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Experimental Infection of Turkeys and Chickens with a Clonal Strain of *Tetratrichomonas gallinarum* Induces a Latent Infection in the Absence of Clinical Signs and Lesions

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Summary

The pathogenicity of a mono-eukaryotic culture of *Tetratrichomonas gallinarum* in specific pathogen free (SPF) chickens and turkeys was studied. Two experiments of identical design were performed: the first with SPF chickens and the second with commercial turkeys. Each experiment included three groups. Groups 1 and 2 each contained 12 infected and three in-contact birds. The birds in these groups were infected on the first day of life, either cloacally (group 1) or orally (group 2). Group 3 consisted of four control birds. Re-isolation of the parasite from cloacal swabs was performed to verify the excretion of *T. gallinarum*. The infected birds excreted trichomonads from the second day post-infection. Spread of the flagellate from infected to in-contact birds was detected after 5 days post-infection (dpi), based on the re-isolation of the protozoa. No clinical signs or deaths were recorded in chickens or turkeys. Three birds were killed at 4, 8, 14 and 21 dpi and various tissues were collected for pathological examination. No gross lesions were noted. Protozoal DNA was demonstrated in the oesophagus, duodenum, jejunum, caecum, liver, lung, bursa of Fabricius and brain by polymerase chain reaction and in-situ hybridization. No antibodies were detected in the serum of infected birds by enzyme linked immunosorbent assay. Microscopical changes were only present in the caecum, where there was sloughing of the epithelium associated with the presence of numerous flagellates on the epithelial surface, within the crypts of Lieberkühn and in the lamina propria. These changes were found in caecal samples from infected and in-contact birds. These studies have demonstrated the rapid transmission of *T. gallinarum* between both turkeys and chickens and the establishment of a latent infection in both species.

Keywords: chickens; in-situ hybridization; latent infection; *Tetratrichomonas gallinarum*; turkeys

Introduction

Tetratrichomonas gallinarum, a member of the Trichomonadidae, was first described by Martin and Robertson (1911). The pathogenic potential of this protozoon has been discussed controversial (Allen, 1941; Kemp and Reid, 1965; Norton, 1997), but *T. gallinarum* is a common inhabitant of the intestinal tract of different poultry species (Friedhoff *et al.*, 1991). This controversy may relate to the frequent occurrence of mixed infections with other protists such as *Histomonas meleagridis* and *Blastocystis* spp. (Tyzzer, 1920). Co-infection with *T. gallinarum* and *H. meleagridis* has been demonstrated in the caecum and liver of naturally infected chickens and turkeys with histomonosis (Grabensteiner and Hess, 2006).

Although it is not clear whether *T. gallinarum* should be regarded as a primary pathogen, Weinzirl (1917) proposed *T. gallinarum* as a cause of fatal catarrhal enteritis in 1–4-week-old chicks and turkey poults. Trichomonads are known to induce lesions in the caecum and liver of domestic fowls and turkeys and should be considered as a causative agent of enterohepatitis (Allen, 1941). A further study reported

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the pathogenic potential of *T. gallinarum* in chickens infected cloacally with the caecal content of broiler breeder chickens naturally infected with *T. gallinarum* (Lee, 1972). Lesions induced within the caecum of these birds included loss of microvilli and reduction of the glycocalyx, and complete loss of the polysaccharide matrix was detected electron microscopically. In contrast, the pathogenic potential of *T. gallinarum* was not confirmed by Delappe (1957) who reported that a strain of *T. gallinarum*, which had been isolated originally from a liver lesion of a turkey, together with *H. meleagridis*, produced no symptoms or lesions after oral administration to young chickens and turkey poults. No clinical signs or histopathological changes were detected following rectal inoculation of turkeys with a *T. gallinarum* culture (Goedbloed and Bool, 1962). Kemp and Reid (1965) considered *T. gallinarum* as a non-pathogenic organism based on the absence of gross lesions and lack of mortality in infected birds. These authors did not report a decrease in body weight or drop in egg production in chickens and turkeys infected experimentally with a strain of *T. gallinarum* obtained from naturally infected birds.

The aim of the present study was to determine whether *T. gallinarum* isolated from turkeys could induce infection in chickens or turkeys and, if such infection was induced, whether the flagellate could be transmitted to non-infected in-contact birds. Additionally, the study aimed to investigate the clinical and pathological changes and the serological response following cloacal or oral infection of the host birds.

Materials and Methods

Animals

The study involved two experiments of identical design: the first with specific pathogen free (SPF) chickens and the second with turkeys obtained from a commercial hatchery. All birds were housed on deep litter (wood shavings) in rooms under negative pressure. On the first day of life each bird was individually identified by a numbered tag (Swiftack, Heartland Animal Health Inc., Fair Play, Missouri). Water and unmedicated feed for chickens (chicken starter feed) and turkeys (turkey starter feed) were provided *ad libidum*. Both experiments were approved by the institutional ethics committee and registered by Austrian law (license numbers 68.205/0017-BrGT/2005 and 68.205/0026-II/10b/2008).

Experimental Design

In both experiments, two groups of 15 birds (groups 1 and 2) were housed in one room in separate pens. Twelve birds in each of these groups were infected on the first day of life, while the other three were kept as in-contact birds. Birds of group 1 were infected cloacally, whereas those of group 2 were inoculated via the oral route. Four control birds (group 3) were kept in another room separate from the infected birds. Clinical examination was performed daily. All birds were weighed once each week throughout the experiments.

Culture of T. gallinarum

The clonal culture *T. gallinarum*/turkey/Germany/4114-C5/05 was established as described by Hess *et al.* (2006). The flagellate was isolated from a flock of turkeys in Germany that suffered from histomonosis. Following axenization this clone was grown in modified *Trichomonas vaginalis* medium (TV; Amin *et al.*, 2010). Each bird was infected with 10^5 trichomonads determined by a Neubauer cell counting chamber (Reichert, Buffalo, New York). The required number of live cells was adjusted in 300 µl modified TV medium for each bird. After infection, the birds were deprived of feed for 5 h. All birds were infected with the same passage number (18×) after thawing the clone from liquid nitrogen and propagating it for two passages.

Re-isolation of the Parasite

For re-isolation of the parasite, cloacal swabs were taken and incubated in 2 ml Eppendorf tubes containing 1.5 ml of fresh Medium 199 supplemented with Earle's salts + L-Glutamine + 25 mM HEPES + L-Amino acids (Gibco; Invitrogen, Lofer, Austria). 15% fetal calf serum (FCS; Gibco) and 2 mg of rice starch (Sigma Aldrich, Steinheim, Germany). The tubes were subsequently incubated at 40°C until the microscopical investigation. This sampling procedure was performed at intervals of 2–3 days throughout the experiments. The re-isolated *T. gallinarum* cells were examined microscopically after 48 and 96 h of incubation.

Serology

Blood samples were collected from all birds in both experiments weekly for the duration of the experiments. Samples were kept at 4°C for 24 h and then centrifuged (3,300g) for 12 min (Hettich Rotanta 460; Tuttlingen, Germany). The sera were stored at −20°C until tested. Serum antibody specific for *T. gallinarum* was detected by indirect enzyme linked immunosorbent assay (ELISA; Windisch and Hess, 2009) with a polyclonal rabbit serum raised against *T. gallinarum* as a positive control. The production of the rabbit antiserum specific for *T. gallinarum*

was performed by a protocol described for preparation of antiserum specific for *H. meleagridis* (Bilic *et al*., 2009). The wells of 96-well microtitre plates were coated directly with 10,000 cells of *T. gallinarum* suspended in 100 µl carbonate buffer (pH 9.6). For determination of the anti-trichomonal antibodies, the serum samples were diluted 1 in 500 in blocking buffer (Starting Block™ T20 PBS; Pierce, Rockford, USA). Additionally, 20 serum samples from non-infected chickens or turkeys were tested for determination of the cut-off value, which was defined as the mean absorbance plus three times the standard deviation of these samples.

Blood Samples

In parallel to collecting blood samples for serological investigation, blood was also collected into 1.3 ml microtubes containing anticoagulant (1.6 mg EDTA/ml blood; Sarstedt, Nümbrecht, Germany) once weekly from all birds and additionally at the time the birds were killed. These samples were stored at −20°C for analysis by polymerase chain reaction (PCR).

Necropsy Examination

At 4, 8, 14 and 21 days post-infection (dpi) three birds from each infected group (groups 1 and 2) and one bird from the control group (group 3) were killed, starting with the lowest tag number in ascending order. All in-contact birds of groups 1 and 2 were killed at the termination of the experiment at 21 dpi. A gross necropsy examination was performed and tissue samples were taken from the oesophagus, duodenum, jejunum, caecum, lung, liver, bursa of Fabricius and brain and stored at −20°C for analysis by PCR. DNA was extracted from these samples with the Dneasy® Tissue Kit (Qiagen, Vienna, Austria). According to manufacturer's instructions, 25 mg of each organ and 10 µl of blood were used for extraction. PCR was performed following the protocol of Grabensteiner and Hess (2006). Samples from these organs were also fixed in 10% neutral buffered formalin and subsequently embedded in paraffin wax for histological investigation. For detection of the flagellates and to identify microscopical lesions in the tissues, haematoxylin and eosin (HE) stain was used. Furthermore, periodic acid−Schiff (PAS) and in-situ hybridization (ISH) for definitive demonstration of the protozoan or its nucleic acid were applied. ISH was performed following the protocol of Liebhart *et al*. (2006). To avoid cross contamination during necropsy examination, autoclaved scissors and disposable forceps were used for sampling.

Results

Clinical Signs

None of the infected chickens or turkeys died or showed any clinical abnormalities except for one in-contact chicken (group 2), which was killed at 5 dpi due to illness (complete filling of the crop with fluid and inability to move), and one orally infected turkey, which was killed at 2 dpi due to inability to move and poor condition. There was no difference in body weight gain between the control birds and the infected or in-contact birds throughout both experiments.

Re-isolation

The results of re-isolations are summarized in Table 1. Live trichomonads were observed in the cloacal swabs from some directly infected birds of both animal experiments after 2 dpi. The first positive re-isolations from one in-contact chicken and all in-contact turkeys occurred at 5 dpi. Examination of cloacal swabs revealed the presence of *T. gallinarum* in all infected and in-contact surviving birds in both animal experiments at least once. The percentage of positive re-isolation of *T. gallinarum* from infected and in-contact birds was 100% after 5 and 16 dpi in turkeys and chickens, respectively. Constant excretion of live parasites from the majority of birds was observed after 2 dpi. One turkey and four chickens had negative re-isolation results from a single cloacal swab only. All swabs examined from control birds (group 3) remained negative for the entire period of the experiments (data not shown).

Serology

In chickens absorbance values above 0.66 were considered positive, whereas the cut-off value of 0.41 was adopted for turkeys. All investigated serum samples from both infected chickens and turkeys had an antibody titre lower than these cut-off values.

Gross Pathology

No gross lesions were observed in any organ of the killed birds. Filling of the crop with fluid was noted in the chicken killed at 5 dpi, while severe emaciation with congestion of all internal organs was found during post-mortem examination of the turkey killed at 2 dpi.

Detection of Protozoa

PCR: No differences were noted, regardless of the route of infection, between samples processed from

Table 1
Re-isolation of live *Tetratrichomonas gallinarum* trophozoites from cloacal swabs from SPF chickens and turkeys

	Chickens		*Turkeys*	
	Intra-cloacal	*Oral*	*Intra-cloacal*	*Oral*
0 dpi				
Infected	0	0	0	0
In-contact	0	0	0	0
2 dpi				
Infected	10*/12†	8/12	12/12	11/12
In-contact	0/3‡	0/3	0/3	0/3
5 dpi				
Infected	9/9	9/9	9/9	8/8
In-contact	0/3	1/3	3/3	3/3
7 dpi				
Infected	9/9	9/9	9/9	8/8
In-contact	2/3	0/2	3/3	3/3
9 dpi				
Infected	6/6	6/6	5/6	5/5
In-contact	2/3	0/2	3/3	3/3
12 dpi				
Infected	6/6	5/6	5/6	5/5
In-contact	2/3	2/2	3/3	3/3
14 dpi				
Infected	6/6	6/6	6/6	5/5
In-contact	2/3	2/2	3/3	3/3
16 dpi				
Infected	3/3	3/3	3/3	2/2
In-contact	3/3	2/2	3/3	3/3
19 dpi				
Infected	3/3	3/3	3/3	2/2
In-contact	3/3	2/2	3/3	3/3
21 dpi				
Infected	3/3	3/3	3/3	2/2
In-contact	3/3	2/2	3/3	3/3

*Total number of positive birds for re-isolation.
†Total number of infected birds.
‡Total number of in-contact birds.

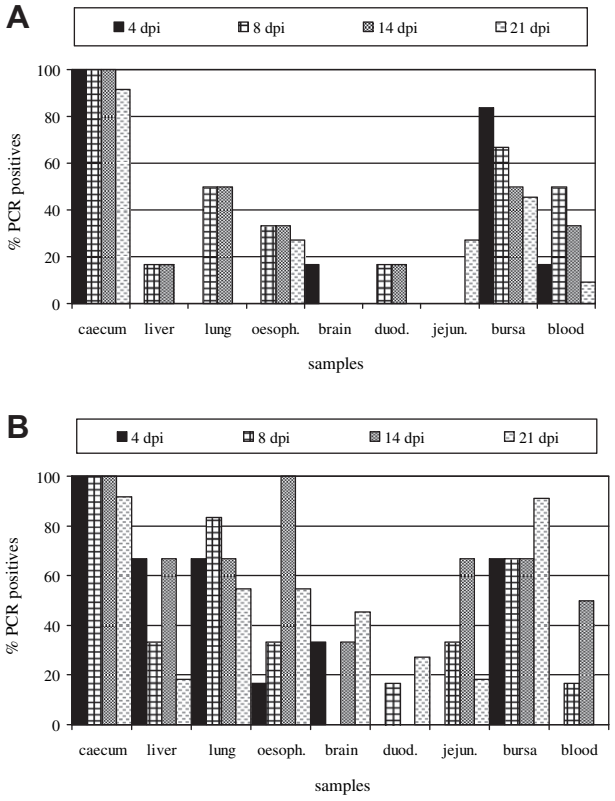

Fig. 1. Percentage of PCR positive signals in different organs of infected chickens (A) and turkeys (B) at different times post-infection, irrespective of the route of infection. Oesoph, oesophagus; duod, duodenum; jejun, jejunum.

chickens or turkeys. The PCR results for both animal experiments are displayed in Fig. 1. Positive PCR results were obtained from different organs (caecum, duodenum, jejunum, bursa of Fabricius, lung, liver, oesophagus and brain) from both chickens and turkeys with different degree. The highest rate of positive PCR signals (96.7%) were found in the caecal samples from both SPF chickens and turkeys independent of the time point post-infection. Furthermore, positive PCR results were also found in 56.7% and 73.3% of samples processed from the bursa of Fabricius of chickens and turkeys, respectively. The lowest number of positive PCR signals in samples from chickens and turkeys was recorded in the brain (3.3%) and the duodenum (13.3%), respectively. DNA of trichomonads was detected in the blood of 23.3% of chickens and turkeys infected by either route. Overall, the percentage of positive PCR signals from all organs was higher in turkeys than chickens. PCR from organs (caecum, duodenum, jejunum, bursa of Fabricius, lung, liver, oesophagus and brain) and blood of the control birds was negative in both experiments (data not shown).

Histological examination: Trichomonad cells were found microscopically in the tissues after HE and PAS staining or ISH. No specific colouration of trichomonads or their organelles was obtained using conventional stains. Therefore, it was difficult to identify the flagellates with HE and PAS stains, especially in organs, that contained only a few protozoal cells. In contrast, protozoa were clearly detected as dark blue-labelled cells following ISH. The histological examination of the caecum of all infected and in-contact birds showed numerous trichomonads on the epithelial surface, within the lumen of crypts of Lieberkühn and in the lamina propria, and these changes were associated with desquamation of the lining epithelium (Fig. 2). Although individual trichomonads reached the muscularis mucosa, the majority of the flagellates

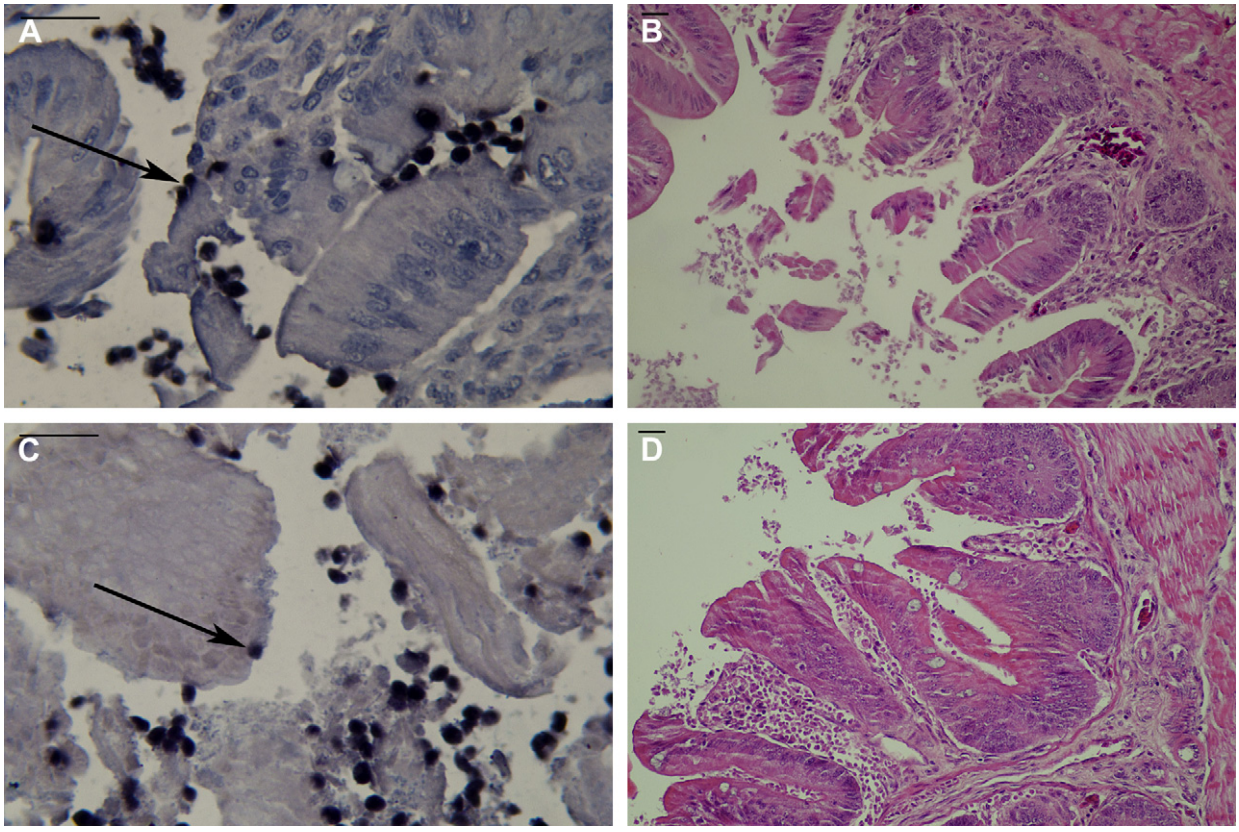

Fig. 2. ISH and HE staining of the caecal tissue of a cloacally infected chicken (A and B) and orally infected turkey (C and D). Trichomonads detected by ISH appear dark blue (arrow). Bar, 20 µm.

did not extend to the muscularis externa of the caecal tissue. The results of ISH confirmed the PCR findings, as nucleic acid of trichomonads was also detected in the oesophagus, duodenum, jejunum, liver, lung, bursa of Fabricius and brain of turkeys (Table 2). In general, ISH revealed that there was no specific predilection site for trichomonads in different organs. For example, trichomonad cells were scattered in the lung tissue (Fig. 3A). Additionally, protozoal cells were found either adherent to the atrial wall or in the bronchial lumen and in the pulmonary blood vessels. In the liver, the flagellates were located within hepatic vessels (Fig. 3B). Furthermore, trichomonad cells were also found in the hepatic parenchyma. Moreover, some trichomonads were observed in the lymphoid follicles of the bursa of Fabricius (Fig. 3C) as well as in the interfollicular connective tissue of this organ (Fig. 3D). Protozoal nucleic acid was noticed within the cerebellar parenchyma. Consequently, extravascular location of trichomonads was present in all of these organs, indicating emigration of the protozoa into the tissues. Uneven distribution of the protozoon cells was seen in the oesophagus, duodenum and jejunum. Although protozoal cells were noted in these organs, there were no pathological changes present. The same organs of chickens were examined using ISH, but trichomonads were only found in the caecum, jejunum, lung, liver and bursa of Fabricius. All investigated organs from the control groups of both chickens and turkeys revealed no ISH signals.

Table 2
Nucleic acid of *Tetratrichomonas gallinarum* in tissue samples from SPF chickens and turkeys detected by PCR and ISH

	Chickens			*Turkeys*		
	PCR	*ISH*	*Rate of agreement*	*PCR*	*ISH*	*Rate of agreement*
Caecum	29*/30†	11/11	11‡/11	29/30	18/19	18/18
Duodenum	2/30	0/13	11/13	4/30	4/19	15/19
Jejunum	3/30	1/13	10/13	8/30	2/19	13/19
Bursa of Fabricius	17/30	13/21	9/21	22/30	17/19	13/19
Lung	6/30	2/5	2/2	17/30	3/11	3/11
Liver	2/30	2/3	3/3	12/30	1/11	4/11
Oesophagus	7/30	0/7	0/7	15/30	5/19	12/19
Brain	1/30	0/2	1/2	13/30	1/11	5/11

*Number of positive samples.
†Number of investigated samples.
‡Identical results obtained by PCR and ISH.

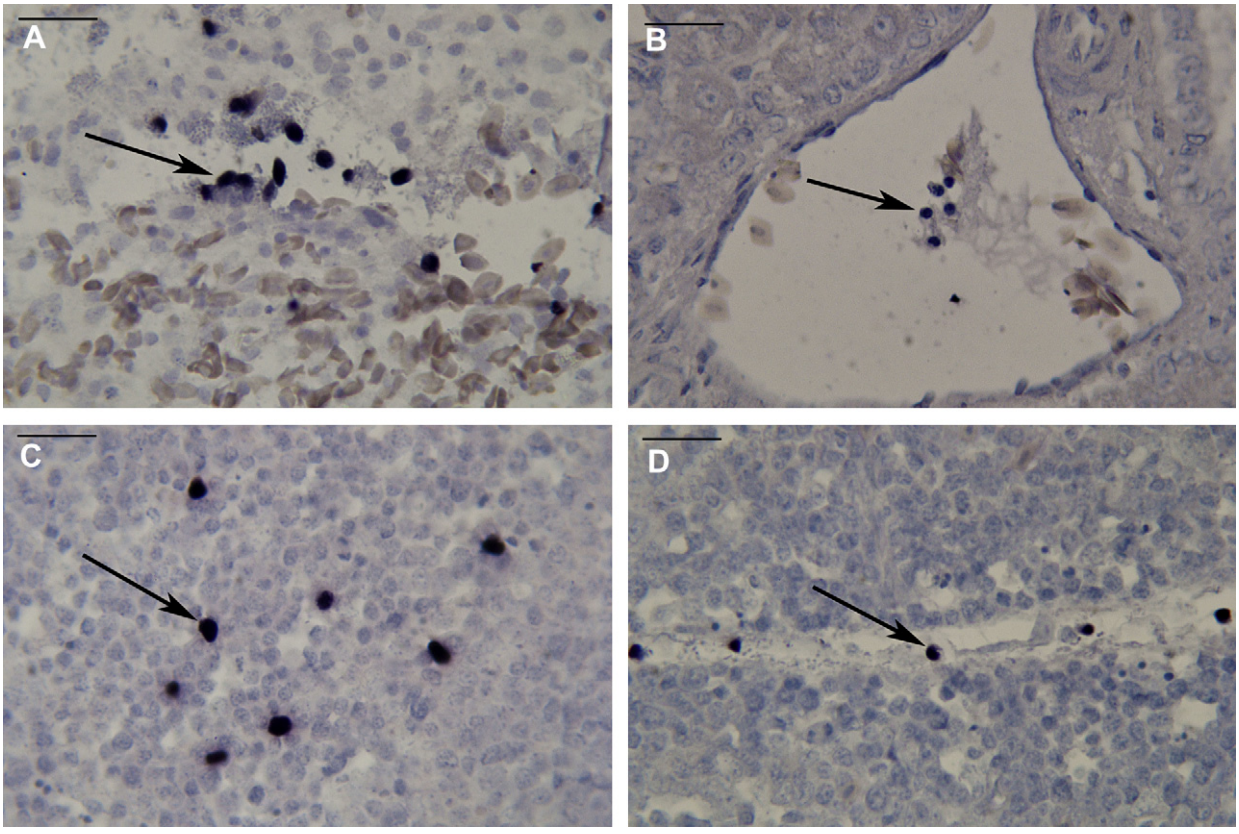

Fig. 3. Detection of the nucleic acid of trichomonads (arrow) by ISH in the lung (A), liver (B) and the bursa of Fabricius (C and D) of infected turkeys. Bar, 20 μm.

Discussion

Until now, the pathogenicity of *T. gallinarum* in poultry has been controversial. The present study reports the infection of turkeys and chickens with a clonal culture of *T. gallinarum* derived from a turkey. Infection and spread of the parasite to other in-contact chickens and turkeys were demonstrated, but notably all of the infected birds remained clinically asymptomatic.

The *T. gallinarum* culture used in these experiments was found to be infectious and of low pathogenicity, as all of the infected and in-contact birds excreted the organism throughout the experiments, while no clinical signs or gross lesions were detected. Some mild changes associated with the presence of numerous protozoa were present in the caecal mucosa. In the present study, the infective dose employed was 10^5 trichomonads, which was lower than that used in an earlier investigation (Kulda *et al.*, 1974). Although these authors used a higher concentration of protozoal cells, they never demonstrated the flagellates in any tissue other than the caecum. In contrast, in the present study the nucleic acid of the flagellates was demonstrated in the caecum, duodenum, jejunum, oesophagus, liver, lung, bursa of Fabricius and brain. Variations in the pathogenicity of *T. gallinarum* in different studies could be based on the existence of different strains among this species, as recorded by Cepicka *et al.* (2005). Consequently, it remains to be elucidated whether another clonal strain of *T. gallinarum* would be able to produce more severe pathogenic effects.

Two different routes of infection (cloacal and oral) were used, and these had no influence on the outcome of the study. Clearly, trichomonad cells survived the passage from the crop to the caecum, an acid environment supposed to have a negative effect on the protozoa according to in-vitro data (Theodorides, 1964; Friedhoff *et al.*, 1991). The ability of the flagellates to form pseudocysts could be an explanation for this (Friedhoff *et al.*, 1991). In addition, trophozoites of *T. gallinarum* displayed pseudocyst formation *in vitro* (Hess *et al.*, 2006). Furthermore, after intra-cloacal inoculation the parasite has been shown to reach the caecum by retrograde peristaltic movement (Browne, 1922). Rapid spread of the parasite to in-contact birds, independent of the route of infection, was noticed in both chickens and turkeys. The infected birds shed the protozoal cells without

showing any clinical signs. This may be due to the continuous infection of the birds through faecal–oral transmission. The infection with *T. gallinarum* was not eliminated from the birds, resulting in a latent infection.

Even though the caecum was the main target organ invaded by the protozoan cells, the nucleic acid of the flagellates was also detected in the oesophagus, duodenum, jejunum, liver, lung, bursa of Fabricius and brain. The neutral pH of the caecum is considered the most suitable environment for the growth of *T. gallinarum* (Buckner *et al.*, 1944). Furthermore, the anaerobic conditions in the caecum are considered to promote the growth of *T. gallinarum* because the parasite is able to grow and ferment various sugars under such conditions (Theodorides, 1964). These facts could explain the superior growth and proliferation of trichomonads in the caecum compared with other organs. The presence of protozoa in the digestive tract could be explained by the faecal–oral transmission, whereas the spread into lung and liver most likely occurred via the bloodstream because the DNA of *T. gallinarum* was detected by PCR in the blood of many birds, as well as in the hepatic and pulmonary blood vessels by ISH. In addition, co-infection with *H. meleagridis* could contribute to the frequent detection of trichomonads in internal organs, due to the destructive effect of this organism on the caecal tissue (Grabensteiner and Hess, 2006).

The presence of infectious agent within the bursae of both turkeys and chickens could be explained by the direct contact between the bursa of Fabricius and the cloaca, together with the uptake of protozoa via 'cloacal sucking' (Schaffner *et al.*, 1974). Further investigations are required in order to demonstrate whether peripheral macrophages contribute to the presence of protozoal DNA in the brain, as described for *Trypanosoma cruzi* (Silva *et al.*, 2004).

Although the parasite was demonstrated in oesophagus, duodenum, jejunum, liver, lung, bursa of Fabricius and brain by PCR and ISH, no pathological changes were noted in these viscera. Singh *et al.* (2008) hypothesized that parasites require interaction with some inflammatory cells in order to produce pathological changes; however, the isolate of *T. gallinarum* used in these studies did not initiate any inflammatory response in these organs.

Two different stains (HE and PAS) together with ISH were applied to demonstrate trichomonads in the tissues of infected birds. Overall, ISH was found to be more sensitive than histochemical staining, especially in tissues showing marginal occurrence of the parasite. Moreover, ISH is a method that may be applied to the detection of *T. gallinarum* in paraffin wax-embedded tissues, as it provides the opportunity to correlate the histological changes with the presence of the protozoon. ISH signals were considered positive when they were consistent with the size and morphology of trichomonads. Intact trichomonad cells were demonstrated in different organs and the location of trichomonads within the tissue could also be determined. The uneven distribution of the trichomonad cells within tissues could explain the different results obtained by PCR and ISH, especially in those organs that showed only few protozoa.

Despite the wide distribution of protozoal DNA in different organs, no antibodies were detected by ELISA in the serum of infected birds. The absence of antibodies could be explained in different ways. The protozoa were of low virulence and this correlates with the antibody response shown for infection with *H. meleagridis* (Windisch and Hess, 2009). Furthermore, such flagellates need a somewhat longer time period to induce a measurable level of antibodies and this may have been insufficient in the present investigation.

In conclusion, the present study has shown that infection of SPF chickens or turkeys with a clonal culture of *T. gallinarum* does not result in clinical signs, mortality or gross lesions, with the exception of some desquamation of epithelial cells in the caecum. However, all infected birds shed the parasites continuously for the duration of the experiments. These findings suggest that SPF chickens and turkeys behave as asymptomatic latent carriers for the *T. gallinarum* isolate used in this experiment.

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