diameter, with or without central openings in the mucosa. The entire wall of the caecum was thickened, giving it a corrugated appearance. Thin worms were visible in the caecal lumen. The spleen, liver, and kidneys were swollen and congested.

Parasitological examination

Direct microscopic examination of fecal samples revealed ellipsoidal eggs with a thick and smooth outer shell and unsegmented contents similar to those of Heterakis species. About 20 and 18 parasites could be visualized in the lumen of male and female pheasants, respectively. There were multifocal nodules with parasites on the cecal mucosa (5 to 7/cm²). Additionally, there were no other species of parasites found. Morphological analysis, based on the shape of the oesophagus and the size of the spicules, of parasites recovered from the lumen as well as the caecal nodules revealed an oesophagus with a prominent posterior bulb and unequal length of spicules similar to *H. gallinarum* (Figs. 1a and b).

Fig. 1: Species identification based on shape of oesophagus and size of spicules. (**a**) Anterior end of roundworm revealing large characteristic posterior bulb (×40), and (**b**) Posterior end of roundworm revealing large circular precloacal sucker (arrow) and unequal spicules (arrowheads), (×40)

Histopathology

Histopathological examination of the caecal sections revealed a granulomatous response with areas of caseation and necrotic fragments of the parasite in the center and infiltrates of multinucleated giant cells and histiocytes at the periphery (Figs. 2a and b). The nodules were localized in tunica submucosa, muscularis, and serosa whorls (Fig. 2c) and, at times, showed distinct reactive fibroblastic proliferation in the form of whorls, which was confirmed by Masson trichrome staining (Fig. 2d). The mucosa overlying the nodules was observed to have atrophied epithelium with shortened and denuded villi and mononuclear inflammatory cells in the lamina propria. The lumen showed several segments of adult parasite structures (Fig. 2c). Morphologically, the cases were diagnosed as the chronic severe multifocal granulomatous typhilitis. Other visceral organs like the liver and spleen showed moderately congested blood vessels.

DNA extraction and PCR amplification

Genomic DNA was extracted from parasites collected from the cecum (10 each of free parasites as well as teased out parasites from the nodules) using a commercially available DNA isolation kit (DNeasy Blood and Tissue Kit™, Qiagen, Germany) as per the manufacturer's protocol. PCR amplification targeting the internal transcribed spacer (ITS) ITS1-5.8 rRNA-ITS2 region was done using published primers and a thermal profile (Bobrek *et al*., 2018). PCR amplification targeting the ITS1-5.8 rRNA-ITS2 region yielded an amplicon size of approximately 1.1 kb, specific for *H. gallinarum*.

Sequencing and sequence analysis

The desired amplicon size was sliced, and gel purification was done using a commercially available gel purification kit (QIAquick® Gel Extraction Kit, Qiagen, Germany) as per the manufacturer's protocol. The

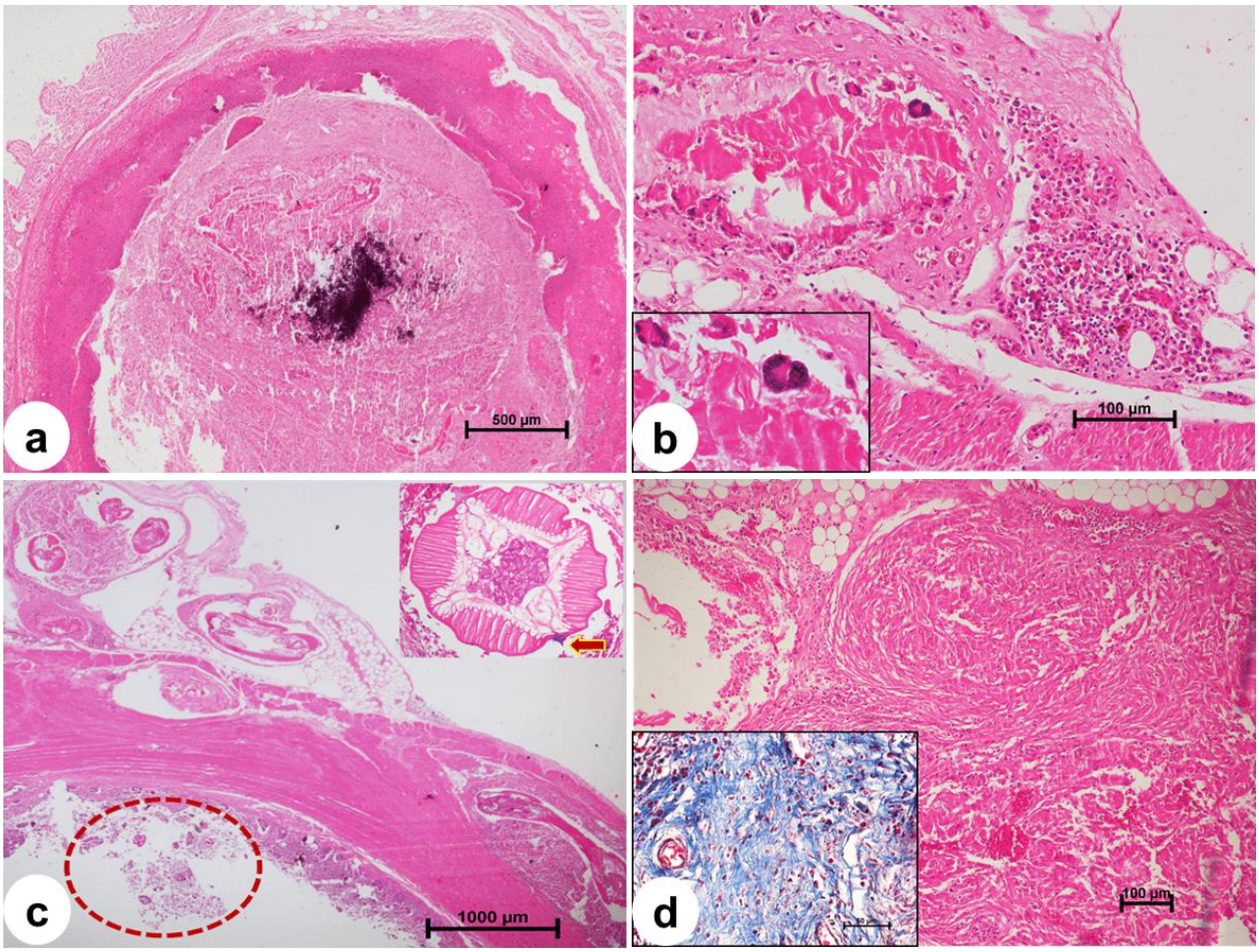

Fig. 2: Histopathological lesions in caecum. (**a**) Cecal sections showing thickened and necrotic walls due to granulomatous response with areas of caseation and necrotic fragments of parasite in the center, (H&E, ×40), (**b**) Serosal surface of the cecum showing granulomatous response surrounded by multinucleated giant cells and histiocytes at the periphery, (H&E, ×200) (Inset: Multinucleated giant cells), (**c**) Cecum showing nodules in tunica serosa, muscularis, and submucosa with cross sections of parasites in the lumen, the mucosal lining epithelium showed shortened and denuded villi, (H&E, ×20) (Inset: Cross section of adult parasite with prominent lateral alae indicated by arrow), and (**d**) Distinct reactive fibroblastic proliferation in the form of whorls was seen alongside the parasitic nodules, (H&E, ×100), which was confirmed by Masson trichrome staining (Inset: MST ×200)

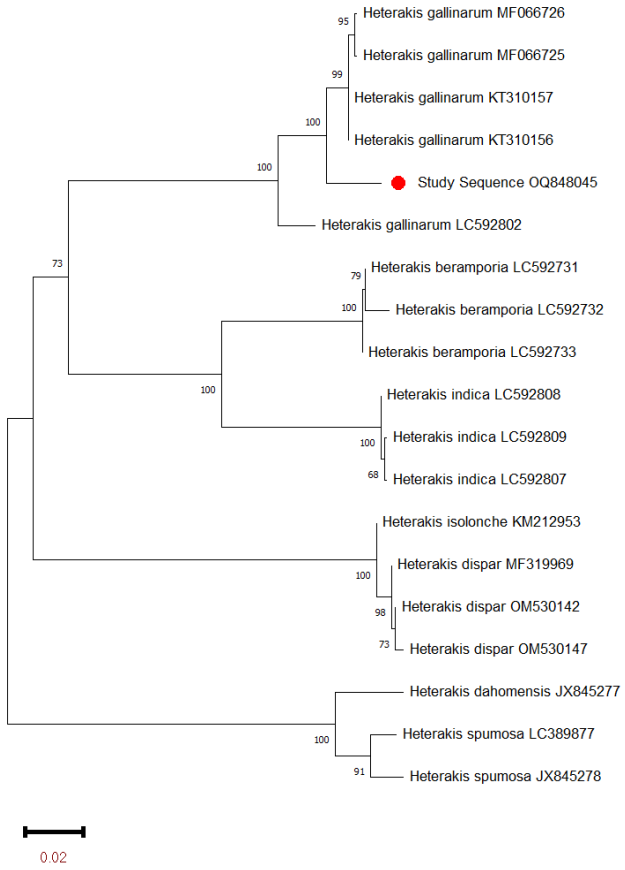

Fig. 3: Phylogenetic analysis: The evolutionary history was inferred using the Maximum Likelihood tree based on the Tamura-Nei model. The *Heterakis gallinarum* query sequences (OQ848045) shown with solid circle and other reference sequences available in the Genbank. Bootstrap values are shown next to the branches in the phylogenetic tree

purified PCR products of the ITS region were sent for custom sequencing in both directions (5´-3´ and 3´-5´) using the automated sequencer, Applied Biosystem 3100 (Eurofins, Bengaluru, India). The specificity of the sequences with respect to the ITS region was determined using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The query nucleotide sequences were aligned with corresponding ITS sequences available in GenBank using the multiple alignment program Clustal Omega (http://www.ebi.ac.uk/clustalomega/). For sequence alignment analysis, the sequences representing the ITS region were compared with those available in the GenBank databases of the National Center for Biotechnology Information (NCBI). The phylogenetic relationship based on the nucleotide sequences of the ITS region was analyzed. From the aligned query nucleotide sequences, the phylogenetic tree was constructed with full- and partial-length sequences of *28 rRNA* genes from different *Heterakis* species obtained from the NCBI and GenBank with the MEGA11 program using the Maximum Likelihood method based on the Tamura-Nei model (Kumar *et al*., 2018). Upon BLAST analysis and sequence alignment, the specificity of the sequences was found to be maximally identical (97.7%) with *H. gallinarum* sequences available in the GenBank. The phylogenetic analysis found a close relationship between our ITS sequence and the sequences of *H. gallinarum* chicken isolates from China and Tunisia (Fig. 3).

Accession number

The aligned partial-length ITS sequence of *H. gallinarum* sequences was submitted to the GenBank database and is available under the accession number OQ848045.

Discussion

Heterakidiosis caused by *H. gallinarum* was reported in domestic chickens from different parts of India. A prevalence rate of 20.80%-24.18%, 14.08% was reported from Kashmir (North India) and Meghalaya (North-Eastern India), respectively (Ara *et al*., 2021; Das *et al*., 2022). Furthermore, sporadic cases were reported from chickens and guinea fowl from Tamil Nadu, southern India (Vijayalingam *et al*., 2020; Marudhai *et al*., 2022). This observation is significant since it is the first report of *H. gallinarum*-associated nodular typhlitis in pheasants in India, to our knowledge. In the literature, only a few cases of *H. gallinarum*-associated nodular typhlitis in pheasants have been documented (Meads and Taylor, 1963; Menezes, 2003). Because the current occurrence was observed in pheasants raised on soil, it was thought that earthworms acted as parasite carriers. The nodular typhlitis in pheasants is characterized by the formation of inflammatory, granulomatous, and/or neoplastic nodules located in the cecal wall attributable to the variations in the cellular reactions, maturity of the nodules, Heterakis species involved, and co-infecton status (Callinan, 1987; Menezes, 2003). In both carcasses, the granulomas were limited to the caeca and had a similar granulomatous gross and microscopic appearance to those previously described in pheasants (Meads and Taylor, 1963; Menezes, 2003). Other researchers obtained similar results in *H. gallinarum*-infected guinea fowls (Khan *et al*., 1994) and domestic chickens (Kaushik and Deorani, 1969; Mutalib and Riddell, 1982; Riddell and Gajadhar, 1988). The PCR reaction amplifying the ITS region is considered a good tool for differentiating *Heterakis* species (Bobrek *et al*., 2018). The primers based on the 18S-ITS1-5.8S-ITS2-28S region take advantage of the intraspecific variability of the ITS regions and the conserved sequences of the 18S, 5.8S, and 28S regions. So, this PCR-based species level identification system used in this study can be an efficient tool for early and specific diagnosis of *H. gallinarum* infection. Further, the close phylogenetic relationship between the study sequence and previously reported *H. gallinarum* chicken isolates suggests cross species infection. As the pheasants were raised in semi intensive soil system, contamination with chicken litter or earthworm carrying larval stages of *H. gallinarum* can be anticipated.

Despite the widespread belief that *H. gallinarum*

develops primarily inside the cecal lumen, a tissue phase has been reported (Madsen, 1962), which is most likely the result of host resistance (Kaushik and Deorani, 1969). The strains of *H. gallinarum* that are not well adapted to the new host population are likely to cause more severe disease forms, as observed in young chukar partridges infected with larval strains of *H. gallinarum* retrieved from other domestic birds, which developed severe peritonitis due to perforation of the cecal wall by the parasites (Lund and Chute, 1972). Similarly, the *H. gallinarum* strain that infected the pheasants in this study was likely not acclimated to the golden pheasants, resulting in a higher pathogenicity, as reported in earlier findings.

It is concluded that the caecal lesions were caused solely by the nematode *H. gallinarum*, as no other species was found during parasitological or molecular detection. As a result, the findings point to a possible fatal outcome of *H. gallinarum* infection in non-adapted host species in the case of heavy infection.

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Conflict of interest

Authors have no conflict of interests to declare.

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