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Prevalence, risk factors of infection and molecular characterization of trichomonads in puppies from French breeding kennels

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ABSTRACT

The trichomonad species *Tritrichomonas fetus* and *Pentatrichomonas hominis* were recently identified in the feces of dogs with diarrhea. However the prevalence and pathogenicity of these parasites in the canine population still remained poorly resolved. Therefore the aim of the present study was (1) to determine the prevalence of trichomonads infecting puppies living in French breeding kennels, (2) to confirm the predominance of *P. hominis* in dogs, (3) to investigate the genetic diversity of *P. hominis* isolates identified in the French canine population and (4) to evaluate the risk factors for infection by *P. hominis* and the influence of the parasite on feces consistency. A total of 215 both diarrheic and non-diarrheic puppies from 25 French breeding kennels were included in this epidemiological survey. Fecal samples from each puppy were examined for 6 gastrointestinal pathogens: parvovirus type 2 (CPV2), coronavirus, *Toxocara canis*, *Cystoisospora ohioensis*-complex, *Cystoisospora canis*, and *Giardia intestinalis*. A part of each collected stool was also tested for the presence of motile trichomonads by microscopy after culturing. The prevalence of trichomonad infection was 15.8% (34/215) among puppies and 20% (5/25) among breeding kennels. DNA from 26 of the 34 positive samples was successfully amplified using a trichomonad-specific primer pair. Analysis of the sequences of PCR products indicated that *P. hominis* was the only trichomonad infecting the canine population. All the puppies infected with *P. hominis* belonged to large breed dogs. Moreover, puppies from large breeding kennels, excreting a high level of *G. intestinalis* and/or excreting a high level of *C. canis* oocysts showed a higher probability of being positive for *P. hominis* infection. Univariate analysis also revealed an increased risk for *P. hominis* infection in puppies with abnormal feces. However, in a multivariate analysis, CPV2 was the only gastrointestinal pathogen associated with abnormal feces. Since enteropathogens were commonly found in dogs infected by *P. hominis*, the pathogenic potential of this trichomonad species remained uncertain and has to be further evaluated by experimental infection studies.

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1. Introduction

Most of parabasalid protists belonging to both classes Trichomonadea (including *Trichomonas*, *Tetratrichomonas*, and *Pentatrichomonas* species) and Tritrichomonadea (including the genus *Tritrichomonas*) and commonly known as trichomonads (Cepicka et al., 2010), are typified by the presence of a well-developed cytoskeleton consisting of three to five anterior flagella, a recurrent flagellum associated with the undulating membrane varying in degree of development and length, two microtubular sheets (axostyle and pelta), and striated fibers (parabasal fibers and costa) (Brugerolle and Lee, 2001). Most of these anaerobic microorganisms are frequently found in the digestive or reproductive systems of humans and a wide range of animals including pets. With the main exceptions of *Trichomonas vaginalis* and *Tritrichomonas fetus* which are the known etiologic agents of human and bovine trichomonosis, respectively, trichomonad species are generally regarded as commensal organisms in mammals (Honigberg, 1978). However, *T. fetus* was also recognized as a primary agent of feline trichomonosis causing large-bowel diarrhea (Gookin et al., 1999, 2001; Levy et al., 2003; Bissett et al., 2008; Profizi et al., 2013) and trichomonads were occasionally observed in the feces of dogs with diarrhea (Brumpt, 1925; Simic, 1932a,b; O'Donnell, 1954; Burrows and William, 1967; SreemanNarayana, 1976; Lopez et al., 2006). Trichomonad species observed in fecal samples of dogs were identified as *T. fetus* and *Pentatrichomonas hominis* using molecular tools (Gookin et al., 2005) but their respective prevalence and pathogenicity in the canine population is poorly understood. Recent surveys including only a limited number of animals suggested that *P. hominis* was more frequent than *T. fetus* in diarrheic dogs with trichomonosis (Gookin et al., 2007; Kim et al., 2010; Tolbert et al., 2012). In parallel, concurrent enteropathogens were found to be common in the population of dogs with *P. hominis* infection and might therefore be responsible for their diarrhea (Tolbert et al., 2012). Several authors described *P. hominis* as the probable causative agent of gastrointestinal disturbances in children (Yang et al., 1990; Chunge et al., 1991; Meloni et al., 2011). This parasite has also been found occasionally outside its natural habitat in patients with liver abscess (Jalkobson et al., 1987) or empyema thoracis (Jongwutiwes et al., 2000). Recently Meloni et al. (2011) suggested that the same *P. hominis* species could possibly colonize the gastrointestinal tract of many mammal species, including dogs, bovid, rats, monkeys, and humans. Consequently, this raised the question of the zoonotic potential of *P. hominis* found in dogs and of the existence of host-specific genotypes as shown amongst *T. fetus* isolates from cats and cattle (Slapeta et al., 2010; Reinmann et al., 2012).

Despite the potential significance of *P. hominis* as a pathogen in mammals, no epidemiological study of this parasite in dogs has been published so far in France. Therefore the aim of the present study was (1) to determine the prevalence of trichomonads infecting puppies living in French breeding kennels, (2) to confirm the predominance of *P. hominis* in dogs, (3) to investigate the genetic diversity of *P. hominis* isolates identified in the French canine population and (4) to evaluate the risk factors for infection by *P. hominis* and the influence of the parasite on feces consistency.

2. Material and methods

2.1. Study population and data collection

A total of 215 puppies from 25 breeding kennels located in the North of France were included in this study and sampled between May and September 2009. A standardized questionnaire was designed to collect information about each participating puppy concerning age, breed, and size of the kennel. According to the expected mean adult body weight of each breed of dog, puppies were divided into two groups: small breed dogs (breed with a mean adult body weight <25 kg) and large breed dogs (breed with a mean adult body weight >25 kg). For data analysis, kennels were also divided into three groups depending on their size: small size kennels (i.e. kennels producing 10 puppies per year or less), medium size kennels (i.e. kennels producing between 10 and 50 puppies per year) and large size kennels (i.e. kennels producing 50 puppies per year or more).

2.2. Evaluation of feces consistency

For each puppy, fecal consistency was evaluated by a single operator (AG) using a 13-point scale, based on the texture and shape of the feces (from liquid to hard and dry) (Grellet et al., 2012). Feces were classified as abnormal according to Grellet et al. (2012). Briefly, a fecal score $\leq$5 was considered abnormal for large breed puppies. For small breed puppies, fecal scores $\leq$6 and $\leq$7 for 4–5 weeks old puppies and older puppies, respectively, were classified as poor. A rectal swab was performed for each puppy immediately after the collection of stools for detection of canine parvovirus type 2 (CPV2) and canine coronavirus (CCV). The swab was stored at −20 °C until DNA extraction.

2.3. CCV and CPV2 detection

CCV and CPV2 excretion was evaluated using molecular methods as already described (Grellet et al., 2012). Briefly, total nucleic acids were extracted from the rectal swabs using the Nucleospin Extract II kit (Macherey Nagel, Hoerdt, France) as recommended by the supplier and eluted in 400 μl of NE buffer. CPV2 and CCV detection was performed by real-time polymerase chain reaction (PCR) and reverse-transcriptase PCR (RT-PCR), respectively, with a 7900 HT sequence detection system (Applied Biosystems, Villebon sur Yvette, France). Five microliters of eluted DNA was used for both real-time PCR and RT-PCR and the absence of PCR inhibitors was checked for each DNA template. The detection threshold of CPV2 by real-time PCR (i.e. the lower number of genomic copies detected in 95% of runs) was 4 copies of genomic DNA. The quantification threshold was 200 copies. These values were determined by Probit analysis as recommended by the European Pharmacopeia for PCR assays assessment. The detection and quantification thresholds of CVV by RT-PCR were 36 and 200 copies of genomic RNA, respectively. Results from duplicate

analyses (mean of the two results) were expressed semi-quantitatively as viral load levels. CPV2 and CCV loads were defined as low under $10^{5.7}$ copies and $10^{5.3}$ copies (Grellet et al., 2012).

2.4. Trichomonad detection and molecular identification

Detection of trichomonads was performed by culturing of fecal samples using the commercially available system InPouch™ TF test (BioMed Diagnostics, Oregon, USA) as already described by Gookin et al. (2003). Approximately 0.1 g of feces was collected and immediately inoculated into the InPouch™ TF bags. The sealed pouches were incubated in an upright position in the dark at room temperature. Cultured samples were examined 48 h post inoculation for the presence of motile trichomonads under the microscope at 100× magnification. Subsequently, microscopic evaluation was performed daily by the same evaluator for 11 days. Observation of ≥1 motile trichomonad was considered a positive result. After 11 days, all pouches were stored at −20 °C until DNA extraction. In order to analyze the genetic diversity of trichomonad isolates identified in this study, DNA from culture positive samples was amplified using the trichomonad-specific sense primer TRICHO-F and antisense primer TRICHO-R targeting the ITS1-5.8S rRNA-ITS2 region as previously described (Jongwutiwes et al., 2000; Duboucher et al., 2006). Briefly, PCR was carried out for 40 cycles with a 50 μl volume according to the standard conditions for Platinum *Taq* high-fidelity DNA polymerase (Invitrogen, Groningen, The Netherlands). Negative and positive controls were included in the series. The PCR product was separated by agarose gel electrophoresis, purified, and sequenced on both strands. The nucleotide sequences obtained in this study have been deposited in GenBank under accession numbers KC623939 to KC623941. These sequences were aligned with all of the trichomonad ITS1-5.8S rRNA-ITS2 sequences available in databases using the BioEdit v7.0.1 package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

2.5. Intestinal parasites detection

A part of each collected stool was examined macroscopically and stored at 4 °C prior to be processed by the standard McMaster flotation technique using saturated magnesium sulphate solution (density: 1.28 g/ml) (Bauer et al., 2010). All eggs and oocysts of parasites detected by this quantitative method were identified according to morphological characteristics under light microscopy by a single operator (BP) (Baek et al., 1993; Levine and Ivens, 1965). For the analysis of the helminth *Toxocara canis*, three levels of excretion were defined: negative (no excretion of eggs observed), low level of excretion (puppies excreting less than 1000 eggs/g of feces) and high level of excretion (puppies excreting more than 1000 eggs/g of feces). Three levels of excretion were also defined for the coccidia *Cystoisospora canis* and *Cystoisospora ohioensis*-complex (Barta et al., 2005): negative (no excretion of oocysts observed), low level (puppies excreting less than 5000 oocysts/g of feces) and high level (puppies excreting more than 5000 oocysts/g of feces). Each fecal sample was also tested for diagnosis of copro-antigens of the diplomonad protozoan *Giardia intestinalis* using the ProSpecT-*Giardia* Microplate Assay (Remel, France) (Decock et al., 2003; Mekaru et al., 2007; Rimhanen-Finne et al., 2007). At the time of analysis, the stored fecal samples and reagent diluents were brought to room temperature and 100 mg of feces was added to 1 ml of diluent. After homogenization, samples were centrifuged 5 min at 500 g, the supernatant was collected and analyzed according to the manufacturer's recommendations. Negative and positive controls provided with the kit were performed with each assay. The optical density (OD) of each sample was measured by spectrometry at 450 nm and was interpreted according to the manufacturer's instructions (i.e., OD values >0.05 were considered positive).

2.6. Statistical analysis

Statistical analyses were performed with the SAS version 9.3 software (SAS Institute Inc., Cary, NC, USA). The statistical procedure SAS PROC MIXED was used to fit a hierarchical linear model as already described (Singer, 1998). The influence of several factors on trichomonad infectious status was assessed. This model included 9 fixed effects: size of the breeding kennel, age of puppy, dog size as well as CPV2, CCV, *T. canis*, *C. canis*, *C. ohioensis*-complex, and *G. intestinalis* infection. As data on puppies were nested within naturally occurring hierarchies (puppies within litter, litters within breeding kennels), litter variable nested within breeding kennel, written as litter (breeding kennel), was defined as a random term. Trichomonad infectious status was encoded as 0 (uninfected) or 1 (infected). For each factor and each level, coefficient estimation was evaluated. A positive coefficient indicate an increased risk of becoming "infected" and reversely.

In parallel, Chi square test was used in a first step to assess univariate associations between trichomonad infection and abnormal feces. In a second step the statistical procedure SAS PROC MIXED was used to evaluate the influence of several factors on fecal score. This model included 7 fixed effects: size of the breeding kennel, CPV2, CCV, *T. canis*, *C. canis*, *C. ohioensis*-complex, *G. Intestinalis* and trichomonad infection. Data on puppies were nested within naturally occurring hierarchies as described above. Feces consistency was encoded as 0 (normal) or 1 (abnormal).

3. Results

3.1. Characteristics of the sample population

Freshly voided fecal specimens and completed questionnaires were collected from 215 puppies representing 25 French breeding kennels. The mean number of samples collected in each kennel was 9 (range, 2–18) and the age of dogs ranged from 5 to 14 weeks (median age, 8 weeks). 156 puppies (72.6%) belonged to large size breeds and 100 puppies (46.5%) were from breeding kennels producing less than 30 puppies per year. Abnormal feces were collected from 27.9% (60/125) of the puppies.

3.2. Prevalence of trichomonad infection and molecular identification

Among the 215 canine fecal samples included in this study, 34 were positive by culturing. By using this method of detection, the overall prevalence of trichomonad infection in our canine population was 15.8% (34/215) among puppies and 20% (5/25) among breeding kennels (Table 1). DNA from the 34 positive samples by culturing was subsequently amplified using trichomonad-specific primers. Among these DNA samples, 8 of them were not amplified by PCR due probably either to the presence of PCR inhibitors in fecal samples or the low number of parasites observed in some InPouch-TF bags. The PCR products of the remaining 26 isolates were purified and both strands sequenced and exhibited 99.3–100% identity (Fig. 1). The sequences obtained from these samples were highly similar since they only differed at 2 variable positions and were classified into three types of sequences called types 1, 2, and 3. Types 1, 2 and 3 included sequences obtained from 18, 7, and 1 of our 26 canine samples, respectively. In the common part of our alignment (309 positions), the types 1, 2 and 3 sequences also exhibited 98.5 to 100% identity to homologous sequences from *P. hominis* strains available from different hosts including human, bovid, pigs, and dogs (Fig. 1). These results unequivocally identified trichomonads found in the feces of dogs as *P. hominis*. In addition, even if the *P. hominis* sequences available from different hosts differed by 17 single nucleotide polymorphisms (SNPs) mainly in the ITS1 and 5.8S rRNA regions (Fig. 1), none of these SNPs was only shared by isolates from the same host.

3.3. Factors associated with the prevalence of P. hominis infection

In our canine population, all the puppies infected with *P. hominis* were classified into the large breed dogs group (Table 1). From our statistical analyses, *P. hominis* infection was significantly associated with 3 factors: the size of the kennel ($P = 0.04$), *G. intestinalis* infection ($P = 0.014$), and *C. canis* infection ($P = 0.002$) (Tables 1 and 2). Puppies from large breeding kennels showed a higher probability of being positive for *P. hominis* than puppies from small breeding kennels ($P = 0.012$) (Tables 1 and 2). A significant higher prevalence of *P. hominis* was also observed in puppies excreting a high level of *G. intestinalis* than puppies excreting a low level of *G. intestinalis* or not infected by this protozoa (Tables 1 and 2). Puppies excreting a high level of *C. canis* oocysts also presented a higher prevalence of *P. hominis* than puppies not excreting *C. canis* (Tables 1 and 2).

3.4. Feces quality and trichomonad infection

The univariate test (Chi square test) showed that puppies infected by *P. hominis* presented significantly more abnormal feces than puppies not infected by this parasite (27% (16/60) vs. 12% (18/155); $P = 0.007$). However this association between *P. hominis* infection and abnormal feces was not found in the multivariate risk factor analysis ($P = 0.402$) (Table 3). Only CPV2 excretion was significantly associated with an abnormal fecal score (Table 3). Indeed, puppies excreting medium to high viral load of CPV2 presented a significantly higher frequency of abnormal feces than negative puppies or puppies with a low excretion ($P = 0.011$) (Table 3).

4. Discussion

To our knowledge, the present study represents the first investigation of the prevalence of trichomonads in the French canine population as well as in a canine population of both diarrheic and non-diarrheic animals. From our data, 15.8% (34/215) of examined puppies and 20% (5/25) of breeding kennels tested positive for trichomonad infection by culturing. The InPouch^{TM} TF was described as a culture system for sensitive and specific culture of *T. fetus* from animal feces (Gookin et al., 2003). According to these authors, neither *G. intestinalis* nor *P. hominis* would be able to survive in this culture system. However Ceplecha et al. (2013) successfully cultivated *P. hominis* in the InPouch^{TM} TF medium after inoculation with cat feces. Accordingly, as *P. hominis* is hardly distinguishable from other trichomonads such as *T. fetus* on the basis of light microscopic observation (Brugerolle and Lee, 2001; Dufernez et al., 2007), molecular identification by means of genes amplification followed by sequencing was used in the present study to unequivocal differentiate trichomonad species found by culturing in dogs feces and prevent misdiagnosis. In our study, *P. hominis* was the only trichomonad species identified in the InPouch^{TM} TF system using molecular tools. Consequently, this study confirms that *P. hominis*, as *T. fetus*, can survive in this culture system.

Using a trichomonad-specific primer pair, DNAs from 26 of the 34 positive samples by culturing were amplified and sequenced. The sequences of the PCR products showed only two variable positions allowing the identification of three types of sequences called types 1 to 3. This low number of differences between PCR products suggested that they were all derived from the same strain and was likely due to the expected variation within the multiple copies of the ITS and RNA genes in any given genome. These sequences showed a high degree of similarity (98.5–100%) to sequences of *P. hominis* strains from different hosts including human, cattle, pigs, and dogs indicating that they all belonged to the same species. Thereby, our data reinforced the hypothesis that the same *P. hominis* species could be able to colonize the digestive tract of several mammal hosts (Meloni et al., 2011). Besides, even if the identification of the trichomonad species cannot be definitely confirmed, Simic (1932b) demonstrated that canine strains of the so-called *Trichomonas intestinalis* (synonym of *P.hominis* according to Honigberg, 1963) were able to infect humans after oral ingestion of the parasite. In addition the same trichomonads found in the feces of these human, were able to reinfect dogs. Accordingly, since *P. hominis* has been frequently identified in a wide range of animals including pets, this parasite may represent a significant zoonotic source of infection for humans. Interestingly, our sequence alignment revealed 17 variable positions or SNPs between *P. hominis* sequences from different hosts. Nevertheless, none of these SNPs was specific to a taxonomic group of hosts as is the case for the parasite *T. fetus*.

Table 1
Characteristics of puppies with *Pentatrichomonas hominis* infection ($n = 215$).

Variable	Levels % (ni/n)[a]		
Puppy breed size	Small breed dogs 21.8 (34/156)	Large breed dogs 0 (0/59)	
Age	<8 weeks of age 17.4 (19/109)	≥8 weeks of age 14.2 (15/106)	
Kennel size	Small 0 (0/42)	Medium 2.4 (2/84)	Large 36 (32/89)
Canine coronavirus	Negative 13.6 (14/103)	Low 12.5 (11/88)	High 37.5 (9/24)
Canine parvovirus type 2	Negative 1.1 (1/90)	Low 19.7 (14/71)	High 35.2 (19/54)
Toxocara canis	Negative 14.4 (25/174)	Low 31.8 (7/22)	High 10.5 (2/19)
Cystoisospora ohioensis-complex	Negative 21 (30/143)	Low 6.1 (2/33)	High 5.1 (2/39)
Cystoisospora canis	Negative 6.4 (12/187)	Low 69.2 (9/13)	High 88.7 (13/15)
Giardia intestinalis	Negative 4.7 (6/128)	Low 18.8 (6/32)	High 40 (22/55)

[a] ni/n = number of puppies infected by trichomonads/total number of puppies in the category considered.

Indeed, a single SNP located in the ITS2 locus was able to discriminate bovine isolates of *T. fetus* from feline isolates (Slapeta et al., 2010; Reinmann et al., 2012).

Although *P. hominis* was already described in dogs feces (Gookin et al., 2005; Kim et al., 2010; Tolbert et al., 2012), no epidemiological data was currently available in the literature regarding the prevalence of *P. hominis* in a large population of both diarrheic and non-diarrheic dogs. To date, the prevalence of *P. hominis* was only determined in two small populations of 14 (Kim et al., 2010) and 38 diarrheic dogs (Tolbert et al., 2012). In these two latter populations, *P. hominis* was identified in feces of 3/14 (21.4%) and 18/38 dogs (47.4%), respectively. Moreover, *P. hominis* was largely predominant since *T. fetus* was only identified in 0/14 and 2/38 dogs, respectively. In the present survey, *P. hominis* was the only trichomonad identified in the canine population using trichomonad-specific PCR assay. As previously suggested (Kim et al., 2010; Tolbert et al., 2012), our data confirmed that most commonly trichomonosis in dogs is due to *P. hominis* infection.

Table 2
Risk factor assessment of *Pentatrichomonas hominis* infection evaluated by a hierarchical linear model ($n = 215$, 2009, France).

Variable Levels	*P* value of overall variable	*P* value for each level	Estimate (lower estimate; upper estimate)
Puppy breed size	0.057		
Small breed dogs			0.149 [−0.004; 0.303]
Large breed dogs			
Age	0.585		
<8 weeks of age			0.039 [−0.101; 0.178]
≥8 weeks of age			–
Kennel size	0.04		
Small		0.2	0.142 [−0.075; 0.359]
Medium		0.012	0.315 [0.07; 0.56]
Large			
Canine coronavirus	0.199		
Negative		–	–
Low		0.106	0.062 [−0.013, 0.137]
High		0.659	−0.055 [−0.298; 0.189]
Canine parvovirus	0.560		
Negative		–	–
Low		0.5	0.031 [−0.06; 0.123]
High		0.666	−0.04 [−0.222; 0.142]
Toxocara canis	0.705		
Negative		–	–
Low		0.47	0.044 [−0.076; 0.165]
High		0.478	0.051 [−0.09; 0.191]
Cystoisospora ohioensis-complex	0.119		
Negative			
Low		–	–
High		0.04	−0.122 [−0.249; −0.006]
		0.13	−0.106 [−0.243; 0.032]
Cystoisospora canis	0.002		
Negative		–	–
Low		0.001	0.289 [0.115; 0.463]
High		0.001	0.324 [0.129; 0.518]
Giardia intestinalis	0.014		
Negative		–	–
Low		0.559	−0.031 [−0.135; 0.015]
High		0.03	0.148 [0.015; 0.281]

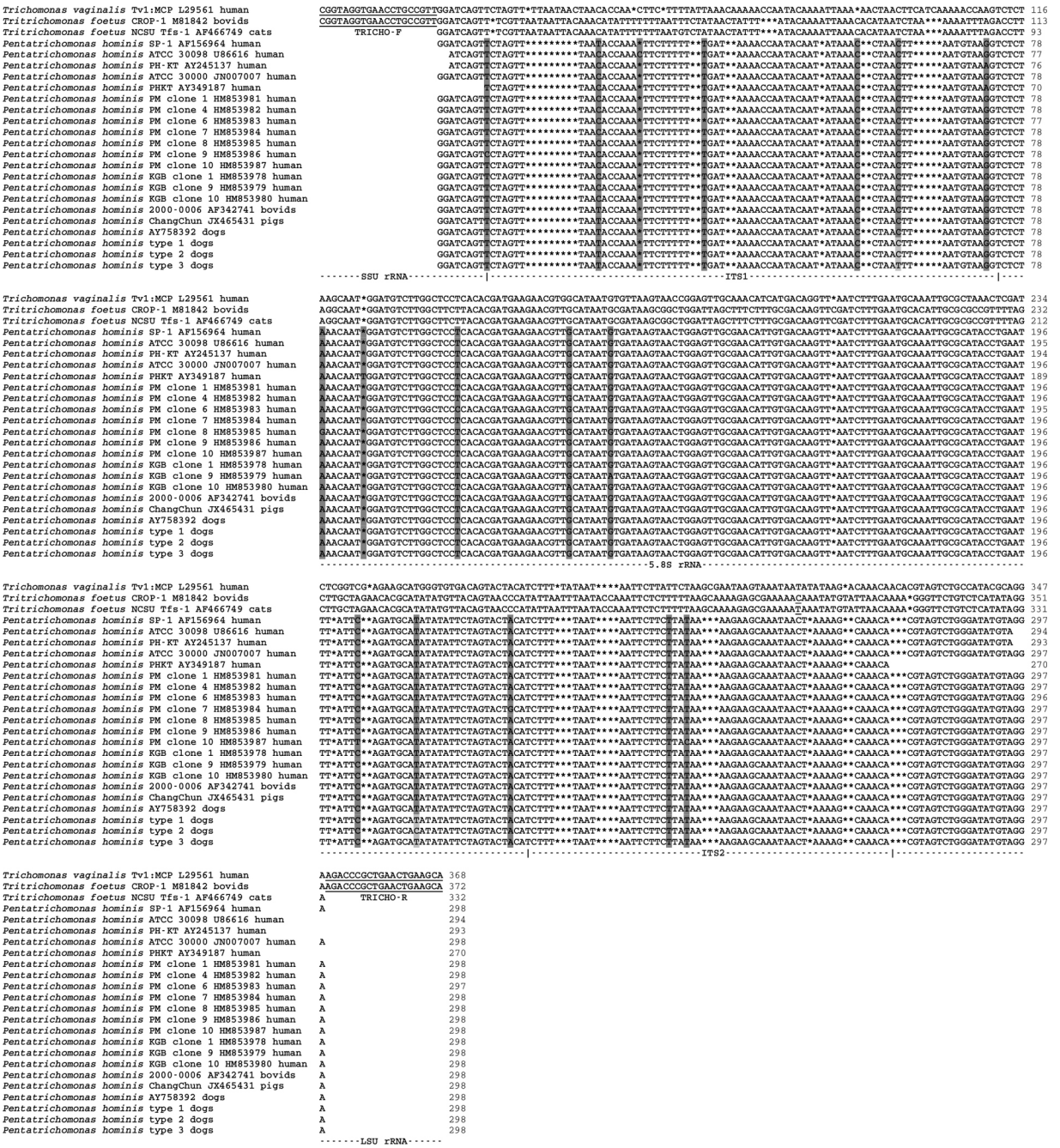

Fig. 1. Alignment of sequences of the ITS1-5.8S rRNA-ITS2 region of isolates obtained in this study (types 1, 2 and 3) and those of trichomonad strains and species of interest. Differences between the sequences of the type 1, type 2, and type 3 isolates (2 variable positions) and differences between these isolates and the homologous sequences of *P. hominis* strains isolated from different hosts (17 variable positions) are shaded in light and dark gray, respectively. The SNP between feline and bovine *T. fetus* isolates is underlined in the ITS2 region. Sequences of the trichomonad-specific primers used in this study are underlined. Gaps are represented by asterisks. SSU, small subunit; LSU, large subunit.

The high prevalence of dogs infected by *P. hominis* in our study could be explained by the age of the canine population analyzed that only included puppies from 5 to 14 weeks. Indeed, as previously reported, dogs presenting with *P. hominis* infection and diarrhea are generally younger than 9 months (Gookin et al., 2007; Kim et al., 2010). Several years ago, by infecting dogs with canine trichomonad strains isolated from stool and likely corresponding to *P. hominis* (see above), Simic (1932b) concluded that dogs were more easily infected and for a longer period when they were young. In infected dogs of less than one month, severe clinical signs were observed and the animal died of the infection whereas adult dogs seemed not to be infected. All these observations suggested that

Table 3
Risk factor of abnormal feces in puppies evaluated by a hierarchical linear model ($n = 215$, 2009, France).

Variable levels	*P* value of overall variable	*P* value for each level	Estimate (lower estimate; upper estimate)
P. hominis	0.402		
Negative			0.1 [-0.136; 0.336]
Positive			
Kennel size	0.286		
Small		0.118	–
Medium		0.467	0.198 [−0.051; −0.447]
Large			0.120 [−0.205; −0.445]
Canine coronavirus	0.835		
Negative		–	–
Low		0.696	0.026 [−0.104; 0.155]
High		0.697	−0.066 [−0.402; 0.269]
Canine parvovirus	0.011		
Negative		–	–
Low		0.9	−0.01 [−0.171; 0.150]
High		0.012	0.358 [0.08; 0.637]
Toxocara canis	0.987		
Negative		–	–
Low		0.872	0.017 [−0.193; 0.227]
High		0.947	0.009 [−0.245; 0.263]
Cystoisospora ohioensis-complex	0.339		
Negative			
Low		–	–
High		0.251	−0.112 [−0.305; 0.08]
		0.866	0.017 [−0.186; 0.22]
Cystoisospora canis	0.923		
Negative		–	–
Low		0.69	−0.06 [−0.357; 0.237]
High		0.84	−0.033 [−0.352; 0.287]
Giardia intestinalis	0.633		
Negative		–	–
Low		0.384	0.08 [−0.101; 0.26]
High		0.964	0.004 [−0.203; 0.212]

puppies were more susceptible to this parasite infection than adults probably due to an immature immune system. However, the relationship between *P. hominis* infection and the young age of animals was not confirmed in the recent study by Tolbert et al. (2012) in which age of the infected dogs ranged from 10 weeks to 10 years.

From our study, large breeding kennels also showed an increased risk of *P. hominis* infection. This result was in concordance with the study by Gookin et al. (2004) in which high housing density (low number of square meter of facility area per cat) was identified as a likely risk factor of infection for *T. fetus* infection in cats. Consequently, the density of animals in large breeding kennels and catteries could promote the environmental contamination and subsequently the spread of trichomonad infections. Some trichomonad species such as *T. fetus* do not form true cysts (Brugerolle, 1973; Mattern et al., 1973) but during unfavorable environmental conditions, the trophozoites can display a pseudocyst stage probably allowing a prolonged survival of the parasite (Pereira-Neves et al., 2011). Regarding *P. hominis* this species could form pseudocysts allowing to the parasite to survive during several days in the environment (Meloni et al., 2011). All these data suggest a direct fecal-oral transmission of *P. hominis* in pets but also a possible indirect infection through a contaminated environment.

In the present survey, all the puppies infected by *P. hominis* were also infected by at least one other enteropathogen including helminth, parasitic protozoa, and virus. In the handful of case reports of dogs with *P. hominis* infection, coexisting intestinal infections were also reported (Vanparijs et al., 1991; Gookin et al., 2005; Yamamoto et al., 2009; Tolbert et al., 2012). In the univariate model presented in this study, *P. hominis* infection was significantly associated with abnormal feces supporting the hypothesis of the potential clinical significance of this parasite infection as an associated cause of diarrhea in domestic dogs. This association was not confirmed in the multivariate risk factor analysis indicating the necessity to evaluate enteritic co-infection in infected puppies before to suspect a pathogenic effect of *P. hominis*. This lack of association between *P. hominis* and abnormal feces could be linked to the high prevalence of other enteropathogens in our population of puppies thereby masking the real pathogenicity of the trichomonad parasite. Another explanation might be the lack of effect of *P. hominis* alone on feces quality. In that case, *P. hominis* would be a commensal that might overgrow in the digestive tract of dogs with other causes for their diarrhea. However it is known that trichomonads could increase the severity of diarrhea in case of coinfection. Indeed severe diarrhea and increased shedding of trichomonads were described in four cats concurrently

infected with *Cryptosporidium* species and *T. fetus* (Gookin et al., 2001). As concurrent enteropathogen, CPV2 was frequently isolated in puppies (Marshall et al., 1984; Vanparijs et al., 1991; Naylor et al., 2001). In the present study, a deterioration of the fecal score was recorded in dogs with high amounts of fecal CPV2. This result was in accordance with previous studies showing a major impact of CPV2 on feces quality at weaning (Potgieter et al., 1981; Carman and Povey, 1982; Schulz et al., 2008).

In conclusion, we have shown for the first time that trichomonads are widespread parasites in puppies living in 25 French breeding kennels and that *P. hominis* infection is the most common cause of trichomonosis in this population of dogs. Our comparative analyses of *P. hominis* sequences obtained from numerous isolates did not recognize host-specific genotypes suggesting that this trichomonad species likely presents a high zoonotic potential and is therefore able to colonize many mammal species. According to our data, young age of the animals and high density of dogs housed in breeding kennels represented risk factors for *P. hominis* infection. In addition, the association between abnormal feces and *P. hominis* infection remained unresolved mainly due to the common presence of other enteropathogens potentially responsible for clinical signs of diarrhea. Consequently, experimental infection studies would be helpful to clarify the pathogenic significance of *P. hominis* infection in dogs.

Conflict of interest statement

The authors declare there are no conflicts of interest.

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