PENTATRICHOMONAS HOMINIS COINFECTION IN A PUPPY FROM A SLOVENIAN ANIMAL SHELTER

Maja Brložnik[1], Siniša Faraguna[2], Brigita Slavec[3], Rok Kostanjšek[4], Aleksandra Vergles Rataj[5], Igor Gruntar[5]

[1]PRVA-K, Small Animal Clinic, Gorkičeva 6, Ljubljana

[2]Student of the Veterinary Faculty, Gerbičeva 60, Ljubljana

[3]Institue for Health Care of Poultry, Veterinary Faculty, Gerbičeva 60, Ljubljana

[4]Department of Biology, Biotechnical Faculty, Jamnikarjeva 101, Ljubljana

[5]Institute of Microbiology and Parasitology, Veterinary Faculty, Gerbičeva 60, Ljubljana

Corresponding author:

Dr. Gruntar Igor, DVM, Institute of Microbiology and Parasitology, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1115 Ljubljana, Slovenia.

E-mail: Igor.Gruntar@vf.uni-lj.si. Telephone: +38614779168

ABSTRACT

A 3-month-old dog from a Slovenian animal shelter presented with bloody, soft, foamy and malodorous diarrhea. Clinical examination, hematology and serum biochemistry were unremarkable. Ultrasonography of abdomen showed prominent mesenteric lymph nodes and the presence of echogenic content within the small intestine.

Light microscopy of native smear and wet mount darkfield microscopy examination of the fecal material showed motile trichomonad-like organisms with a particular circular motion. Flotation and SAF method using light microscopy revealed eggs of nematode *Toxocara canis* and protozoan oocysts *Isospora* spp. Trichomonad-like organisms were successfully isolated and cultivated in axenic culture. Light microscopy of Giemsa stained trichomonads allowed for the identification of a number of flagella, and *Pentatrichomonas hominis (P. hominis)* was presumptively diagnosed. The diagnosis was confirmed by the Polymerase Chain Reaction (PCR) followed by DNA sequencing and by the Scanning Electron Microscopy (SEM) of cultured trichomonad isolates. The PCR and sequencing results confirmed a 99% homology of our *P. hominis* isolates with isolates from other studies, originating both from humans and from animals, which suggests that *P. hominis* could have zoonotic potential and could have been transmitted from animals to people via the per-oral route. This is also the first report on *P. hominis* involvement in clinical disease in dogs in Slovenia.

Keywords: *Pentatrichomonas hominis*, isolation, cultivation, PCR, DNA sequencing, scanning electron microscopy

1. INTRODUCTION

Young dogs are commonly infested with different zoonotic intestinal parasites. Conventional canine intestinal parasites of zoonotic importance like *Toxocara*, *Taenia, Ancylostoma*, *Giardia, Cryptosporidium*, etc. have been studied thoroughly, but less is known about zoonotic trichomonad species *Pentatrichomonas hominis (P. hominis)*, formerly *Trichomonas intestinalis* and *Trichomonas hominis* (1). This flagellated protozoan has recently been identified in feces of dogs with diarrhea (2, 3, 4, 5). Pathogenicity of the parasite remains unclear. Due to the lack of evidence of cases where *P. hominis* was the only infecting agent, this trichomonad is presumed to be a commensal organism that may overgrow in patients with other causes of diarrhea. As enteropathogens have always been found in dogs infected by *P. hominis* (6), the pathogenic potential of this trichomonad species has to be further evaluated by experimental infection studies (4). For such trials an axenic P. *hominis* culture of dog origin is needed, which, to the best of our knowledge, has not yet been available. In humans, *P. hominis* has been reported as the causative agent of gastrointestinal disturbances in children (7, 8, 9). Therefore the assessment of the zoonotic potential of canine *P. hominis* is important. It is also necessary to establish whether host-specific genotypes exist as demonstrated among *Tritrichomonas foetus* isolates from cats and cattle (10, 11). To prove this, the characterization of as many isolates as possible from diverse hosts should be performed. A last year study (12), however, suggests that even when using the high-resolution gene locus of the ITS (internal transcribed spacer) regions, all *P. hominis* strains from diverse hosts are genetically identical. This means that zoonotic transmission between humans and mammals may be occurring in the area investigated. Consequently, further research is required to clarify the role *of P. hominis* in human and animal disease. This article provides the first description of *P. hominis* involved in a clinical disease in dogs in Slovenia. The trichomonad was

68 successfully isolated and then cultivated in the axenic culture. The presumptive diagnosis
69 based on light microscopy was confirmed by the SEM and PCR assay.

70 **2. MATERIALS AND METHODS**

71 **Case Description and Sampling**

72 A 3-month old, 9 kg mixed breed dog from a Slovenian animal shelter presented with a
73 bloody, soft, foamy and malodorous diarrhea. Clinical examination, hematology, blood
74 serum biochemistry and abdominal ultrasonography were performed.
75 The fecal sample was analyzed by light microscopy, bacteriological examination, flotation,
76 sedimentation and the SAF (Sodium acetate-acetic acid-formalin solution) method. The
77 isolation of trichomonads followed. SEM and PCR were used for definitive diagnosis.

78 **Isolation of Trichomonads**

79 Trichomonads were isolated using the following procedure: a set of 3 tubes containing
80 Modified Diamond's growth medium (MDM) was inoculated with a loopful (approx. 0.1 g)
81 of fecal sample and incubated at 37°C for seven days (13). In parallel, another set of tubes
82 was inoculated in the same way, this time with MDM supplemented with meropenem (6
83 µg/ml; MeMDM), to provide additional prevention against bacterial contamination (14).
84 The inoculated tubes were checked for trichomonad growth at intervals during this
85 period. An aliquot was taken from the bottom of the tube and wet mount-examined by
86 darkfield microscopy. When motile flagellates were observed, an aliquot (0.1 ml) of fresh
87 culture was transferred to fresh MDMs/MeMDMs.

88 **Staining of Isolated Trichomonads**

89 Thin smears of cultivated trichomonad suspension were air-dried, fixed and stained with
90 Giemsa, trichrome and methylene blue stain.

91

Scanning Electron Microscopy

For scanning electron microscopy, the trichomonads in the cultivation suspension were washed with Phosphate Buffered Saline (PBS) by centrifugation at 100 g for 5 min before overnight fixation in the combination of 1% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C. Fixed cells were transferred to a pre-cleaned cover slides, washed by PBS and postfixed in 2% OsO_4 for one hour at 4°C. After being washed in deionized water, the cells on the slides were dehydrated in a graded series of ethanols, then dried by hexamethyldisilazane (HMDS), mounted on aluminum stubs and coated with platinum, as described above (15). The samples were examined with the JEOL JSM-7500F field emission scanning electron microscope.

Molecular Diagnosis

For the molecular detection of trichomonads, two hundred microliters of protozoal culture suspension were used. Total DNA was extracted using the QIAamp® Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions for Blood and body fluid spin protocol. Finally, the DNA was eluted with 100 µl of AE buffer and stored at -20 °C until examination. Specific pairs of primers TFR1 and TFR2 amplifying the 350 bp long ITS1-5.8S-ITS2 region were used (16). PCR was performed in a total volume of 20 µl containing 10 µl of 2X Thermo Scientific DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, CA, USA), 0.8 µl of each primer (0.4 µM), 6.4 µl of nuclease-free water and 2 µl of extracted DNA. The reaction was performed on an ABI 2720 Thermo Cycler (Applied Biosystems, Foster City, CA, USA). The cycling profile included initial denaturation at 95°C for 5 min, which was followed by 40 cycles of heat denaturation at 94°C for 30 s, oligonucleotide annealing at 55°C for 1 min, oligonucleotide extension at 72°C for 1 min, and final oligonucleotide extension step at 72°C for 10 min. The extracted DNA of *Trichomonas gallinae* was used as positive control in the PCR assay.

The PCR products were analyzed by electrophoresis on a 1.8% ethidium bromide stained agarose gel. DNA fragments were excised from the gel and, after being purified with Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA), sent for sequencing to Macrogen laboratory (Macrogen Inc, Amsterdam, the Netherlands).

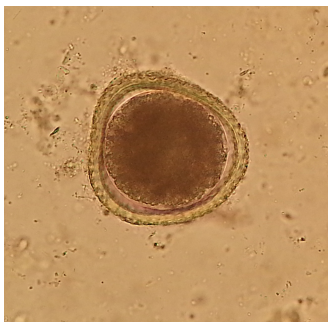
The nucleotide sequences were downloaded using Chromas software (Technelysium Pty Ltd., Queensland, Australia), and the nucleotide sequence data were analyzed by BLAST (17) to find similar sequences in Genbank NCBI sequence database.

3. RESULTS

Clinical examination of the diarrheic 3-month old mixed breed dog was unremarkable. Hematology and serum biochemistry did not reveal any abnormalities Ultrasonography of abdomen showed prominent mesenteric lymph nodes and the presence of echogenic content within the small intestine.

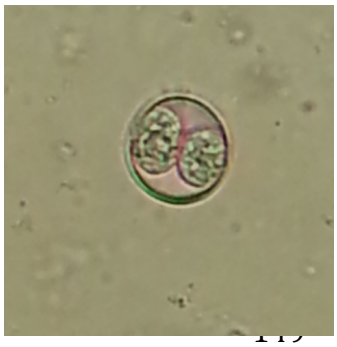
Light microscopy of the native fecal smear and the darkfield microscopy (wet mount examination) of the diarrheic material showed motile trichomonad-like organisms with a particular circular motion. Flotation and SAF method using light microscopy revealed *Toxocara canis* nematode eggs (Figure 1), *Isospora* sp. oocysts (Figure 2) and the above-mentioned trichomonad species. Bacteriological analyses of fecal samples were negative for the aerobic and anaerobic bacteria. Trichomonad isolation and cultivation attempts were successful. Numerous motile trichomonads were observed after seven days incubation in MDM (xenic culture; with other organisms - bacteria - present). In MeMDM, the trichomonads were less abundant but other organisms were absent (axenic culture). The cultures were stored using 10% dimethylsulphoxide (18). The isolated trichomonads were stained with Giemsa, trichrome and methylene blue stain. When examined by light microscopy, the Giemsa stain improved the visibility and enabled the enumeration of flagella (Figure 3).

142 Figure 1: *Toxocara canis* egg, size 90 x 80 µm, flotation method, x400.



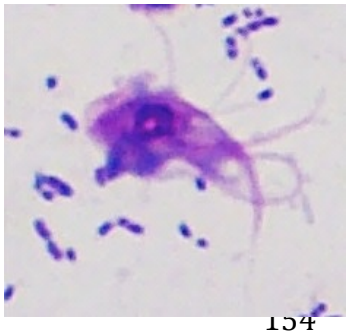
143

144 Figure 2: *Isospora* sp. oocyst, size 23 x 18 µm, flotation method, x400.



149

150 Figure 3: *Pentatrichomonas hominis* trophozoite, Giemsa, x1000.

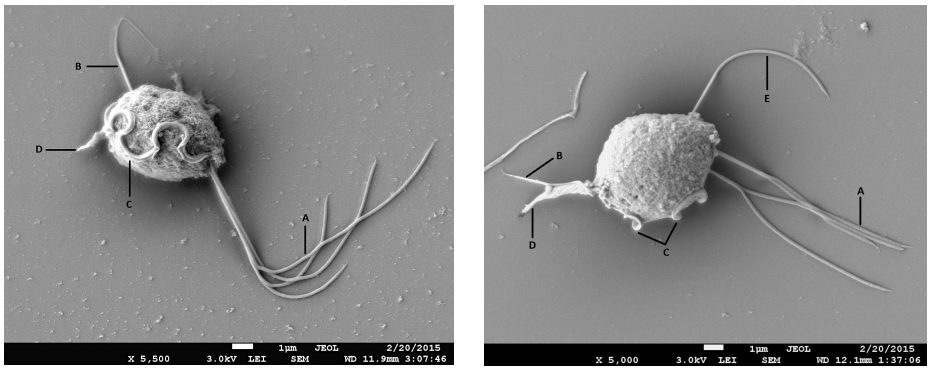


154

155 SEM and PCR assay confirmed the diagnosis of *P. hominis.* SEM observations revealed the
156 presence of five flagella in the anterior part of the trichomonad (four flagella in a group
157 and a single independent flagellum) and one in the posterior part (Figure 4). The latter run
158 from the anterior part, alongside the cell in a posterior direction, forming a distinct
159 undulating membrane displaying three undulations. It ended freely at its distal end. The
160 axostyle was observed as a discrete tip in the posterior region of the cell.
161 With PCR and sequencing, high similarity of obtained sequence with *P. hominis* was
162 confirmed. The comparison of the 300 bp long sequence of the complete ITS1-5.8S-ITS2

gene had 100% homology with *P. hominis* isolate from empyema thoracis (Accession No. AF156964), and 99% homologies with the *P. hominis* isolates from a human (Acc. No. JN007007) and a dog (Acc. No. KJ404270).

Figure 4: *Pentatrichomonas hominis* trophozoite, A – anterior flagella, B – posterior flagellum, *C* – undulating membrane, *D* – axostyle, E – independent flagellum, SEM x 550.



The dog was treated with metronidazole 200 mg/12 h p/o for 5 days and Dehinel plus® (febantel 150 mg, pyrantel embonate 144 mg, praziquantel 50 mg) 1 tbl/day for 5 days. Dog's condition improved immediately, and the fecal exam was negative two weeks after the treatment. Since then, the dog has been asymptomatic for one year.

4. DISCUSSION

P. hominis, a flagellated protozoan of the order *Trichomonadida*, inhabits the large intestine of many mammalian hosts, including humans (6, 7, 8, 9, 19). Its prevalence is low in developed countries (20), but gets much higher in subtropical and tropical zones (21). In dogs, the prevalence of trichomonad infection in a study analyzing 215 puppies from French kennels was 15.8% (4). This study also reports that *P. hominis* was the only trichomonad infecting the studied canine population, whereas some older papers suggest that *P. hominis* was far more frequent than *Tritrichomonas foetus* in diarrheic dogs suffering from trichomonosis (2, 3, 6).

In a majority of studies (2, 3, 4), the reported age of dogs with trichomonosis ranged from 7 weeks to 6 months which is consistent with the age of the 3-month-old dog in our case. A

187 more recent study reported that trichomonosis was diagnosed also in 10-year-old dogs
188 (6).
189 Differential diagnoses for a bloody, soft, foamy and malodorous diarrhea are dietary
190 intolerance, infection, partial obstruction, motility disorders, inflammatory/immune-
191 mediated disease, drugs/toxins, idiopathic disease, neoplasia and extra-gastrointestinal
192 disease (22). According to the age of our dog, clinical signs and the results of the clinical
193 investigation, infection was considered the most likely cause. Negative results of
194 bacteriological analyses led to a conclusion that infestation with *P. hominis, Isospora* sp.
195 and *T. canis* was responsible for the symptoms.
196 Microscopical observations of trichomonad-like organisms in native sample helped to
197 make a presumptive diagnosis of trichomonad infection. Microscopy, however, cannot be
198 used to identify the trichomonad species in native samples (23, 24, 25). Flagella are not
199 well discernable in such preparations, but their visibility improves with the use of stains,
200 thus supporting the species differentiation.
201 Trichomonad species can accurately be determined by SEM: our isolate was characterized
202 as a pear-shaped organism with distinctive undulating membrane, an axostyle, a single
203 posterior (recurrent) flagellum and five anterior flagella, comprising the group of four
204 flagella of unequal length and a single independent flagellum. The undulating membrane
205 displayed three undulations. An axostyle was an elongated rod-like structure projecting
206 out of the posterior end to form a pointed spine. The axostyle is an important criterion for
207 distinguishing tritrichomonads from pentatrichomonads (5, 26). In our study, the axostyle
208 of the trichomonad had a relatively heavy terminal segment and was observed as a
209 discrete tip in the posterior region of the cell. By contrast, the axostyle of *T. foetus* has a
210 short conical projection with a small spherical structure at the end (27). Furthermore, it
211 has only 3 anterior flagella. The long undulating membrane is also suggestive of *P. hominis*

because the other trichomonads have a shorter undulating membrane (27).

For the detection of *P. hominis* in biological specimens, a highly specific and sensitive PCR assay can be used (28, 29, 30). In our case, the PCR assay for amplifying of the 5.8S rRNA gene and two flanking internal transcribed spacer regions ITS1 and ITS2 of trichomonads was used. This region is one of the mostly used regions for taxonomic classification of trichomonads (16). Our results confirmed high homology of this isolate with the previously described genetic loci of *P. hominis* from various mammalian hosts (human, dogs, cattle, pigs, cats, goats, water buffaloes), suggesting a low genetic diversity in *P. hominis* isolates and a very broad host range for this species (5, 12, 28, 29, 30).

Therefore, it is to be expected that *P. hominis* strains might be circulating between diverse identified hosts, including humans, where they, under certain circumstances, cause clinical disease (7, 8, 9) or exacerbate symptoms of an existing illness (31). To elucidate actual role and pathogenicity of *P. hominis* in human and animal disease, further research is needed.

5. CONCLUSION

Routine coprological examinations are essential to make a diagnosis in a dog with diarrhea. In the case of trichomonads, SEM or PCR assays are required for the definite diagnosis of trichomonad species. Molecular diagnostic data of trichomonads suggest that *P. hominis* is a zoonotic species with a potential of transmission via the per-oral route from animals to people and vice versa.

Acknowledgments

The authors of this study acknowledge the assistance of Mrs. Darja Faraguna for proofreading. Furthermore, a great thanks goes to the dog and his owner who entrusted us with diagnostics and treatment.

REFERENCES:

1. Saunders Comprehensive Veterinary Dictionary, 3rd ed. St. Louis: Elsevier, 2007. http://medical-

dictionary.thefreedictionary.com/Pentatrichomonas+hominis+(syn.+Trichomonas+hominis%2c+T.+intestinalis) (14.10.2015)

2. Gookin JL, Stauffer SH, Coccaro MR, Marcotte MJ, Levy MG. Optimization of a species-specific polymerase chain reaction assay for identification of *Pentatrichomonas hominis* in canine fecal specimens. Am J Vet Res 2007; 68: 783–7.
3. Kim YA, Kim HY, Cho SH, Cheun HI, Yu JR, Lee SE. PCR detection and molecular characterization of *Pentatrichomonas hominis* from feces of dogs with diarrhea in the Republic of Korea. Korean J Parasitol 2010; 48: 9–13.
4. Grellet A, Polack B, Feugier A, et al. Prevalence, risk factors of infection and molecular characterization of trichomonads in puppies from French breeding kennels. Vet Parasitol 2013; 197: 418–26.
5. Li WC, Gong PT, Ying M, Li JH, et al. *Pentatrichomonas hominis:* first isolation from the feces of a dog with diarrhea in China. Parasitol Res 2014; 113: 1795–1801.
6. Tolbert MK, Leutenegger CM, Lobetti R, Birrell J, Gookin JL. Species identification of trichomonads and associated coinfections in dogs with diarrhea and suspected trichomonosis. Vet Parasitol 2012; 187: 319–22.
7. Yang CR, Meng ZD, Wang X, Li YL, Zhang YX, Zhao QP. Diarrhoea surveillance in children aged under 5 years in a rural area of Hebei Province, China. J Diarrhoeal Dis Res 1990; 8: 155–9.
8. Chunge RN, Nagelkerke N, Karumba PN, et al. Longitudinal study of young children in Kenya: intestinal parasitic infection with special reference to *Giardia lamblia*, its prevalence, incidence and duration, and its association with diarrhoe and with other parasites. Acta Trop 1991; 50: 39–49.
9. Meloni D, Mantini C, Goustille J, et al. Molecular indentification of *Pentatrichomonas hominis* in two patients with gastrointestinal symptoms. J Clin Pathol 2011; 64: 933–5.
10. Šlapeta J, Craig S, McDonell D, Emery D. *Tritrichomonas foetus* from domestic cats and cattle are genetically distinct. Exp Parasitol 2010; 126: 209–13.
11. Reinmann K, Müller N, Kuhnert P, et al. *Tritrichomonas foetus* isolates from cats and cattle show minor genetic differences in unrelated loci ITS-2 and EF-1*α*. Vet Parasitol 2012; 185: 138–44.
12. Kamaruddin M, Tokoro M, Rahman M, et al. Molecular characterization of various trichomonad species isolated from humans and related mammals in Indonesia. Korean J Parasitol 2014; 52: 471–8.
13. OIE Manual of diagnostic tests and vaccines for terrestrial animals, 2012. http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/2.04.17_TRICHOMONOSIS.pdf (14.10.2015).
14. Amin A, Neubauer C, Liebhart D, Grabensteiner E, Hess M. Axenization and optimization of in vitro growth of clonal cultures of *Tetratrichomonas gallinarum* and *Trichomonas gallinae*. Exp Parasitol 2010; 124: 202–8.

15. Kostanjšek R, Pirc-Marolt T. Pathogenesis, tissue distribution and host response to *Rhabdochlamydia porcellionis* infection in rough woodlouse *Porcellio scaber*. J Invertebr Pathol 2015; 125: 56-67.
16. Felleisen RS. Comparative sequence analysis of 5.8S rRNA genes and internal transcribed spacer (ITS) regions of trichomonadid protozoa. Parasitol 1997; 115: 111–9.
17. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990; 215: 403–10.
18. Miyake Y, Karanis P, Uga S. Cryopreservation of protozoan parasites. Cryobiology 2004; 48: 1–7.
19. Gookin JL, Stauffer SH, Levy MG. Identification of *Pentatrichomonas hominis* in feline fecal samples by polymerase chain reaction assay. Vet Parasitol 2007; 145: 11–5.
20. Ackers JP. Trichomonads. In: Gillespie SH, Pearson RD, eds. Principles and Practice of Clinical Parasitology. Chichester: John Wiley & Sons, 2001: 258–60.
21. Saksirisampant W, Nuchprayoon S, Wiwanitkit V, Yenthakam S, Ampavasiri A. Intestinal parasitic infestations among children in an orphanage in Pathum Thani province. J Med Assoc Thai 2003; 86: 263-70.
22. Gough A. Differential diagnosis in small animal medicine. Oxford: Blackwell, 2007: 26–31.
23. Romatowski J. *Pentatrichomonas hominis* infection in four kittens. J Am Vet Med Assoc 2000; 216: 1270–2.
24. Gookin JL. Trichomoniasis. In: Greene CE, ed. Infectious diseases of the dog and cat, 4th ed. St. Luis: Saunders, 2012: 797–801.
25. Rivera WL, Lupisan AJ, Baking JM. Ultrastructural study of a tetratrichomonad isolated from pig fecal samples. Parasitol Res 2008; 103: 1311–6.
26. Levy MG, Gookin JL, Poore M, Birkenheuer AJ, Dykstra MJ, Litaker RW. *Tritrichomonas foetus* and not *Pentatrichomonas hominis* is the etiologic agent of feline trichomonal diarrhea. J Parasitol 2003; 89: 99–104.
27. Warton A, Honigberg BM. Structure of trichomonads as revealed by scanning electron microscopy. J Protozool 1979; 26: 56–62.
28. Crucitti T, Abdellati S, Ross DA, Changalucha J, van Dyck E, Buve A. Detection of *Pentatrichomonas hominis* DNA in biological specimens by PCR. Lett Appl Microbiol 2004; 38: 510–6.
29. Gookin JL, Birkenheuer AJ, St John V, Spector M, Levy MG. Molecular characterization of trichomonads from feces of dogs with diarrhea. J Parasitol 2005; 91: 939–43.
30. Mostegl MM, Wetscher A, Richter B, Nedorost N, Dinhopl N, Weissenböck H. Detection of *Tritrichomonas foetus* and *Pentatrichomonas hominis* in intestinal tissue specimens of cats by chromogenic in situ hybridization. Vet Parasitol 2012; 183: 209–14.
31. Jongwutiwes S, Silachamroon U, Putaporntip C. *Pentatrichomonas hominis* in empyema thoracis. Trans R Soc Trop Med Hyg 2000; 94: 185–6.