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First report of typhlitis / typhlohepatitis caused by *Tetratrichomonas gallinarum* in three duck species

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Abstract

Two Red-breasted Mergansers (*Mergus serrator*), one Hooded Merganser (*Lophodytes cucullatus*), and one Common Eider (*Somateria mollissima*) from a German zoological collection died of necrotizing typhlitis / typhlohepatitis within two years. Using a newly established chromogenic in-situ hybridization assay, numerous intralesional trophozoites of *Tetratrichomonas gallinarum* could be detected in formalin-fixed and paraffin-embedded tissues from caeca and livers of the affected birds. Partial sequencing of the 18S rRNA-gene revealed two unique nucleotide sequences very similar to *T. gallinarum* strains isolated from avian and human hosts. One turkey kept in the same zoological collection succumbed to histomonosis (blackhead disease) confirmed with chromogenic ISH at the time of the first duck fatalities. This turkey also harboured *T. gallinarum* trophozoites within necrotic cell debris in the caecal lumen, which might be epidemiologically related to the *T. gallinarum* infections in the ducks.

Keywords

Anatidae; *Tetratrichomonas gallinarum*; typhlohepatitis; protozoal parasitosis

Introduction

The flagellated protozoan parasite *Tetratrichomonas gallinarum* (former name *Trichomonas gallinarum*) has been described to cause typhlohepatitis in turkeys morphologically very similar to histomonosis (blackhead disease) either as monoinfection or as mixed infection with *Histomonas meleagridis* (Allen, 1941). Oral inoculation of cultured tetratrichomonads obtained from turkeys that had died of the disease induced characteristic macroscopic lesions in poults. These consisted of cheesy cores of blood-stained tissue debris in the caeca and granular, cream-colored, well defined necrotic areas in the liver that were level with or elevated above the surface. In comparison, typical lesions from *H. meleagridis* looked very similar but missed the granular appearance and were slightly depressed below the liver surface. *T. gallinarum* showed a significantly lower pathogenicity than *H. meleagridis* in inoculated poults and the disease took a rather chronic course (Allen, 1941).

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Furthermore, *T. gallinarum* has been found to be associated with clinically relevant infections in various bird species, including manifestations at extraintestinal sites (Patton *et al.*, 1996; Crespo *et al.*, 2001). There have been repeated attempts to clarify the pathogenicity of *T. gallinarum* with the help of experimental infections, mostly in turkeys and chickens, with various outcomes and inconsistent pathological lesions (Allen, 1941; Kemp & Reid, 1965; Lee, 1972; Kulda, 1974; Norton, 1997).

There are only few reports of intestinal disease caused by trichomonads in ducks. For example, *Tetratrichomonas anatis* has been reported to cause mucopurulent sinusitis and catarrhal rhinitis, tracheitis and enteritis in juvenile ducks (Tsai, 1997). Also, next to bacteria and other protozoa, three different species of trichomonads were found to be associated with necrotic enteritis of breeder ducks by Leibovitz (1973), but *T. gallinarum* was not among them. In ducks and geese an experimental infection by inoculation of *T. gallinarum* cultures did not lead to pathological changes in the caeca in spite of large protozoal numbers (Pecka, 1991). Thus, clinically relevant intestinal infections of duck species with *T. gallinarum* have not been described previously.

Red-breasted Mergansers (*Mergus serrator*), Hooded Mergansers (*Lophodytes cucullatus*), and Common Eiders (*Somateria mollissima*) belong to the tribe Mergini, family Anatidae, order Anseriformes, and live along the continental coasts of the Northern hemisphere.

Materials and Methods

Standard pathology

Within two years two Red-breasted Mergansers (*Mergus serrator*), one Hooded Merganser (*Lophodytes cucullatus*), and one Common Eider (*Somateria mollissima*) kept in a zoological garden in northeastern Germany died suddenly and were submitted for necropsy. A domestic turkey from the same zoo was submitted for necropsy together with the first Red-breasted Merganser after sudden death at the same day. Sex, age and clinical history for all birds included in this study are listed in table 1.

Tissue samples taken at necropsy from all birds including liver and caeca were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. For pathohistological examination hematoxylin-eosin (HE) stain as well as periodic acid-Schiff (PAS) reaction following standard protocols were used. Native caecal tissue from the second Merganser was additionally preserved by freezing at −20° C.

Standard etiological examinations

Routine diagnostic workup included bacteriology of lung, liver and intestine using standard culture media and coproscopical examination of intestinal contents for the detection endoparasites. In addition, chlamydial infections, avian influenza viruses (AIV) and avian paramyxovirus serotype 1 (APMV-1) were excluded by PCR using standard protocols (Spackman *et al.*, 2002; Wise *et al.*, 2004; Ehricht *et al.*, 2006). In the second Red-breasted Merganser, mycological investigation of lung and air sacs was carried out using standard culture media.

Chromogenic in-situ hybridization

Histological sections from liver and caecum of all birds were processed for in-situ hybridization (ISH) according to Liebhart *et al.* (2006) using the therein described probe specific for *H. meleagridis*.

Another ISH assay with a protocol similar to the assay mentioned above but specific for *T. gallinarum* was established. The parameters remained unchanged except for an extended colour reaction of one hour. The sequence of the digoxigenin-labelled oligonucleotide probe designed complementary to the 18S ribosomal RNA (rRNA)-gene of *T. gallinarum* was: 5′-TCACCGCACTGGAAAGGTGCGATCCTATTCACAATGG-3′. This sequence was checked for its specificity with Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov/blast.cgi). A culture of *T. gallinarum* isolated from the caecal contents of turkeys was used as positive control (Liebhart *et al.*, 2006). Cross-reactivity was further checked directly on various embedded cultures or tissue samples including several species of flagellates, other protozoan parasites, bacteria, fungi and viruses as listed in Mostegl *et al.* (2010) except for *T. gallinarum*.

In addition, the tissues from the turkey were investigated with double-coloured ISH using probes specific for *H. meleagridis* and *T. gallinarum* (Liebhart *et al.*, 2006), and a previously described protocol (Richter *et al.*, 2008).

Polymerase chain reaction and sequencing

From the first Red-breasted Merganser, DNA was extracted from paraffin-embedded hepatic tissue with a commercial kit (nexttec Genomic DNA isolation kit, Biozym Biotech Trading GmbH, Vienna, Austria). A standard polymerase chain reaction (PCR) resulting in a relatively short amplicon of 153 basepairs suitable for DNA amplification from formalin-fixed, paraffin-embedded tissue was established. The primers targeted the 18S rRNA-gene and their sequences were: F: 5′-GACCTGTCTAGCGTTGATTC-3′ and R: 5′-AGGACATCACGGACCTGTTA-3′. The annealing temperature was calculated to be 60°C and a commercial master mix was used (5Prime HotMasterMix, Eppendorf Austria GmbH, Vienna, Austria). The resulting amplicons were sequenced.

To further identify the parasites, another PCR was established, which was specific for trichomonads and included a region of higher genetic variability of the 18S rRNA-gene among different tetratrichomonad species and strains. DNA was extracted from two different localizations of frozen native caecal tissue from the second Red-breasted Merganser using the same commercial kit as described above. A standard PCR assay with an annealing temperature of 54°C was carried out with the following primers: F: 5′-AGGCACGTCATTCGACTGAG-3′ and R: 5′-ACTGCGCTGAGTCATTCYTG-3′. The resulting amplicons of 431 basepairs were cloned in competent *E. coli* using the TOPO TA cloning Kit (Invitrogen GmbH, Lofer, Austria) and the plasmids were extracted with Pure Link Quick Plasmid Mini Prep Kit (Invitrogen GmbH, Lofer, Austria) from four different bacterial colonies. A nucleotide sequencing reaction for each colony followed.

The resulting sequences were submitted to Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov/blast.cgi) and aligned.

Accession numbers

The sequence derived from the paraffin-embedded tissue from the first Red-breasted Merganser and the two sequences from the clones derived from the caecal tissue of the second Red-breasted Merganser have been deposited in Genbank under the accession numbers HM162407 – HM162409.

Results

Standard pathology and standard etiological examinations

All three Mergansers and the Common Eider had a necrotizing typhlitis. The caecal lumina were filled with concentrically laminated fibrinous exsudate and necrotic debris with a brittle to firm consistency (Fig. 1a). The mucosal and submucosal layers of the caecal wall were completely necrotic down to the tunica muscularis. In addition, the first Red-breasted Merganser, the Hooded Merganser, and the Common Eider had a multifocal to coalescing necrotizing hepatitis (Fig. 1b). The necrotic foci were uniformly pale and slightly elevated above the liver surface. They had a maximum size of approximately 1 mm and were lacking a typical target shape with a darker central zone as seen in histomonosis.

In caecal and hepatic lesions from all ducks, except for the second Red-breasted Merganser, protozoa were detected histologically. They were round to oval with a size of 6 to 11μm and distinctly PAS positive. In the caeca, they showed a strong tendency for tissue-invasion and were found in all layers of the caecal wall. In the liver, they were seen especially at the border of necrotic foci (Fig. 2).

The caecal lesions in the second Red-breasted Merganser had a subacute character in comparison with the other ducks, and protozoa could not be identified in HE and PAS stained slides.

At gross as well as histopathology, the turkey showed a necrotizing typhlohepatitis consistent with histomonosis (blackhead disease). The liver contained typical target shaped concentric necrotic lesions of up to 1 cm in diameter with a dark depressed centre surrounded by a pale, slightly raised border zone. Histopathologically, numerous PAS-positive round to oval protozoa of 11 to 17 μm diameter were found in liver and caeca. The protozoa were present in high numbers in the necrotic debris filling the caecal lumen and invading the deeper layers of the caecal wall. Identical protozoa were present in massive amounts within the necrotic foci in the liver.

Additional pathological findings and the results of the standard etiological examinations for all birds included in this study are listed in table 2.

Chromogenic in-situ hybridization

All protozoa present in the liver and / or caeca from the Mergansers and the Common Eider showed a positive signal with the *T. gallinarum* probe (Fig. 3) and no signal with the *H. meleagridis* probe. Positive signals were visible mainly inside or at the border of necrotic lesions in both organs. In the second Merganser with the subacute typhlitis, low numbers of tetratrichomonads were located inside the necrotic cell debris but they did not invade the tissue as in the other ducks.

ISH from the tissues of the turkey were positive for *H. meleagridis* in liver and caeca. In addition, tetratrichomonads were present in the caeca but not in the liver. The double-coloured ISH showed two distinctly coloured types of protozoa in the caeca (Fig. 4). The histomonads were primarily located at the border between the necrotic debris and the inflamed caecal tissue and some were found to be invading the caecal wall. They had a size of 12 to 16 μm. The tetratrichomonads on the other hand were located closer to the lumen mainly within the necrotic debris. With a size of 5 to 8 μm they were generally smaller than the histomonads.

Polymerase chain reaction and sequencing

The 153 bp sequence derived from the paraffin-embedded tissue from the first Red-breasted Merganser showed a 96% similarity with 13 *T. gallinarum* sequences from various birds, mainly Gallifomes and Anseriformes, four *Tetratrichomonas* spp. sequences from humans as well as two from unidentified hosts, and one termite gut symbiont, when submitted for BLAST search. Sequences from other strains of *T. gallinarum* and other *Tetratrichomonas* species followed with less similarity. All four clones derived from the caecal tissue of the second Red-breasted Merganser had a length of 431 basepairs. Three of them had exactly the same sequence, being 98% similar to eight *T. gallinarum* sequences from Galliformes, Anseriformes and one unidentified bird, and two human *Tetratrichomonas* sp. sequences. All of these were found amongst those with the highest similarity from the other PCR assay described above, too. The fourth clone showed four differing nucleotides when compared to the other clones resulting in a slightly decreased similarity of 97% to sequences from tetratrichomonads mainly consistent with those mentioned with the first Merganser above.

An alignment of the resulting sequences from the bacterial clones compared to one from *T. gallinarum* from the GenBank database is shown in figure 5.

Discussion

This is the first report of fatal necrotizing typhlitis / typhlohepatitis in duck species associated with *T. gallinarum* similar to tetratrichomonosis in turkeys. The partially sequenced *T. gallinarum* strains show unique nucleotide sequences in parts of the 18S rRNA-gene compared to sequences listed in the GenBank database. Tetratrichomonads as well as histomonads were specifically detected intralesionally with previously reported and newly established chromogenic ISH assays conducted on formalin-fixed and paraffin-embedded tissue samples.

The pathogenicity of different *T. gallinarum* strains may vary among avian host species, such as turkeys, chickens and ducks. Also, based on the results from experimental infections and molecular genetic studies, protozoa phenotypically identified as *T. gallinarum* seem to form a heterogenous group regarding their virulence and genotype, and there may be cryptic species hidden within this group (Cepicka *et al.*, 2005). This variability has to be kept in mind, since in natural infections as well as in conventional cultures used for experimental infections multiple morphologically undistinguishable strains may be present.

Bacteria or other parasites, such as *Eimeria* spp., may play an important role in the pathogenicity of *T. gallinarum* (Norton, 1997). Similarly, in infections with the more virulent *H. meleagridis* in turkeys, the presence of caecal bacteria plays an important role in the development of clinical disease (McDougald, 2005). The birds described in this study had no concurrent parasitic infection at the time of their death. Except for *Campylobacter* spp. from the Red-breasted Mergansers, the enteric bacterial flora of these birds included mainly coliform bacteria and *Escherichia coli*, which might have had an influence on the pathogenicity of the tetratrichomonads. All affected birds were juvenile or subadult individuals, which might have had an additional influence on the course of the disease. Also, as seaducks they all belonged to the tribe Mergini, while ducks belonging to other tribes kept in the same collection have been unaffected.

In turkeys, coinfections of *H. meleagridis* and *T. gallinarum* occur frequently (Allen, 1941; Kulda, 1974). In the present study, a coinfection with both parasites was demonstrated in the caecum of the turkey, which died from blackhead disease. Using double-coloured ISH, the tissue distribution of both parasites could be assessed and compared directly to each other. Since the tetratrichomonads were present only within the caecal necrotic debris and not at

the interface between necrosis and inflamed tissue or in the liver lesions as were the histomonads, it is presumed, that the present strains of tetratrichomonads revealed no high pathogenicity for the turkey. Thus, turkeys might be subclinical carriers of some strains of *T. gallinarum*, which may have the potential to induce disease in various duck species living in the same zoological collection. Further studies are required to elucidate the complete epidemiology that led to the recurrent fatal diseases in the ducks presented in this article.

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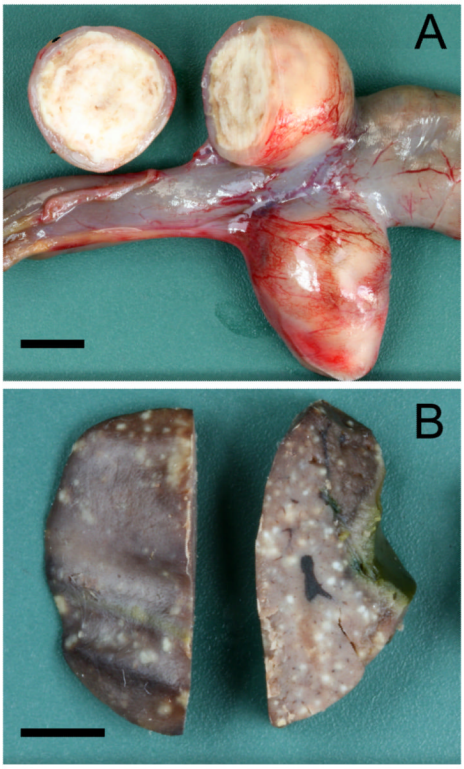

Figure 1.
1a: Photograph of the caecum from the second Red-breasted Merganser with concentrically laminated fibrinous and necrotic material. Bar = 0.5 cm. 1b: Photograph of the liver from the first Red-breasted Merganser with multifocal uniformly pale tissue necrosis. Bar = 0.5 cm.

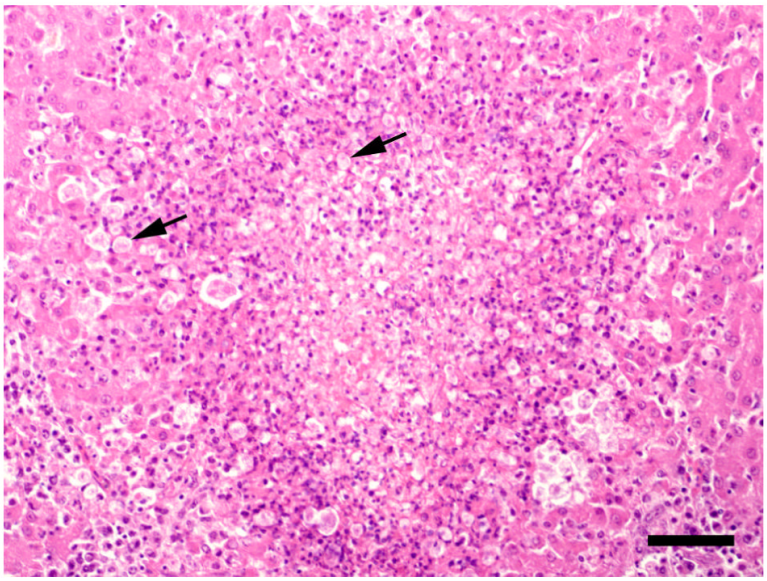

Figure 2.
Tissue section from the liver of the first Red-breasted Merganser, focal tissue necrosis and inflammation associated with numerous tetratrichomonads (arrows). HE stain. Bar = 50 μm.

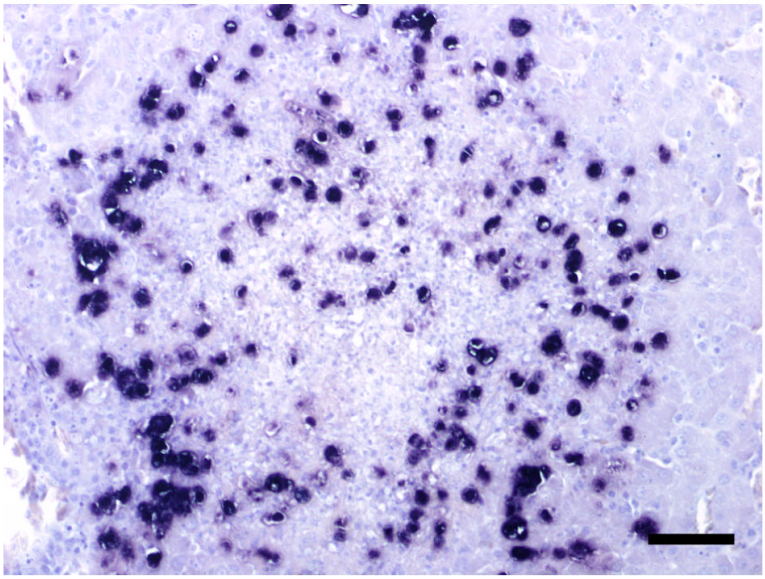

Figure 3.
Tissue section from the liver of the first Red-breasted Merganser, *T. gallinarum* are clearly visible as dark purple spots. ISH. Bar = 50 μm.

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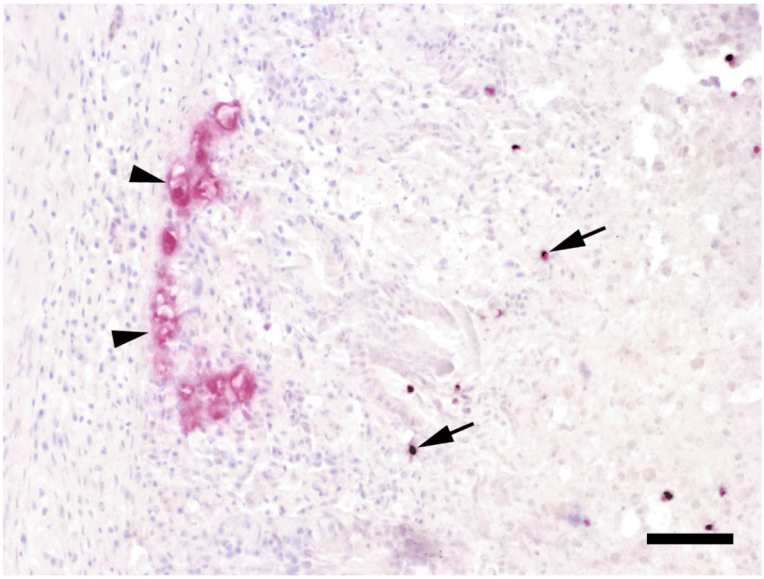

Figure 4.
Tissue section from the caecum of the turkey, on the left hand side *H. meleagridis* stained in red (arrowheads) invade the mucosal tissue, on the right hand side scattered *T. gallinarum* stained dark purple (arrows) can be seen closer to the caecal lumen. Double-coloured ISH. Bar = 50 μm.

Figure 5.
Alignment of partial 18S rRNA sequences of *T. gallinarum* from the GenBank and the second Red-breasted Merganser.

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Table 1

Sex, age and clinical history

Animal	Sex	Age (weeks)	Clinical history
Red-breasted Merganser No. 1 (*Mergus serrator*)	female	3	seemed healthy, sudden death
Red-breasted Merganser No. 2 (*Mergus serrator*)	male	12	reduced growth, sudden death
Hooded Merganser (*Lophodytes cucullatus*)	male	10	stopped grooming, sudden death
Common Eider (*Somateria mollissima*)	female	24	lameness, sudden death
Turkey (*Meleagris gallopavo* f. domestica)	male	12	seemed healthy, sudden death

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Table 2

Results of standard etiological examinations and additional pathological findings

Animal	Bacteriology / Mycology	Coproscopy	Concurrent Pathology	PCR*
Red-breasted Merganser No. 1	*Campylobacter jejuni*	neg.	none	neg.
Red-breasted Merganser No. 2	*Campylobacter coli, Aspergillus fumigatus*	neg.	Aspergillosis, Amyloidosis	neg.
Hooded Merganser	no pathogenic bacteria	neg.	Amyloidosis	neg.
Common Eider	no pathogenic bacteria	neg.	none	neg.
Turkey	no pathogenic bacteria	neg.	none	neg.

** *Chlamydia* spp., *Chlamydophila* spp., avian influenzaviruses, avian paramyxovirus serotype*