

## REVIEW ARTICLE

# Trichomonads in birds – a review

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### SUMMARY

Members of the family Trichomonadidae, mainly *Trichomonas gallinae* and *Tetratrichomonas gallinarum*, represent important parasites in birds with worldwide presence, since being reported in the 19th century. Especially Columbiformes, Falconiformes and Strigiformes can be severely affected by trichomonads, whereas the majority of infections in Galliformes and Anatiformes are subclinical although severe infections are occasionally reported. With the recent appearance of deadly infections in wild Passeriformes the protozoan parasite *T. gallinae* obtained greater attention which will be addressed in this review. Although light microscopy remains the method of choice to confirm the presence of trichomonads molecular studies were introduced in recent years, in order to characterize the parasites and to establish relationships between isolates. Isolation of trichomonads is a prerequisite for detailed *in vitro* and *in vivo* studies and different media are reported to obtain suitable material. The limited information about virulence factors will be reviewed in context with the pathogenicity of trichomonads which varies greatly, indicating certain strain heterogeneity of the parasites. Options for treatment characterized by the leading role of imidazoles whose activity is sometimes hampered by resistant parasites remains a challenge for the future. Introducing more standardized genetic studies and investigations concentrating on the host-pathogen interaction should be helpful to elucidate virulence factors which might lead to new concepts of treatment.

Key words: *Trichomonas gallinae*, *Tetratrichomonas gallinarum*, morphology, epidemiology and transmission, pathogenicity, diagnostics, genetics, treatment, review.

### INTRODUCTION

Flagellates of the family *Trichomonadidae*, order Trichomonadida, are amitochondriate, microaerophilic protozoa that mostly live as parasites in the intestine or in the urogenital tract of humans and animals (Brugerolle and Müller, 2000). In birds, two trichomonad species, *Trichomonas gallinae* and *Tetratrichomonas gallinarum*, are most commonly found.

*Trichomonas gallinae*, present in lesions of the upper digestive tract of pigeons, was firstly reported by Rivolta (1878), who named it *Cercomonas gallinae*. Because some flagellates were also found in a pigeon liver in connection with caseous hepatitis the pathogen was also called *Cercomonas hepaticum*. Later on, Stabler (1938) introduced the name *Trichomonas gallinae* for trichomonads that colonize the crop of pigeons. *Trichomonas gallinae* is the only trichomonad species with a non-ambiguous, proven

pathogenic potential for birds (Bondurant and Honigberg, 1994).

*Tetratrichomonas gallinarum* was originally reported by Martin and Robertson (1911) as *Trichomonas gallinarum*, while the actual name *Tetratrichomonas gallinarum* was established following a taxonomic scheme applied by Honigberg (1963). *Tetratrichomonas gallinarum* is commonly found in the large intestine of gallinaceous and anseriform birds (McDougald, 2008).

In the past, any flagellates present in the upper part of the digestive tract anterior to the gizzard and in the tissue of the head, thorax or abdomen of a bird were considered to be *T. gallinae* (Stabler, 1954). In comparison, *T. gallinarum* is most often found in the lower part of the intestinal tract, principally the caeca. Additionally, different species of trichomonads were also classified on the basis of the hosts they infected (Levine, 1985). Recently, the introduction of molecular methods and the development of clonal cultures have proved to be useful in more accurate identification and classification of these protozoa.

### MORPHOLOGY

Representatives of the order Trichomonadida are unicellular organisms with a single nucleus.

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These flagellates are characterized by the presence of a single karyomastigont, five to six flagella, undulating membrane of lamelliform-type and the B-type costa (Cepicka *et al.* 2010). Trichomonads lack classic mitochondria as sites of oxidative fermentation, but instead possess specialized organelles named hydrogenosomes (Müller, 1993). These energy-generating organelles use a fermentative pathway for pyruvate metabolism and not the Krebs cycle as classical mitochondria.

Trichomonads exist at the trophozoite stage *in vitro* under favourable incubation conditions and move with the help of flagella (Stabler, 1954). Much has been learned about the morphology of *T. gallinae* and *T. gallinarum* by light microscopy, even though several investigations reported contradicting sizes of trophozoites (Stabler, 1941, 1954; McDowell, 1953; Abraham and Honigberg, 1964; Theodorides and Olson, 1965; Honigberg, 1978; Bondurant and Honigberg, 1994). These variations in size could be attributed to the inherent constitution of these flagellates based upon physicochemical changes in their growth environment, or due to distortions caused by the various fixatives used during preparation (Theodorides and Olson, 1965).

In general, the morphological variations among *T. gallinae* and *T. gallinarum* are mainly represented by the presence or absence of a protruding flagellum behind the posterior end of the body. *Trichomonas gallinae* trophozoites vary in shape reaching from ovoidal to pyriform with a size of about 7–11  $\mu\text{m}$ . They are provided with four free anterior flagellae and a fifth recurrent one, which does not become free at the posterior pole as it extends for only two-thirds of the body length (Tasca and De Carli, 2003; Mehlhorn *et al.* 2009). The nucleus is ovoid with a size of 2.5–3  $\mu\text{m}$ . The axostyle consists of a row of microtubules running from the region of the apical basal bodies to the posterior end of the cell. Flagellated stages contain food vacuoles, hydrogenosomes, a costa-like structure, and glycogen granules beside lacunes of endoplasmic reticulum. In addition, spherical, non-flagellated and cyst-like stages occur. In general, the trophozoites of *T. gallinarum* reflect a similar constitution as *T. gallinae* but they appear mostly pear-shaped and range in size from 6 to 15  $\mu\text{m}$  which was described to be roughly the same size as red blood cells (Clark *et al.* 2003). They also have four free anterior flagella and a fifth recurrent one, which becomes free at the posterior pole in contrast to that of *T. gallinae*. The anterior flagella were found to be approximately 8–13  $\mu\text{m}$  in length (Bondurant and Honigberg, 1994). Another clearly visible difference to *T. gallinae* is the occurrence of a sphere of lacunes of the endoplasmic reticulum surrounding the nucleus with its typical perinuclear membranes in a regular distance. Furthermore, food vacuoles appear very large (Mehlhorn *et al.* 2009).

## EPIDEMIOLOGY AND TRANSMISSION

### *Trichomonas gallinae*

The parasite *T. gallinae* is of veterinary and economic importance, as it causes avian trichomonosis, a disease with important medical and commercial implications. Avian trichomonosis has been reported from several continents and is considered a major disease of numerous avian species, especially columbiformes and falconiformes (Stabler, 1954). In pigeons, the disease is also called canker. The rock pigeon (*Columba livia*) is the primary host of *T. gallinae* and has been considered responsible for the worldwide distribution of this protozoal infection (Stabler, 1954; Harmon *et al.* 1987). Similarly, other species within the *Columbidae*, like doves (e.g. *Streptopelia decaocto*) and feral or wood pigeons (e.g. *Columba palumbus*) are important hosts as well (Bondurant and Honigberg, 1994).

Raptors, like hawks, eagles and falcons, are also susceptible to infection by *T. gallinae* (Krone *et al.* 2005) and may develop trichomonosis which is also termed 'frounce' in these birds (McDougald, 2008). Work and Hale (1996) reported severe trichomonosis-induced mortality in owls. A very detailed listing of bird species from the orders Columbiformes, Falconiformes and Strigiformes was published recently by Forrester and Foster (2008).

Since 2005 avian trichomonosis has been recognized as an emerging infectious disease of wild finches in the UK (Robinson *et al.* 2010), which further spread as a consequence of bird migration (Lawson *et al.* 2011b). Later on, several outbreaks were recorded in Southern Fennoscandia, Northern Germany, Eastern Canada, the British Isles, France and Slovenia (Peters *et al.* 2009; Forzan *et al.* 2010; Neimanis *et al.* 2010; Gourlay *et al.* 2011; Lawson *et al.* 2012; Zadavec *et al.* 2012; Lehikoinen *et al.* 2013), including Passeriformes like *Lonchura oryzivora*, *Taeniopygia guttata*, canaries and psittacines. Park (2011) documented the infection by *T. gallinae* in several novel species including lorikeets, corvids and a cuckoo species, plus its distinctive presentation in southern boobook owls (*Ninox boobook*). Trichomonosis, characterized by morbidity and mortality was also reported in free-ranging house finches (*Carpodacus mexicanus*), mockingbirds (*Mimus polyglottos*) and corvids (scrub jay: *Aphelocoma californica*; crow: *Corvus brachyrhynchos*; raven: *Corvus corax*) in northern California (Anderson *et al.* 2009). In comparison to the species of birds mentioned before only a few natural occurrences of trichomonosis have been reported in gallinaceous birds like turkeys (Hawn, 1937) and chickens (Levine and Brandly, 1939).

Avian trichomonosis has been reported from almost every major land mass, indicating a worldwide prevalence of the parasite (Forrester and Foster, 2008). In addition to the worldwide distribution,

especially in *Columbidae*, reports of prevalence vary greatly, ranging from 5.6% (Schulz *et al.* 2005) in Mourning Doves (*Zenaida macroura*) to 34.2% in wintering woodpigeons (*C. palumbus*) (Villanua *et al.* 2006) and up to 95% in White-winged Doves (*Zenaida asiatica*) (Conti and Forrester, 1981).

The threatening role for endangered species has been reported several times, supported by the ability of the parasite to infect numerous avian species (Höfle *et al.* 2004; Bunbury *et al.* 2007; Hegemann *et al.* 2007). This seems to be particularly important for birds of prey that nest near urban areas. Due to the loss of habitat their traditional prey is mainly replaced by urban pigeons. A noticeable variation was found in the prevalence of the parasite in Cooper's hawk (*Accipiter cooperi*) in Arizona (Boal *et al.* 1998). The infection rate in nestlings of couples breeding far from urban areas was only 9%, in comparison to 85% in birds from urban areas. According to the authors, this was due to the increased consumption of urban columbiformes. This observation was further corroborated by Estes and Mannan (2003), who determined that 57% of the urban Cooper's hawks' diet consisted of columbiformes compared with 4% in rural areas. The same was observed in goshawk nestlings (*Accipiter gentilis*) close to urban areas in Europe, with 100% prevalence in Poland (Wieliczko *et al.* 2003) and 65% in Germany (Krone *et al.* 2005). On the Iberian Peninsula, studies about the prevalence of *T. gallinae* focused on Bonelli's eagle (*Hieraetus fasciatus*), a vulnerable species, whose population on the Peninsula accounts for 75–93% of the total European population (Real and Manosa, 1997). In 1993, trichomonosis was one of the most important single nestling mortality factors for Bonelli's eagle, accounting for 22% of total chick mortality. In southern Portugal *T. gallinae* was demonstrated in 50% of the Bonelli's eagle chicks analysed by Höfle *et al.* (2000). In northeast Spain, Real *et al.* (2000) found the parasite present in 36% of the raptors. In all cases, a high percentage of pigeons were observed in the eagle's diet. A remarkable difference in the prevalence of trichomonosis in captive and wild birds was recorded in Saudi Arabia by Bailey *et al.* (2000), who detected a prevalence of 35% in wild pigeons and 68% in captive birds. However, this trend was not so pronounced in Australia, where the prevalence of trichomonosis was almost equal between captive pigeons (49%) and wild birds (46%) (McKeon *et al.* 1997). Altogether, in numerous reports avian trichomonosis could be linked to the bird species and the way birds are kept or the biology of a certain bird species.

Great variations could be noticed between studies focusing on the prevalence depending on the parameters (season, age or bird species) applied in the investigations. The occurrence of avian trichomonosis among pigeon nestlings has been reported throughout the year, but marked seasonal

fluctuations were also recorded. No clear seasonal link could be established as outbreaks in pigeons and doves occur throughout the whole year (Gerhold *et al.* 2007a; Begum *et al.* 2008). In comparison, seasonal patterns of disease occurrence appear to be prominent in finches where trichomonosis has recently emerged (Neimanis *et al.* 2010; Robinson *et al.* 2010; Lawson *et al.* 2011b). In the UK, finch mortalities begin in July, with peaks reached in late summer and early autumn. Climatic factors were initially thought to have played a role in the emergence of the disease in the UK (Anonymous, 2006), but this hypothesis was all but neglected due to the inconsistency between weather events and trichomonosis outbreaks. Despite reports suggesting the seasonality of the disease, climatological data have not been assessed to determine the potential role of weather conditions on the emergence of trichomonosis in a particular region. However, more recently, dry weather and low rainfall have been suggested as factors involved in the emergence of the disease in finches in the UK (Simpson and Molenaar, 2006). In agreement with this, the prevalence of *T. gallinae* infection in doves in Mauritius was found to be higher at sites and times of warmer temperatures and lower rainfall (Bunbury *et al.* 2007).

Due to the extreme fragility of *T. gallinae* trophozoites, it was long believed that the parasite was unable to survive outside the host "for more than the briefest periods" (Stabler, 1954). Furthermore, an intermediate host (live vector) is unknown among these protozoa (Stabler, 1954; McDougald, 2008). In view of this, the flagellate displays only a low tenacity in an ambient environment and dies quickly outside the host. It is generally believed that water and bird feed are sources for the transmission of the parasite. *Trichomonas gallinae* survives only for a short period in tap water (Bondurant and Honigberg, 1994) and at least 8 hours in carcasses (Erwin *et al.* 2000). Moreover, *T. gallinae* could survive outside the host for up to 120 h under certain laboratory conditions (Amin *et al.* 2010).

However, a direct contact seems most efficient to establish an infection and the best example for this is the transmission of parasites via the crop milk from infected parent birds to the nestlings during first feeding (Stabler, 1954). In adult pigeons, the infection can occur during courtship while raptors can be infected from prey animals carrying the parasite. The infection of turkeys and chickens happens mainly via drinking water contaminated by pigeons (Bondurant and Honigberg, 1994). Conclusively, a wet environment seems to be generally required by trichomonad flagellates to persist in their motile form, so persistent drying of buildings and housing facilities following washing will enhance the control of a trichomonad infection.

*Trichomonas gallinae* is unable to form true cysts, even though cyst-like stages (pseudocysts) were reported (Tasca and De Carli, 2003; Mehlhorn *et al.* 2009). These pseudocysts may provide another route of transmission and an environmentally resistant stage during unfavourable conditions. Consequently, pseudocysts apparently enhance transmission and extend survival time outside the host.

#### *Tetratrichomonas gallinarum*

*Tetratrichomonas gallinarum* is a flagellate commonly inhabiting the intestinal tract of different poultry species including chickens, turkeys, guinea fowl, quails, ducks and geese (Levine, 1985; Friedhoff *et al.* 1991; Bondurant and Honigberg, 1994). In older literature, the incidence of *T. gallinarum* in chickens was about 60% in Pennsylvania (McDowell, 1953) and 43% in Russia (Bondarenko, 1964). An influence of the weather on the prevalence of *T. gallinarum* infections was reported by Weinzirl (1917) who found an increase during warmer periods, whereas Leibovitz (1973) mentioned a peak of infection in autumn. Anyhow, prevalence data might be influenced by the different detection methods including variations of the media used for isolation and propagation as outlined below (sub-heading: cultivation of trichomonads).

The flagellate can be transmitted via consumption of contaminated food. Infected birds excrete live parasites as soon as 2 days post infection, as proven recently by experimental infection of chickens and turkeys with an axenic clonal culture of *T. gallinarum* (Amin *et al.* 2011). In addition to trophozoites, pseudocysts of *T. gallinarum* are reported *in vivo* and *in vitro* which might protect the parasite during fecal oral transmission (Friedhoff *et al.* 1991; Mehlhorn *et al.* 2009).

#### PATHOGENICITY

##### *Trichomonas gallinae*

Pindak *et al.* (1986) noticed the deficiency of an easy procedure by which the pathogenicity of the organism can be determined, a statement still valid today. The assignment of trichomonad isolates as pathogenic or non-pathogenic is mainly based on the severity of the symptoms induced in the host from which the particular strain is isolated.

The preferred site for *T. gallinae* is the upper digestive tract including the mouth, pharynx, oesophagus and crop, with the parasite rarely found posterior to the proventriculus (Cauthen, 1936). Consequently, the excretion of the protozoa via droppings is very limited. Moreover, the flagellate is able to enter the head sinuses and invade the brain and eye regions and can be detected in tears

(Bondurant and Honigberg, 1994). Jaquette (1950) demonstrated that *T. gallinae* reached the abdominal viscera presumably via the blood, but not via the gut and the bile duct. Moreover, systemic trichomonosis involving the liver, lung, heart, pancreas, air sacs and pericardium has been documented (Stabler and Engley, 1946). The severity of pathologic lesions of *T. gallinae* in the upper digestive tract varies from a mild inflammation of the mucosa to caseous areas that block the oesophageal lumen (Stabler, 1954). Narcisi *et al.* (1991) reported that a virulent strain of *T. gallinae* was able to create diphtheritic membranes of wet canker, associated with fibrinous lesions in internal organs such as the liver, lungs and peritoneum, resulting in high mortality. The authors also described that vascular congestion of the tongue, liver and lung was detected already 4 days post infection (dpi). Moreover, necrosis of the epithelial cells and submucosa of the oropharyngeal regions was observed on the 5th and 6th dpi. Trichomonads were only found attached to the epithelium of this localization. Additionally, purulent exudates containing mainly heterophils were noticed after one week in the oropharynx, crop and the lungs. Histopathological changes associated with *T. gallinae* infection in the liver were characterized by a vascular congestion with perivascular cuffing, observed as early as 4 dpi. Fatty degeneration of the hepatocytes at 7 dpi was found before complete necrosis of the hepatic cells in the presence of trichomonads occurred. Furthermore, degenerative lesions were detected in the kidneys and genitalia of the infected pigeons. Additionally, the caseous masses may appear in intestinal and gizzard surfaces, substernal membranes and pericardium (Stabler, 1954). The myocardium also may become caseous as an extension from the pericardium. *Trichomonas gallinae* strains of moderate virulence are often associated with caseous abscesses in the upper digestive tract and oropharyngeal region, whereas no visible lesions are produced by avirulent strains of *T. gallinae* (Cole and Friend, 1999). Stabler (1948) reported that 80–90% of adult pigeons were infected without showing any clinical signs of the disease. The author assumed in a later report that most of these birds became immunized as a result of exposure to an avirulent strain of the parasite, enabling them to act as a constant source of infection for their progenies (Stabler, 1954).

It seems that the severity of the disease depends on the susceptibility of the infected birds together with the pathogenic potential of the incriminated strain and the stage of infection (Cooper and Petty, 1988; Cole and Friend, 1999). It was also thought that variations in virulence are related to the antigenic composition of the parasite (Stepkowski and Honigberg, 1972; Dwyer, 1974). Even though genetic data indicate a certain variation between isolates, no correlation with virulence was



established (Gerhold *et al.* 2008; Anderson *et al.* 2009; Grabensteiner *et al.* 2010; Ecco *et al.* 2012; Stimmelmayer *et al.* 2012; Chi *et al.* 2013).

Early studies indicated that the virulence of *in vitro* grown *T. gallinae* could be determined by producing lesions in mice at the site of subcutaneous inoculations (Honigberg, 1961; Frost and Honigberg, 1962). The use of haemolytic activity was demonstrated as unsuitable for determining the virulence of *T. gallinae* (Gerhold *et al.* 2007b) contrary to reports about the virulence of *Trichomonas vaginalis* (Krieger *et al.* 1983). Double-stranded RNA (dsRNA) virus particles, detected in *T. vaginalis*, were assumed to be a virulence factor by Wang *et al.* (1987). However, these particles were not detected by transmission electron microscope and dsRNA segments were not visualized in agarose gel electrophoresis of extracted RNA from 12 *T. gallinae* isolates recovered from wild birds (Gerhold *et al.* 2009).

Only a few studies investigated the behaviour of the parasite in cell cultures. Honigberg *et al.* (1964) examined the effect of a virulent (Jones' Barn) and an avirulent (Lahore) strain of *T. gallinae* on trypsin-dispersed chick liver cell cultures. The authors showed that there were significant differences in the behaviour of the two strains, while the effect of a cell-free filtrate obtained from an actively growing virulent trichomonad strain on liver cell cultures was relatively small. Kulda (1967) demonstrated abnormal changes in a monkey kidney cell line caused by trichomonads but ultrafiltrates obtained from cultures with high protozoal numbers had no effect on this cell line. Recently, we were able to demonstrate that genetically different *T. gallinae* isolates caused diverse magnitude of cytopathic effects on LMH and QT35 monolayers (Amin *et al.* 2012a). In contrast to other studies, which were focused on the direct interaction of *T. gallinae* with cell cultures, we demonstrated that the destruction of monolayers was the consequence of both direct and indirect interaction of the parasite with the cells. Consequently, it seems that tissue cultures are a practical and sophisticated approach to study the pathogenicity of different axenic *T. gallinae* isolates.

Little information is available about the mechanism by which *T. gallinae* causes pathological changes in its hosts. In comparison, it could be shown that glycosidase, neuraminidase and certain peptidases are present in extracts of related trichomonads (North, 1982; Lockwood *et al.* 1984; Provenzano and Alderete, 1995; Thomford *et al.* 1996). Until recently, the role of secreted products by *T. gallinae* in growth media and their function in host-pathogen interaction have not been clarified. Amin *et al.* (2012b) identified proteolytic proteins secreted by *T. gallinae*, which contributed to the detachment of a cell monolayer as mentioned above. In that study, it was shown that the addition of specific peptidase inhibitors such as TLCK and E-64 to the cell-free

filtrate partially inhibited the destruction of the monolayer. This result implies the presence of peptidases in the filtrate and their involvement in the cytopathogenic effect. The application of multiple molecular techniques led to the identification of four different Clan CA, family C1, cathepsin L-like cysteine peptidases in the pool of proteins secreted by *T. gallinae*.

#### *Tetratrichomonas gallinarum*

*Tetratrichomonas gallinarum* frequently occurs in mixed infections with other protozoa, especially *Histomonas meleagridis* and *Blastocystis* spp., due to its presence in the large intestine (Tyzzer, 1920). Various studies investigated the pathogenicity of *T. gallinarum* either in naturally infected chickens and turkeys or via experimental infection, with contradicting outcomes (Allen, 1941; Goedbloed and Bool, 1962; Kemp and Reid, 1965; Lee, 1972; Patton and Patton, 1996; Norton, 1997; Richter *et al.* 2010; Amin *et al.* 2011). However, it needs to be mentioned that these investigations could have been significantly influenced by some other concurrent infections due to limited characterization of samples obtained from naturally infected birds. Very often those samples are contaminated with bacteria or other protozoan parasites, mainly *H. meleagridis*. Accordingly, the pathogenicity of *T. gallinarum* in poultry has been discussed controversially which is elaborated in detail below, and the pathogenic effect of *T. gallinarum* alone as a primary pathogen remains in dispute.

Allen (1941) reported *T. gallinarum*-induced lesions in the caecum and liver of domestic fowls and turkeys and that the parasite may be a possible causative agent of enterohepatitis. This suggestion disagreed with the findings of Tyzzer (1920, 1934), who already proved that *H. meleagridis* was the true aetiologic agent of such an infection. The pathogenic potential of *T. gallinarum* was demonstrated by Lee (1972) following cloacal infection of 3–6-week-old chickens with the caecal content of naturally infected broilers harbouring *T. gallinarum*. Although the infected chickens appeared healthy, a loss of microvilli and reduction of glycocalyx with complete loss of the polysaccharide matrix was noticed. Yellow, frothy liquid caecal content as well as small raised papulae on the mucosal surface of the caeca were observed in both chickens and turkeys experimentally infected with an emulsion containing *T. gallinarum* and bacteria (Norton, 1997). Additionally, severe necrotic enteritis of the duodenum and the jejunum of turkeys were reported in that study. A pathogenic potential of *T. gallinarum* in ducks was noticed by Crespo *et al.* (2001) characterized by a decrease in egg production and an increase of mortality of female ducks. Interestingly, the male

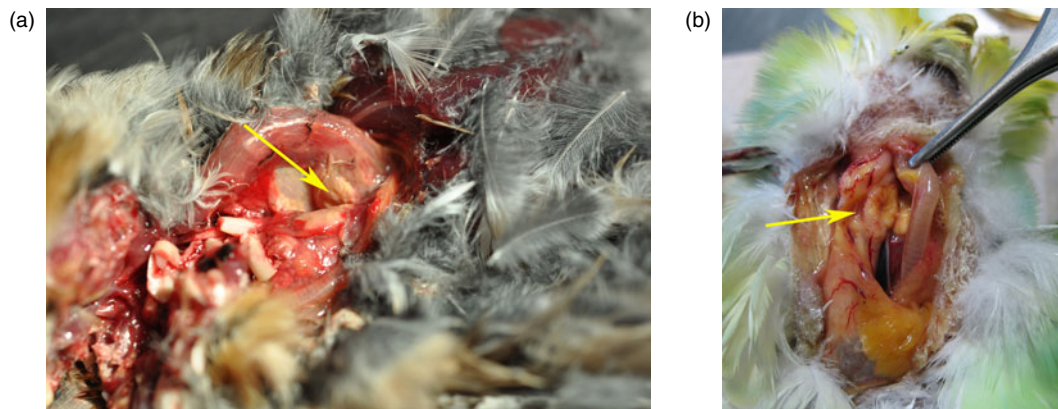


Fig. 1. Caseous lesions that can block the lumen of the oesophagus in (a) Budgerigar (*Melopsittacus undulatus*) and (b) Hawfinch (*Coccothraustes, Coccothraustes*) that died due to trichomonosis.

ducks from the same naturally infected flock remained clinically normal. Another recent study also reported the pathogenic potential of *T. gallinarum* in ducks suffering from acute typhlohepatitis (Richter *et al.* 2010). In wild birds, three reports connected *T. gallinarum* with pathological changes. Patton and Patton (1996) reported *T. gallinarum* as the likely trichomonad found in the brain of a mockingbird (*M. polyglottos*) suffering from encephalitis. As none of the molecular methods were used to characterize samples from the diseased bird it cannot be excluded that *T. gallinae* was responsible for the infection. Two recent reports of tetratrachomonosis in a Waldrapp ibis (*Geronticus eremita*) (Laing *et al.* 2013) and an American white pelican (*Pelecanus erythrorhynchos*) (Burns *et al.* 2013), described necrotizing hepatitis or hepatitis/splenitis in the affected birds. Both reports applied molecular methods for identification of protozoan parasite present in the samples, eliminating the possible confusion with *T. gallinae*.

In contrast to this, a collection of other reports could not demonstrate a pathogenic potential of *T. gallinarum*. Goedbloed and Bool (1962) were unable to produce any clinical signs or histopathological changes in turkeys following rectal inoculation with a *T. gallinarum* culture. Kemp and Reid (1965) reported that chickens and turkeys infected with a certain strain of *T. gallinarum* obtained from naturally infected birds showed no mortality, gross lesions, or even decrease in body weight. Furthermore, *in vitro* studies failed to demonstrate the pathogenic potential of *T. gallinarum*. Kulda (1967) demonstrated that the parasite was able to grow in a monkey kidney cell culture without producing any cytopathogenic effect. In another study, a subcutaneous mouse assay did not reflect any pathological changes following injection with different strains of *T. gallinarum* (Kulda *et al.* 1974). Recently, it has been proven that genetically different *T. gallinarum* clones and their cell-free filtrate had no destructive effect on cell cultures (permanent chicken liver

(LMH) and a permanent quail fibroblast (QT35) cell line) (Amin *et al.* 2012a). One of the investigated clones used in these studies was also used to infect turkeys and specified pathogen-free chickens without producing any clinical signs, macroscopic or microscopic lesions (Amin *et al.* 2011). Even though being non-pathogenic *T. gallinarum* was transmitted rapidly between infected birds and a latent infection was established.

#### DIAGNOSTIC OPTIONS

##### *Clinical signs and post-mortem investigations*

Clinical signs associated with avian trichomonosis are loss of appetite, vomiting, ruffled feathers, diarrhoea, dysphagia, dyspnoea, weight loss, increased thirst, inability to stand or to maintain balance and a pendulous crop (Narcisi *et al.* 1991). A greenish fluid or whitish fibrinous material may be accumulating in the mouth and crop as demonstrated in Fig. 1. These materials may also exude from the beak of the infected bird (Stabler, 1947). Death may occur within 3 weeks of infection.

Post-mortem investigations could play an important role in the diagnosis of trichomonads, especially *T. gallinae* due to the pathognomonic lesions characterized by the presence of yellowish soft caseous material in the oropharynx of infected birds. However, *T. gallinae* infections may be confused with some other pathological conditions that result in similar lesions. For example, infections with avian poxviruses, fungi (*Candida* sp., *Aspergillus* sp.) and nematodes (*Capillaria* sp.), as well as the presence of sialoliths (salivary stones), a non-specific pharyngo-oesophagitis or Vitamin A deficiency could result in similar oral lesions (Levine, 1985). Confusions with pigeon herpesvirus, avian paramyxovirus or fowl adenovirus were demonstrated when trichomonads affect the internal organs, and bacterial infections of the navel might be confused with navel canker caused by *T. gallinae* (Vogel, 1992).

Additionally, trichomonosis could be confused with other infectious diseases that are characterized by granuloma formation such as tuberculosis, mycoplasmosis, salmonellosis and coligranuloma (Friedhoff, 1982). In general, trichomonosis should be a differential diagnosis for birds showing regurgitation or upper gastrointestinal abscesses (Park, 2011). Apart from this, infected birds can also remain asymptomatic due to the infection with avirulent strains of trichomonads or due to a lower susceptibility as seen in older birds. Therefore, diagnosis is established by microscopic examination of samples from infected birds with definite identification of its nucleic acids.

#### MICROSCOPICAL EXAMINATIONS

##### *Wet mount preparation*

Diagnosing trichomonads depends traditionally on direct microscopic observation of motile protozoa via wet mount preparation (i.e. immediate examination of glass slides). Sample material can be obtained via swabbing the cloacae in case of *T. gallinarum* (Allen, 1941) or the oral cavity for *T. gallinae* (Honigberg, 1978). Trichomonads appear as elongated, oval shapes, which move briskly. The wet mount sample smeared on a glass slide can be stained with Giemsa as demonstrated for *T. gallinae* (Borji *et al.* 2011). However, the sensitivity to detect trichomonads in wet mount preparations is low, especially if the number of parasites in the host is marginal. In this case, inoculation of swabs into a suitable growth medium and their incubation at optimal temperature was shown to be helpful to enrich the number of trichomonads.

##### *Cultivation of trichomonads*

Growth medium for the detection of trichomonads has been shown to be more sensitive than wet mount preparations, considering that several investigators reported superior results when both methods were applied (Fouts and Kraus, 1980; Cooper and Petty, 1988; Bunbury *et al.* 2005). Cultivation has been the gold standard for detection of trichomonads as it is easy to interpret and gives valid results, even in poorly infected birds. *Trichomonas gallinae* grows in a variety of media (Forrester and Foster, 2008). Several investigations used InPouch™ TF Kits (BioMed Diagnostics, White City, OR, USA), a commercial product originally developed to culture *Tritrichomonas foetus* from cattle, which was shown to be very convenient and effective for use in the field (Schulz *et al.* 2005; Bunbury *et al.* 2007; Gerhold *et al.* 2007b). Development of a technique to establish clonal cultures of trichomonads raised the standard of cultivation (Hess *et al.* 2006). Such well-defined cultures were shown to be very useful

for detailed characterization of protozoa (Amin *et al.* 2010; Grabensteiner *et al.* 2010) and they are indispensable for pathogenicity studies (Amin *et al.* 2011). However, due to the complexity of the technique, application of clonal cultures in daily routine is still inconvenient.

Minimal nutritional requirements are essential to obtain good growth results of protozoa *in vitro*. The nutritional requirements and energy metabolism of trichomonads differ from those of the majority of eukaryotic cells. Trichomonad flagellates depend mainly on pre-formed metabolites as nutrients which indicates the absence of essential biosynthetic pathways (Müller, 1990). Undoubtedly, there are various factors which may influence the growth behaviour of flagellates *in vitro*. These issues were fruitfully investigated in the first half of the last century and are reviewed by Stabler (1954), focusing on media supplements that boost *T. gallinae* growth. Higher cell counts of well-defined protozoal cultures are obtained in monoxenic cultures in comparison to axenic ones (Tasca and De Carli, 2001). In this context it was noticed that clonal cultures of trichomonads grown in Medium 199 develop high populations in the presence of bacteria (Hess *et al.* 2006). Different media and techniques have been described to obtain axenic cultures, but most of them are rather laborious and time consuming (Diamond, 1957; Kulda *et al.* 1974). Using different media a standard procedure for axenization of *T. gallinarum* and *T. gallinae* was established recently (Amin *et al.* 2010). Under axenic conditions *T. gallinarum* and *T. gallinae* grew in modified *T. vaginalis* (TV)– and Hollander fluid (HF) medium, respectively. The incubation temperature was shown to influence the growth rate of trichomonads (Theodorides, 1964; Amin *et al.* 2010). Axenically, the most favourable temperature for *T. gallinarum* was 40 °C with higher cell yields than those observed following incubation at 37 °C. In contrast, the number of live cells recorded for *T. gallinae* was higher at 37 °C in comparison to 40 °C of incubation (De Carli *et al.* 1996; De Carli and Tasca, 2002; Tasca and De Carli, 2003; Amin *et al.* 2010). These findings might reflect the clinical environment of both parasites, either in the pharynx or the caeca of birds. Virulence of *T. gallinae* was also reported to be influenced by the incubation temperature and the cultivation method (Stabler *et al.* 1964). Consequently, in order to maintain the virulence and high number of protozoal cells after the axenization process, it is essential to keep a certain optimal temperature for incubation of trichomonad cells.

Until recently, there were no data available about aerobic and anaerobic growth conditions of *T. gallinarum* and *T. gallinae* following axenization. In this context it needs to be considered that trichomonads are amitochondrial anaerobic

protozoa, which gain the required energy by utilizing exogenous and endogenous carbohydrates under both aerobic and anaerobic conditions (Donald and Miklos, 1973). Recently, Amin *et al.* (2010) revealed that *T. gallinarum* and *T. gallinae* were able to multiply under both aerobic and anaerobic conditions in an axenic environment. Interestingly, the growth of these trichomonads under anaerobic and aerobic conditions was very similar and the addition of antibiotics to the axenic cultures of *T. gallinarum* and *T. gallinae* had no adverse effect on the growth. However, adding of antibiotics to the culture medium might lead to *in vitro* attenuation which was speculated as a consequence of direct interaction of the protozoal nucleic acid with antibiotics (Kirk, 1962; Stabler *et al.* 1964). Therefore, antibiotics should only be used until axenic cultures are established in order to minimize any influence of drugs on the virulence in consecutive studies (Bondurant and Honigberg, 1994).

Several studies showed that prolonged axenic cultivation of virulent *T. gallinae* strains caused a loss in pathogenicity (Goldman and Honigberg, 1968; Amin *et al.* 2012a,b). In that respect, it is important to use cultures with low passages if aspects of virulence are investigated. In general, virulent strains of trichomonads grow faster *in vitro* than avirulent ones (Bondurant and Honigberg, 1994).

Cell cultures were reported as a sensitive tool to differentiate between strains of different pathogenicity (Honigberg *et al.* 1964). Recently, it has been proven that both LMH and QT35 cells were able to support and enhance the growth of *T. gallinarum*, an effect even more pronounced for *T. gallinae* (Amin *et al.* 2012a). Obviously, some components of the cells are considered to be necessary nutrients for the protozoa. In agreement with this media from uninfected cells or the media itself did not appear to possess soluble growth factors for trichomonads as mentioned before for *T. vaginalis* (Peterson and Alderete, 1984; Karen *et al.* 1990). In this context, it was demonstrated that the adherence between the parasite and the cells in the culture is helpful for the protozoa to ascertain the delivery of nutrient substances. Additionally, it was confirmed that cell culture matrix as well as special growth media could be used for *in vitro* cultivation of *T. gallinae* after axenization (Amin *et al.* 2010, 2012a).

#### Staining of trichomonads

In most of the protocols applying staining methods for trichomonads, smears are prepared from cultures. The smears are fixed on slides and treated with different staining methods, such as Giemsa, silver, iron-hematoxylin, Malachite green or methylene blue, Papanicolaou, acridine orange or other stains

(Borchardt and Smith, 1991; Kaufman *et al.* 2004). Gram-stained smears were already described by Cree (1968). However, these methods are not of use in routine clinical settings because they are laborious, expensive and dilute the original samples. Furthermore, they might result in false negative result if direct smears are made from birds harbouring only low numbers of parasites (Kaufman *et al.* 2004). In tissues, the use of haematoxylin and eosin (HE) and Periodic-acid Schiff (PAS) stains was proven to be limited for identification of the flagellates, especially in organs that contained only a few protozoal cells (Amin *et al.* 2011).

#### Detection of trichomonad's nucleic acid

The presence of trichomonads, respectively parasite's DNA, can be detected in oral fluids, in tissue taken from the crop, pharynx or from faeces by polymerase chain reaction (PCR). A variety of primers have been described, most of them targeting ITS1-5.8S rRNA-ITS2 and 18S rRNA regions (Felleisen, 1997; Delgado-Viscogliosi *et al.* 2000; Gerbod *et al.* 2004; Cepicka *et al.* 2005; Grabensteiner and Hess, 2006; Lawson *et al.* 2011a; Malik *et al.* 2011; Noda *et al.* 2012). The majority of primers were originally designed to detect all trichomonads, as they were developed for phylogenetic analysis and not for diagnostics. Exceptional to this are primers targeting either the 18S rRNA (Grabensteiner and Hess, 2006) or the Fe-hydrogenase (Lawson *et al.* 2011a). The 18S rRNA primers developed to detect *T. gallinae* and *T. gallinarum*, were successfully applied in a field study reporting a prevalence of up to 31.8% of trichomonads in German poultry flocks (Hauck *et al.* 2010). The Fe-hydrogenase primers were designed to specifically amplify the Fe-hydrogenase gene of *T. gallinae*, which was used to support the classification of strains. Recently, this application demonstrated the potential for detecting fine-scale variations amongst *T. gallinae* strains (Chi *et al.* 2013).

Moreover, *in situ* hybridization (ISH) for definitive demonstration of the protozoan nucleic acid in paraffin-embedded tissues was applied (Liebhart *et al.* 2006). Overall, ISH was found to be more sensitive than histochemical staining (such as PAS and HE), especially in tissues showing marginal occurrence of the parasite. The protozoa were clearly detected as dark blue labelled cells following ISH (Amin *et al.* 2011). Intact trichomonad cells could be demonstrated in different organs and their location within the tissue could be determined. Moreover, ISH provides the opportunity to correlate the histological changes with the presence of the protozoan, offering the option to investigate the virulence of trichomonads on a cellular level.



### Antibody based technique

ELISA has only been used under experimental conditions to detect antibodies against *T. gallinarum* and *T. gallinae* in poultry (Amin *et al.* 2011). It remains to be determined whether such a technique can be used to obtain a more detailed picture about the presence of trichomonads in poultry. Moreover, it would be interesting to employ serology for screening potential carrier birds because birds that survived an infection with *T. gallinae* are considered to act as a latent carrier for the parasite over years. Therefore, it is conceivable that these carrier birds might contribute to the persistent spread of the parasite.

## GENETIC ANALYSIS OF TRICHOMONADS

### *Trichomonas gallinae*

Based on clinical signs the degree of pathogenicity of *T. gallinae* isolates may vary as mentioned above. To determine the genetic polymorphism among *T. gallinae* isolates in different bird species, several studies were performed but none focused explicitly on distinguishing pathogenic from non-pathogenic strains (Gaspar da Silva *et al.* 2007; Gerhold *et al.* 2008; Anderson *et al.* 2009; Sansano-Maestre *et al.* 2009; Grabensteiner *et al.* 2010; Lawson *et al.* 2011a; Ecco *et al.* 2012; Stimmelmayer *et al.* 2012; Chi *et al.* 2013; Lennon *et al.* 2013). Most of the studies analysed the ITS1-5.8S rRNA-ITS2 region and/or 18S rRNA sequences, with exception of two studies, which additionally used either  $\alpha$ -tubulin (Gerhold *et al.* 2008) or Fe-hydrogenase gene sequences (Lawson *et al.* 2011a) (Table 1). Some studies observed only minor or no sequence variations between *T. gallinae* isolates, even though they analysed different bird species from various geographic regions including pink pigeons (*Columba mayeri*) and Madagascar turtle-doves (*Streptopelia picturata*) from the island of Mauritius (Gaspar da Silva *et al.* 2007), domestic pigeons and birds of prey from the east part of the Iberian Peninsula, Spain (Sansano-Maestre *et al.* 2009) or different passerines, columbids and raptors from the UK (Lawson *et al.* 2011a; Chi *et al.* 2013). Furthermore, the ITS1-5.8S rRNA-ITS2 sequences reported in these studies were identical to either one or to both previously reported isolates: *T. gallinae* strain G7 (GenBank Accession No. AY349182) (Kleina *et al.* 2004) and *T. gallinae* strain TG (GenBank Accession No. U86616) described by Felleisen (1997). In order to further investigate the degree of variation among such isolates some studies applied methods like random amplified polymorphic DNA analysis (RAPD) or PCR restriction fragment length polymorphism analysis (PCR-RFLP) (Table 1) (Gaspar da Silva *et al.* 2007; Sansano-Maestre *et al.* 2009; Lawson *et al.* 2011a). However, these methods

just confirmed the data obtained by sequence analysis of the ITS1-5.8S rRNA-ITS2 locus. Interestingly, Sansano-Maestre *et al.* (2009) reported a prevalence of *T. gallinae* isolates with identical sequence to *T. gallinae* strain TG (U86614) in columbiformes, whereas isolates with identical sequence to *T. gallinae* strain G7 (AY349182) were more often found in raptors and all birds that displayed macroscopic lesions. A study analysing *T. gallinae* isolates causing massive mortality of British passerines demonstrated the presence of a single strain in all deceased birds (Lawson *et al.* 2011a). This strain was shown to possess the ITS1-5.8S rRNA-ITS2 sequence identical to a *T. gallinae* G7 strain (AY349182) (Robinson *et al.* 2010). Just recently, studies of Chi *et al.* (2013) and Ganas (pers. communication) demonstrated that by additionally analysing the Fe-hydrogenase locus finer-scale genetic variations could be detected in isolates displaying identical ITS1-5.8S rRNA-ITS2 sequence, arguing for detailed analysis of different loci.

New light was shed by several studies (Gerhold *et al.* 2008; Anderson *et al.* 2009; Grabensteiner *et al.* 2010; Ecco *et al.* 2012; Stimmelmayer *et al.* 2012) reporting greater genetic diversity among *T. gallinae* isolates. Two studies (Gerhold *et al.* 2008; Grabensteiner *et al.* 2010) reported many different sequence groups/types, but used different nomenclature in labelling of these groups. Additional confusion is given by the fact that some sequence groups (assigned as C to E) reported by Gerhold *et al.* (2008) have identical sequence but differ slightly in their length.

The study of Anderson *et al.* (2009) described a new flagellate isolated from *M. polyglottos* (EU290650) which was genetically distinct from all previously sequenced trichomonads and resembled more the sequences from tetratrachomonads. Three other studies (Gerhold *et al.* 2008; Grabensteiner *et al.* 2010; Ecco *et al.* 2012), showed that some isolates were more related to the human parasite *T. vaginalis* than to other isolates of *T. gallinae*. Furthermore, in different studies flagellates were detected and defined as *Trichomonas*-like parabasalids as they did not group to any defined strain from the genus *Trichomonas* (Gerhold *et al.* 2008; Grabensteiner *et al.* 2010; Stimmelmayer *et al.* 2012). Grabensteiner *et al.* (2010) and recently Lennon *et al.* (2013) identified an isolate that showed the highest relationship to the human parasite *Trichomonas tenax*. Both studies also showed a closer relationship of *T. tenax* to *T. gallinae* than to the human parasite *T. vaginalis*, an observation already reported by Kleina *et al.* (2004). It remains speculative whether the close relationship between *T. gallinae* and *T. tenax* emerges from the fact that both are implicated in the infections of the upper digestive tract. In contrast to all studies, Grabensteiner *et al.*

Table 1. Molecular typing of *Trichomonas gallinae* isolates

Genomic locus/method	Primers	Number of <i>T. gallinae</i> isolates	Sequence types	Reference
ITS1 – 5.8S rRNA-ITS2	TFR1/TFR2 (Felleisen, 1997)	24	1 sequence <sup>a</sup>	(Gaspar da Silva <i>et al.</i> 2007)
		42	5 sequence groups (A-E) <sup>b</sup>	(Gerhold <i>et al.</i> 2008) <sup>c,d</sup>
		19	1 sequence type <sup>a</sup>	(Anderson <i>et al.</i> 2009)
		34	2 sequences (genotypes A <sup>c</sup> and B <sup>a</sup> )	(Sansano-Maestre <i>et al.</i> 2009)
		52	3 sequence types (ITS-I,-IV,-V)	(Grabensteiner <i>et al.</i> 2010) <sup>c,d,f</sup>
		18	1 sequence	(Robinson <i>et al.</i> 2010)
		68	1 sequence	(Lawson <i>et al.</i> 2011a)
		80	4 sequence types	(Chi <i>et al.</i> 2013)
		14	3 sequences	(Lennon <i>et al.</i> 2013) <sup>f</sup>
		2	1 sequence	(Ecco <i>et al.</i> 2012) <sup>g</sup>
18S rRNA	16S1/16Sr; 514F/1055R, 1055F/1385R (Cepicka <i>et al.</i> 2006) SSU rRNA forward/reverse (Hess <i>et al.</i> 2006) Nested PCR: SSU forward/reverse (Cepicka <i>et al.</i> 2005) TN3forward/TN4reverse (Robinson <i>et al.</i> 2010)	4	4 sequences <sup>b</sup>	(Gerhold <i>et al.</i> 2008) <sup>c,d</sup>
		23	5 sequences	(Grabensteiner <i>et al.</i> 2010) <sup>c,d,f</sup>
		8	1 sequence	(Robinson <i>et al.</i> 2010)
		22	1 sequence	(Lawson <i>et al.</i> 2011a)
alpha-tubulin	$\alpha$ -tubF1/ $\alpha$ -tubR1; trichtubF1/trichtunR1 (Edgcomb <i>et al.</i> 2001)	5	2 sequences	(Gerhold <i>et al.</i> 2008)
		24	7 sequences	(Lawson <i>et al.</i> 2011a)
Fe-hydrogenase	TrichhydFOR/TrichhydREV Nested PCR: TrichhydFOR/TrichhydREV FeHydFOR/ FeHydREV	24	7 sequences	(Lawson <i>et al.</i> 2011a)
		44	9 sequences	(Chi <i>et al.</i> 2013)
RAPD <sup>h</sup> analysis	OPD3, OPD5, OPD7, OPD8 fluorescently labelled OPD3, OPD5, OPD7, OPD8 (Gaspar da Silva <i>et al.</i> 2007)	22	7 groups	(Gaspar da Silva <i>et al.</i> 2007)
		14	10 groups	(Lawson <i>et al.</i> 2011a)
PCR-RFLP <sup>i</sup> analysis	TFR1/TFR2 PCR digested with HaeIII	116	3 groups <sup>j</sup>	(Sansano-Maestre <i>et al.</i> 2009)

<sup>a</sup> Sequence is identical to AY349182 (*T. gallinae* G7).<sup>b</sup> Sequence groups C to E have identical sequence, but their length varies.<sup>c</sup> The study also reports *Trichomonas* spp. sequences.<sup>d</sup> The study additionally reports *Trichomonas* spp. sequences closely related to *Trichomonas vaginalis*.<sup>e</sup> Sequence is identical to U86614 (*T. gallinae* TG).<sup>f</sup> The study additionally reports *Trichomonas* spp. sequence that closely related to *Trichomonas tenax*.<sup>g</sup> The study additionally reports *Simplicimonas* spp.- and *T. vaginalis*-like sequences.<sup>h</sup> RAPD analysis = random amplified polymorphic DNA analysis.<sup>i</sup> PCR-RFLP analysis = PCR restriction fragment length polymorphism analysis.<sup>j</sup> One group was interpreted as mix of other two, i.e. that affected birds were infected with both genotypes.

(2010) employed clonal cultures of isolated trichomonads and by doing this it was possible to demonstrate the co-existence of diverse strains within a single bird.

#### *Tetratrichomonas gallinarum*

Genetic analysis of *T. gallinarum* isolates demonstrated a complexity that goes beyond the usual intraspecific polymorphism generally seen in trichomonad genera of *Trichomonas*, *Tritrichomonas* and *Tetratrichomonas* (Cepicka *et al.* 2005). In their study, Cepicka *et al.* (2005) applied two methods: sequence analysis of 18S rRNA and ITS1-5.8S rRNA-ITS2 and random amplified polymorphic DNA (RAPD) analysis, for analysing isolates obtained from different bird species and humans. The authors report the separation of *T. gallinarum* isolates into five groups (A–E) and 11 subgroups (A1, A2, B1, B2, B3, C1, C2, C3, D1, D2 and E), that, according to the extensive polymorphism, might represent at least three different species: groups A–C, D and E. Hence, these results could be considered as an explanation for controversies on pathogenicity of *T. gallinarum* observed in different reports, even though our initial studies with genetically different isolates did not point in this direction (Amin *et al.* 2012a). Finally, as phylogenetic analysis revealed a close relationship of some human with avian *T. gallinarum* isolates a zoonotic potential of these parasites should be considered.

#### TREATMENT OF AVIAN TRICHOMONOSIS

The first specific chemotherapeutic agents against trichomonads were tested and approved more than 50 years ago. Some drug compounds have been administered either in drinking water or applied topically to the bird's mouth and throat. A certain degree of success has been achieved by using compounds such as acriflavine, weak hydrochloric acid and copper sulphate (Rosenwald, 1944; Jaquette, 1948). Effective antibiotic therapy was reported once in the case of avian trichomonosis, despite the fact that this is not a typical procedure for treatment (Hamilton and Stabler, 1953). Furthermore, after several treatments of a diseased gyrfalcon with broad-spectrum antibiotics (Aureomycin) numerous sites of active infection with *Aspergillus fumigatus*, in addition to canker, were noticed. It was suggested that the antibiotics may cause activation of latent fungal infections and possibly latent trichomonosis. Therefore, applying certain antibiotics to treat trichomonosis might even be unfavourable.

Various nitroimidazoles, including metronidazole, dimetridazole, ronidazole and carnidazole have been considered the standard treatment for avian as well as for human trichomonosis (Franssen and Lumeij, 1992; Kulda, 1999). However, even after

successful treatment captive pigeons can often carry the parasite for a long time. In order to prevent economic losses nitroimidazole drugs are routinely administered to racing pigeons in subtherapeutic doses. This prolonged exposure to nitroimidazole creates the environment for developing resistance to these compounds, as shown for related trichomonads, *T. vaginalis* and *T. foetus* (Kulda *et al.* 1984, 1993). Indeed, in the past, several investigations reported the resistance to nitroimidazole derivatives of *T. gallinae* isolates from pigeons (Lumeij and Zwijnenberg, 1990; Franssen and Lumeij, 1992; Munoz *et al.* 1998). A recent study with different *T. gallinae* clonal cultures obtained from budgerigars and racing pigeons reported significantly different minimal lethal concentrations (MLCs) against four 5-nitroimidazoles (Zimre-Grabensteiner *et al.* 2011). Variations in sensitivities of two genetically different isolates obtained from the same bird were reported, indicating a correlation between *in vitro* results and genetic relationship (Grabensteiner *et al.* 2010; Zimre-Grabensteiner *et al.* 2011). Correlation between *in vitro* and *in vivo* resistance of one *Trichomonas* strain could be demonstrated, underlining the benefit of *in vitro* tests to investigate treatment failures.

In wild birds, treatment is much more problematic and generally not considered an option due to the way of application (Cole and Friend, 1999). In the case of medicated food supplied on bird feeders, medication is based on an estimated food intake of a normal bird per day. However, as birds might not feed only on a single feeder the uptake of a drug could be suboptimal which might lead to resistance development of target *T. gallinae* strains (Munoz *et al.* 1998). Apart from the potential of developing resistant *T. gallinae* strains, implementation of medicated feed could cause harmful effects in non-target bird species. Reece *et al.* (1985) already demonstrated that dimetridazole can be toxic to birds. Indeed, the incidence of population decline of non-target birds was documented for Red-legged Partridges (*Alectoris rufa*), whose population of chicks and adults decreased in the area where *T. gallinae* outbreak in woodpigeons (*C. palumbus*) was treated with dimetridazole via game bird feeders (Höfle *et al.* 2004). In contrast to this, metronidazole derivatives, dimetridazole and ronidazole, were used to treat trichomonosis with limited success in one subpopulation of wild pink pigeons (*C. mayeri*) (Swinnerton *et al.* 2005).

Conclusively, future measurements to prevent *T. gallinae* outbreaks in wild as well as in captive birds should concentrate on actions to reduce sources of infection as already outlined by Forrester and Foster (2008). The major aim would be to prevent attracting birds to feeding places if not necessary. In any case such places should fulfil minimum requirements with regard to sanitary conditions, like

changing of food regularly and disinfection of food places.

#### CONCLUSIONS AND PERSPECTIVES

Amongst the trichomonadidae the flagellate *T. gallinae* is the most important parasite in birds due to its worldwide distribution and pathogenicity, mainly in Columbiformes, Falconiformes and Strigiformes whereas other trichomonads are of limited significance. With the appearance of more severe cases in wild birds in recent years the disease has gained further attention. Epidemiological studies with detailed molecular characterization of the parasites will be of high importance in future studies in order to track more precisely individual strains or clones of *T. gallinae*. This will also shed new light on a possible zoonotic character of trichomonads in general originating from birds. The genetic heterogeneity of isolates causing avian trichomonosis indicates that even though in the past all infections were solely attributed to *T. gallinae* they might have been caused by species more closely related to *T. vaginalis* or *T. tenax*. These observations signify a necessity to implement standardized molecular methods in routine diagnostics. Recent data strengthen the requirement for implementing several genomic loci in typing *T. gallinae* isolates. Ultimately, new developments should concentrate on determining loci that would give strain variation data as well the information on strain's pathogenicity.

A panel of *in vitro* and *in vivo* studies should be applied in future studies to characterize the biology of isolates, including drug sensitivity and host response. Easier systems and models need to be developed in order to gain more principal data about virulence factors and host-pathogen interactions. New technologies such as genomics and proteomics should be applied to resolve genome structures and protein profiles of avian trichomonads. Elucidating the pathogenesis and virulence factors of these parasites will enforce the development of new protection strategies, which is needed considering data about resistance of flagellates against actual treatments. However, the fact that trichomonosis is restricted to certain bird species with its limited economic market may pose a certain hindrance for new developments.

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