Infection, Genetics and Evolution 55 (2017) 93–103

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Novel avian oropharyngeal trichomonads isolated from European turtle doves (*Streptopelia turtur*) and racing pigeons (*Columba livia*): genetic and morphometric characterisation of clonal cultures

M.C. Martínez-Herrero[a], M.M. Garijo-Toledo[a], D. Liebhart[b], P. Ganas[b], R.A. Martínez-Díaz[c], F. Ponce-Gordo[d], A. Carrero-Ruiz[e], M. Hess[b], M.T. Gómez-Muñoz[f,*]

[a] Departamento de Producción y Sanidad Animal, Salud Pública Veterinaria y Ciencia y Tecnología de los Alimentos, Facultad de Veterinaria, Instituto de Ciencias Biomédicas, Universidad CEU Cardenal Herrera, Valencia, Spain
[b] Clinic for Poultry and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, Vienna, Austria
[c] Departamento de Medicina Preventiva, Salud Pública y Microbiología, Facultad de Medicina, Universidad Autónoma de Madrid, Madrid, Spain
[d] Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense de Madrid, Madrid, Spain
[e] GREFA - Grupo de Rehabilitación de la Fauna Autóctona y su Hábitat, Majadahonda, Madrid, Spain
[f] Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain

ARTICLE INFO

Keywords:
Trichomonas gallinae
Genetic variants
Morphology
Oropharyngeal bird trichomonads
Trichomonas sp.

ABSTRACT

Extensive diversity has been described within the avian oropharyngeal trichomonad complex in recent years. In this study we developed clonal cultures from four isolates selected by their different ITS1/5.8S/ITS2 (ITS) genotype and their association with gross lesions of avian trichomonosis. Isolates were obtained from an adult racing pigeon and a nestling of Eurasian eagle owl with macroscopic lesions, and from a juvenile wood pigeon and an European turtle dove without clinical signs. Multi-locus sequence typing analysis of the ITS, small subunit of ribosomal rRNA (SSUrRNA) and Fe-hydrogenase (Fe-hyd) genes together with a morphological study by optical and scanning electron microscopy was performed. No significant differences in the structures were observed with scanning electron microscopy. However, the genetic characterisation revealed novel sequence types for the SSUrRNA region and Fe-hyd gene. Two clones were identified as *Trichomonas gallinae* in the MLST analysis, but the clones from the racing pigeon and European turtle dove showed higher similarity with *Trichomonas tenax* and *Trichomonas canistomae* than with *T. gallinae* at their ITS region, respectively. SSUrRNA sequences grouped all the clones in a clade that includes *T. gallinae*, *T. tenax* and *T. canistomae*. Further diversity was detected within the Fe-hyd locus, with a clear separation from *T. gallinae* of the clones obtained from the racing pigeon and the European turtle dove. In addition, morphometric comparison by optical microscopy with clonal cultures of *T. gallinae* revealed significant statistical differences on axostyle projection length in the clone from the European turtle dove. Morphometric and genetic data indicate that possible new species within the *Trichomonas* genus were detected. Taking in consideration the diversity in *Trichomonas* species present in the oral cavity of birds, a proper genetic analysis is highly recommended when outbreaks occur.

1. Introduction

Avian trichomonosis is one of the most important infectious diseases of wild birds with an emergent status. Latest outbreaks documented in passeriform species affected several countries with important consequences on the dynamics of avian populations (Forzán et al., 2010; Ganas et al., 2014; Lawson et al., 2011; Neimanis et al., 2010). *Trichomonas gallinae* (Rivolta 1878) is the causative agent of these mortality episodes, a flagellated protozoan unable to survive for long periods of time outside the host. Trophozoites from 6.2–20 μm in length are the parasitic stage of this parabasalid, with four anterior flagella and a fifth recurrent one that forms the undulating membrane (BonDurant and Honigberg, 1994; Mehlhorn et al., 2009; Stabler, 1941). Furthermore, pseudocysts formation has been described under suboptimal *in vitro* conditions, but it has not been detected in natural infections. This stress-triggered morphotype lacks external flagella and undulating membrane, which seem to have been internalized through an invagination process (Tasca and De Carli, 2003).

Direct transmission of trophozoites through contact with infected saliva or regurgitated food is the common route of infection, although

* Corresponding author at: Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Av. Puerta de Hierro s/n, 28040 Madrid, Spain.
E-mail address: mariateg@ucm.es (M.T. Gómez-Muñoz).

http://dx.doi.org/10.1016/j.meegid.2017.08.028
Received 2 August 2016; Received in revised form 9 July 2017; Accepted 28 August 2017
Available online 31 August 2017

contaminated water and carrion-feeding are also important (Erwin et al., 2000; Purple and Gerhold, 2015). Columbiform species are the reservoir host of the protozoan, with an endemic presence of infection (Amin et al., 2014). Besides, they are the primary source of infection for other birds such as raptors that feed on these species or passeriforms that share feeding stations or water sources with them.

In recent years, new investigations of symptomatic cases have changed the etiology of avian trichomonosis as new agents were discovered. Several research groups reported other protozoans that were not genetically identified as *T. gallinae*. In 2009, a new *Trichomonas* sp. was isolated from mockingbirds (*Mimus polyglottos*) in USA, in 2012 a *Simplicomonas*-like organism was detected in green-winged saltators (*Saltator similis*) in Brasil and in 2014 the new species of *Trichomonas stableri* was described from band-tailed pigeons (*Patagioenas fasciata monilis*) in USA (Anderson et al., 2009; Ecco et al., 2012; Girard et al., 2013, 2014). Coinfections of *T. stableri* and *T. gallinae* were found in birds with gross lesions of avian trichomonosis (Girard et al., 2013). In addition, several authors reported strains with higher similarity to *Trichomonas tenax*, *Trichomonas vaginalis* or *Trichomonas canistomae* organisms than with *T. gallinae* (Gerhold et al., 2008; Grabensteiner et al., 2010; Kelly-Clark et al., 2013; Martínez-Herrero et al., 2014). *Trichomonas vaginalis*-like protozoans were isolated from a bearded vulture (*Gypaetus barbatus*) from the Czech Republic and an American bald eagle (*Haliaeetus leucocephalus*) from Canada without clinical signs (Grabensteiner et al., 2010; Kelly-Clark et al., 2013). Later, the description of a novel organism associated with scavenging birds of prey, *Trichomonas gypaetinii*, determined that the previously reported *T. vaginalis*-like protozoa belonged to this new species (Martínez-Díaz et al., 2015).

Despite this increase in the number of genetic variants and species of avian oropharyngeal trichomonads, there is still poor knowledge about the epizoological implications of these newly identified trichomonads. As a result, the diagnosis based on culture or cytology is turning difficult to interpret. Therefore, due to this increasing diversity and considering that both, parasites and commensal organisms, are found within the Trichomonadidae family, the genetic characterisation of avian trichomonosis outbreaks in the nature is strongly recommended. At least one genetic marker useful for their phylogenetic classification, such as the ribosomal region of the ITS1/5.8S/ITS2 (ITS) should be always included (Felleisen, 1997; Kleina et al., 2004; Sansano-Maestre et al., 2016).

The objectives of this study were to characterise four clonal cultures of different oropharyngeal trichomonads. The isolates, *T. gallinae*, *T. canistomae*-like and *T. tenax*-like strains, were selected according to their difference in the ITS genetic profile previously described in other studies (Grabensteiner et al., 2010; Zimre-Grabensteiner et al., 2011; Martínez-Herrero et al., 2014). A multi-locus sequence typing (MLST) approach was used, including the small subunit of ribosomal rRNA (SSUrRNA) and Fe-hydrogenase (Fe-hyd) genes. Also, a morphometric study by optical microscopy as well as a structural evaluation by scanning electron microscopy (SEM) were performed.

2. Material and methods

2.1. Source of the isolates

Oropharyngeal trichomonads were recovered from four different host species, including raptors and columbiformes with or without lesions of avian trichomonosis (Table 1). Birds with macroscopical lesions were a nestling of Eurasian eagle owl (*Bubo bubo*), clone R17-C1, sampled in the wild in collaboration with a bird ringing scientist in Murcia (Spain) and an adult racing pigeon, clone 7895-C2, hospitalized at the Clinical Unit of Avian Medicine, University of Veterinary Medicine, Vienna (Austria). Birds without lesions were juvenile individuals of an European turtle dove, clone P196-C20, and a wood pigeon (*Columba palumbus*), clone P178-C7, from the wildlife recovery centre of "La Granja de El Saler" (Valencia, Spain).

2.2. Culture of the parasite

Two types of culture medium were used for the primary isolation of the parasite. Five milliliters of Trypticase-Yeast-Maltose (TYM) medium, pH 6.5, in 10 ml sterile tubes were used for the isolates from Spain. For 1 l of TYM medium, the composition was as follows: 20 g of trypticase, 10 g of D(+)-maltose, 10 g of yeast extract, 1 g of L-cysteine and 0.1 g of ascorbic acid (all Sigma-Aldrich, St. Louis, Missouri, USA). Filtration through 0.22 μm filters was used for sterilization (Millipore, Billerica, Massachusetts, USA). The culture medium was enriched with 10% of inactivated fetal bovine serum (FBS), and supplemented with the antibiotics ceftiofur, ticarcillin in combination with vancomycin (36 mg/l each) together with nystatin (24 ml/l, 10,000 IU/ml) as antimycotics (Sigma-Aldrich, St. Louis, Missouri, USA).

For the Austrian isolate, medium 199 (M199) with Earle's salts, L-glutamine, 25 mM of HEPES and L-amino acids (Gibco, Thermo Fisher Scientific, Vienna, Austria) was employed supplemented with 15% of inactivated FBS (Gibco, Thermo Fisher Scientific, Vienna, Austria) and 0.22% of rice starch (Carl-Roth, Karlsruhe, Germany) sterilized by dry heat for 1 h at 180 °C prior to use. In addition, a bacterial culture was added to improve the growth of the protozoan, using 0.5 ml of *Escherichia coli* DH5α-T1 strain previously incubated in agitation at 37 °C for 24 h in 9 ml of M199. The cultures were routinely passaged every 48–72 h. An inoculum of 1000 or 500 μl was transferred into 9 ml of a temperated fresh growth medium (M199) with 0.5 ml of bacterial culture prepared as previously described.

2.3. Preparation of clonal cultures

For the establishment of single cell originated cultures, isolates were transferred to M199 with the bacterial strain of *E. coli* DH5α-T1 until optimal growth was achieved. A passage using 1 ml of culture in 9 ml of M199 without rice starch and bacteria was done 24 h prior to the micromanipulation technique. Single trichomonad cells were isolated and transferred to Eppendorf tubes with 0.5 ml of M199, using a micromanipulation method, following the protocol of Hess et al. (2006). For that, an inverted microscope (Diaphot 300, Nikon, Austria) was used with Narishige micromanipulators (Narishige, Japan). Growth was examined 48 h post inoculation by optical microscopy and subcultivation was done in M199 with *E. coli* DH5α-T1. Cryopreservation for long term storage was performed in a cryo freezing container (Mr. Frosty™, ThermoFisher Scientific, Vienna, Austria) at − 180 °C using 5% of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, Missouri, USA) as cryoprotective.

For DNA extraction, 1 ml of every clonal culture in exponential growth phase was used. Cells were centrifuged at 1200 rpm for 3 min to remove the culture medium and washed with sterile PBS (pH 7.2). Silica-based purification of genomic DNA using columns of a commercial kit was employed following the manufacturer's instructions (DNeasy Blood and Tissue Extraction kit, QIAGEN, Valencia, California, USA).

2.4. Genetic characterisation

A MLST approach including the ITS, SSUrRNA and Fe-hyd regions was used to characterise the selected clonal cultures (Table 1). All reactions contained a final volume of 25 μl of: 12.5 μl HotStarTaq Master Mix Kit polymerase (QIAGEN, Hilden, Germany), 8 μl of PCR water (QIAGEN, Hilden, Germany), 1 μl of each primer at 10 μM and 2.5 μl of genomic DNA. Primers for the ITS region were: TFR1 (5′-TGCTTCAGTTCAGCGGGTCTTCC-3′) and TFR2 (5′-CGGTAGGTGAACCTGCCGTTGG-3′), for the SSUrRNA: Hm-Long-f (5′-AGGAAGCACACTATGGTCATAG-3′) and Hm-Long-r (5′-CGTTACCTTGTTACGACTTCTCCTT-3′) and for the Fe-hyd gene: Fe-hyd-for (5′-GTTTGGGATGGCCTCAGAAT-3′)