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Veterinary Parasitology 122 (2004) 193–199
veterinary parasitology
www.elsevier.com/locate/vetpar

Genotype characterisation of *Giardia duodenalis* isolates from domestic and farm animals by *SSU-rRNA* gene sequencing

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Received 26 September 2003; received in revised form 8 March 2004; accepted 2 April 2004

Abstract

In order to investigate the genotypes of *Giardia duodenalis* from domestic and farm animals in Italy, 21 *Giardia* isolates, 17 from dogs, 1 from cat and 3 from dairy calves, were genetically characterised by *SSU-rRNA* gene sequencing. Among dogs, 76.5% of isolates showed the dog-specific genotypes (Assemblages C, D and C/D mixed Assemblage) and 23.5% exhibit potential zoonotic genotypes (Assemblage A and A/C mixed Assemblages). The cat isolate belonged to assemblage A, whereas the sequences among the isolates from calves were found to correspond to hoofed-livestock genotype, namely Assemblage E. These findings suggest that infection of humans by zoonotic genotypes from domestic animals could be of low epidemiological significance, although possible. The present study represents the first contribute to the knowledge of *G. duodenalis* genotypes in domestic and farm animals from Italy.

Keywords: Giardia duodenalis; Dog; Cat; Cattle; PCR; Genotype

1. Introduction

The flagellate protozoan *Giardia* is the etiological agent of giardiasis, one of the most prevalent and widespread intestinal diseases in humans and several vertebrate animal species worldwide. Taxonomy of the genus is mainly based on morphology and, only recently, on

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doi:10.1016/j.vetpar.2004.04.008

genetic evidences. According these criteria, six species have been recognized in the genus *Giardia* until now: *G. agilis* in amphibians, *G. muris* and *G. microti* in rodents, *G. psittaci* and *G. ardeae* in birds and *G. duodenalis* in mammals. *G. duodenalis* (syn *G. intestinalis*; *G. lamblia*) is the only species found in humans as well as in other mammals, including domestic and farm animals such as dogs, cats, cattle, pigs, sheep and horses (Filice, 1952; Monis et al., 1996; Adam, 2001). Although morphologically very similar, isolates of *G. duodenalis* show a large genetic heterogeneity. Molecular characterisation and phylogenetic analysis of isolates from different hosts has revealed the existence of seven major genotypes (Monis et al., 1999, 2003). *Giardia* from humans falls exclusively into Assemblage A and B (Homan et al., 1992; Mayrhofer et al., 1995; Nash and Mowatt, 1992). Some animal derived isolates appear to be similar or identical to human derived genotypes while others represent unique genotypes that seem to be host-specific. There are two dog-specific genotypes, Assemblages C and D (Hopkins et al., 1997; Monis et al., 1998) and a hoofed livestock lineage, defined as Assemblage E, characteristic of isolates from sheep, goats, cattle and pigs (Ey et al., 1997). Recently, Monis et al. (1999) designated two lineages represented by cat and rat isolates, defined as Assemblages F and G, respectively. When considering the zoonotic potential of each genotype, the Assemblages A and B are believed to represent a major risk for human health since both appear to be infective to a wide range of wild and domestic host species (Thompson, 2000). In contrast, the animal-specific genotypes display remarkable host range differences, and there is no epidemiological evidence suggesting they can infect humans. In Italy, although *G. duodenalis* is recognized as a common parasite of several animal species, no information on the identity of *Giardia* isolates, their prevalence, animal and public health impact are available. Moreover, the diagnosis is mainly performed using conventional microscopy, and no genetic characterisation of isolates has been currently performed. In order to address these questions, the present study was carried out to investigate the presence of *Giardia* from domestic and farm animals in a region of Central Italy and to genotype the isolates by *SSU-rRNA* gene sequence analysis.

2. Materials and methods

2.1. Sample collection

All the samples were sent to the Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana for copro-parasitological diagnosis. In some cases parasitological examination was requested just for routine controls, but usually it was aimed to identify the cause of diarrhoea. On dog samples the diagnosis for giardiasis was specifically requested from veterinarians, whereas on samples of different origin it was performed on clinical suspect basis along with other tests for viral and bacterial pathogens. Between January 2002 and April 2003 stool samples from a total of 113 dogs, 10 calves and 1 cat were analysed microscopically by direct fresh Lugol-stained examination for the detection of both *Giardia* trophozoites or cysts. The majority of dog samples and the only one tested cat originated from kennels in the Lazio region (Central Italy). All the calf specimens originated from dairy farms of the same region.

2.2. DNA extraction and nested PCR

DNA was extracted directly from faeces using QIAamp DNA Stool Mini Kit (Qiagen) for PCR and stored at −20 °C. Since double amplifications by PCR was found to be significantly more sensitive than single PCR, isolate genotyping was carried out using a nested PCR. A 292 bp region of the 5′ end of the small subunit (16S) rRNA gene was amplified using the conditions and primers RH11 and RH4, to a final volume of 25 μl, as described by Hopkins et al. (1997). For the nested PCR reaction, a specifically designed internal primer Gia-N: 5′-GTG ATG CCC CGG AAG CCC G -3′ was used. The nested PCR program was as follows: denaturing step at 94 °C for 2 min, followed by 35 cycles of denaturing for 20 s at 94 °C, annealing for 20 s at 65 °C and extension for 20 s at 72 °C, followed by a final extension at 72 °C for 7 min.

2.3. Sequence analysis and genotyping

Bands were excised from agarose gels, purified using NucleoSpin® Extract (Macherey–Nagel) purification Kit and sequenced with the Big Dye Terminator Cycle Sequencing Ready Reaction version 2.0, and the reading was performed using an ABI Prism DNA Sequencer (Perkin Elmer). The obtained sequences were aligned with those available in GenBank for awarding the genotype using Clustal X (Thompson et al., 1997).

3. Results

Stool samples of 17 dogs, three calves and the only one tested cat resulted positive for *Giardia* after microscopic examination. Fifteen of the 17 positive dogs and the cat were coming from kennels. The remaining two dogs (Dogizp16 and Dogizp17 isolates) were companion animals. The three positive calves were bred in two dairy farms in Rome and Viterbo provinces. Samples assessed positive for *Giardia* by microscopy and analysed for molecular characterization are listed in Table 1. The DNA from all the samples was successfully amplified by PCR and sequenced. Summary of the nucleotide variations detected in the diagnostic positions of 5′ end 16S-rRNA gene sequence alignment observed among the different isolates and compared with references strains is reported in Table 2. Among dog isolates, 11 displayed the sequence reported for Assemblage C, 2 for Assemblage A, 1 for Assemblage D whereas three displayed a mixed Assemblage (2 A/C and 1 C/D). In these mixed isolates, peaks corresponding to both Assemblages (A/C or C/D) were present at the position characterizing the genotype and their presence was recognised by the sequence analysis software which identified the nucleotide present as ‘N’ rather than the specific nucleotide for Assemblages A or C or D. The genetic sequence obtained from cat isolate aligned with Assemblage A. The three 16SrDNA sequences from calves revealed the A-G substitution at nucleotide position 92, indicative of the Assemblage E, as evidenced when compared with the hoofed-livestock genotype (AF113902). These results represent the first report of Assemblage C, D and E from *Giardia* animal isolates in Italy.

Table 1
Isolate code, host origin and identification of genotypes of *Giardia duodenalis* isolates studied

Isolate code	Genotype
DOG[a]	
Dogizp1	C
Dogizp2	C
Dogizp3	A/C
Dogizp4	C
Dogizp5	A
Dogizp6	A/C
Dogizp7	A
Dogizp8	C
Dogizp9	C
Dogizp10	C/D
Dogizp11	C
Dogizp12	C
Dogizp13	C
Dogizp14	C
Dogizp15	C
Dogizp16	D
Dogizp17	C
CAT[a]	
Catizp1	A
CALF[a]	
Calfizp1	E
Calfizp2	E
Calfizp3	E

[a] Host origin.

4. Discussion

Giardia is one of the most common parasites of domestic dogs. Prevalences of 10, 30–50 and 100% were previously recorded in well cared-for dogs, pups and kennels dogs, respectively (Barr and Bowman, 1994). In a recent study on the prevalence of canine giardiasis in Central Italy, 22.2% of 436 samples examined resulted infected. Higher prevalences were detected in kennel dogs (32.1%) than in companion ones (12.4%) (Giangaspero et al., 2002). Prevalence observed in the present study (15.0%) do not significantly differ from these data. At present, molecular studies showed that *G. duodenalis* isolates recovered from dogs fall into the two genotype A and B, characteristics of human and several animal isolates, but also evidenced the existence of two genetically distinct dog-specific genotypes, Assemblage C and D (Hopkins et al., 1997; Monis et al., 1998; van Keulen et al., 2002; Abe et al., 2003). In the present study, 76.5% of isolates showed the dog-specific genotypes (Assemblages C, D and C/D mixed Assemblage) and only 23.5% exhibited potential zoonotic genotypes (Assemblage A and A/C mixed Assemblages). Although the number of isolates was too small to provide significant conclusions, the low number of isolates recovered with zoonotic genotype and the presence of dog-specific genotypes D and C in the two companion dog isolates suggest that infection of humans by zoonotic genotypes from dogs could be of low

Table 2
Summary of the nucleotide variations detected in the diagnostic position of 5′ end 16S-rRNA gene sequence alignment

Giardia isolates	Alignment position								
	22	23	24	44	62	72	93	167	171
Assemblage A	G	C	G	G	T	C	A	G	G
Assemblage C	A	T	C	A	A	G	·	T	·
Assemblage D	A	T	C	A	A	A	·	T	A
Assemblage E	·	·	·	·	·	·	G	·	·
Dogizp1	A	T	C	A	A	G	·	T	·
Dogizp2	A	T	C	A	A	G	·	T	·
Dogizp3	·	·	·	A	·	S	·	·	·
Dogizp4	A	T	C	A	A	G	·	T	·
Dogizp5	·	·	·	·	·	·	·	·	·
Dogizp6	A	T	C	A	·	S	·	K	·
Dogizp7	·	·	·	·	·	·	·	·	·
Dogizp8	A	T	C	A	A	G	·	T	·
Dogizp9	A	T	C	A	A	G	·	T	·
Dogizp10	A	T	C	A	A	R	·	T	R
Dogizp11	A	T	C	A	A	G	·	T	·
Dogizp12	A	T	C	A	A	G	·	T	·
Dogizp13	A	T	C	A	A	G	·	T	·
Dogizp14	A	T	C	A	A	G	·	T	·
Dogizp15	A	T	C	A	A	G	·	T	·
Dogizp16	A	T	C	A	A	A	·	T	A
Dogizp17	A	T	C	A	A	G	·	T	·
Catizp1	·	·	·	·	·	·	·	·	·
Calfizp1	·	·	·	·	·	·	G	·	·
Calfizp2	·	·	·	·	·	·	G	·	·
Calfizp3	·	·	·	·	·	·	G	·	·

Dots indicate identity with the first sequence. Polymorphic sites are designated with IUPAC codes. Accession number for reference strains are as follow: Assemblage A (AF199446); Assemblage C (AF199449); Assemblage D (AF199443); Assemblage E (AF199448).

epidemiological significance, although possible. Concerning animal health, it's interesting to note that the majority of positive samples presented manifest diarrhoea, in some cases with watery faeces, where also trophozoites were detected. In about 50% of positive dog samples also other intestinal parasites were detected, mainly *Toxocara* sp., *Trichuris* sp. and *Isospora* sp. Thus it could be concluded that *Giardia*, often concurrent with other monoxenous parasites, represent a real pathogen for dogs, especially when, as in kennels, general sanitary conditions appear to be poor, there is high concentration of individuals, animals are stressed and continuous re-infections are expected to take place. Also the occurrence of mixed Assemblages is probably to charge to high re-infection rate which likely occur among dogs. Hence, giardiasis could constitute a major threat for kennels.

At present isolates from cats corresponded to the zoonotic assemblage A and B (Thompson et al., 2000; van Keulen et al., 2002) or to the cat-specific genotype F (Monis at al., 1999, 2003). Recently McGlade et al. (2003) in a study to determine the prevalence of *Giardia* in domestic cats from the Perth metropolitan area (Australia), found that all but one isolate most closely resembled *G. duodenalis* dog-specific Assemblage D. In the present study the

molecular characterization of the *Giardia* sample from a cat assigned it to zoonotic genotype A. Since cats are kept in the same sanitary kennels than dogs, the same conclusions reported above for dogs could be considered valid.

About farm animals, *Giardia* infections in cattle is of increasing concern due to negative impact on animal health and production and to potential contamination of surface and ground waters and subsequent zoonotic risk (O'Handley et al., 2000). Previous data about *Giardia* genotypes in calves from Western Australia and Western Canada (O'Handley et al., 2000) showed dairy calves to be infected with two Assemblages, A and E. Moreover, genotypic analysis of *Giardia* isolates from beef cattle from Alberta (Canada) showed that the hoofed livestock genotype is predominant, but the zoonotic genotype, Assemblage A, is reported to be as high as 20% (Appelbee et al., 2003). Genetic analysis of *Giardia* isolates from calves examined in this study showed only the presence of Assemblage E. The animals resulted also positive for *Eimeria* sp. but negative for *Cryptosporidium* and *Salmonella* sp. Young animals, like in coccidial infection, are the ones that suffer clinical disease, but adult animals, even in absence of symptoms, could act as eliminators and should be monitored. This is particularly true for intensive farming, due to high concentration of animals over a small area and the subsequent heavy fecalization of the environment.

The present data represent the first contribute to the knowledge of *G. duodenalis* genotypes in domestic and farm animals in Italy. In particular, the correct genotyping of *Giardia* isolates appears of relevant importance to provide accurate information about the risk of zoonotic transmission and host specificity and to determine the sources of infection in outbreak situations. Such an analysis could also help in defining the role of *Giardia* as animal pathogen, having an adverse impact on animal health and production.

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