Genetic identification of *Giardia* isolates from symptomatic and asymptomatic shelter dogs

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SUMMARY

*Giardia* cysts were obtained from a cohort of 20 infected shelter dogs with diarrhoea (n = 10) or asymptomatic (n = 10), and were examined by molecular methods that included DNA extraction, triosephosphate isomerase gene amplification, and DNA sequence analysis. Results showed the presence of Assemblages A and C. Three samples belonged to Assemblage A (subgenotype A1) but the majority (17) to Assemblage C. A degree of genetic polymorphism was evident within Assemblage C with 2 subgenotypes identified (C1 and C2). This suggests that infection of humans by zoonotic Assemblage C from shelter dogs is of limited epidemiological significance, though possible. The relation between presence or absence of diarrhoea and *Giardia* Assemblages or subgenotypes was also investigated by Fisher’s Exact Test. No statistical relation was detected. Therefore, it is concluded that host factors probably play a major role in the clinical manifestation of *Giardia* infections in dogs.

Keywords: *Giardia*, Assemblage, subgenotype, dog, shelter, diarrhoea.

RÉSUMÉ

Identification génétique de souches de *Giardia* chez des chiens diarrhéiques et asymptomatiques.

Des kystes de *Giardia* ont été obtenus à partir d’une cohorte de 20 chiens infectés au refuge avec diarrhée (n=10) ou asymptomatiques (n=10), et ont été examinés par des méthodes moléculaires incluant l’extraction de l’ADN, l’amplification du gène de la triosephosphate isomérase, et l’analyse des séquences d’ADN. Les résultats montré la présence des assemblages A et C. Un degré de polymorphisme génétique était possible dans l’assemblage C avec 2 sub-génotypes identifiés (C1 et C2). Ces résultats suggèrent que l’infection humaine à partir de l’assemblage zoonotique A chez les chiens au refuge est de signification épidémiologique limitée bien que possible. La relation entre la présence ou l’absence de diarrhée et les assemblages ou les sub-génotypes de *Giardia* a été également étudiée par la méthode de Fisher. Aucune relation statistique n’a été détectée. On peut donc en conclure que les facteurs de l’hôte jouent probablement un rôle majeur dans la manifestation clinique de l’infestation par *Giardia* chez le chien.


Introduction

It is known that *Giardia* is a cosmopolitan enteric protozoan with a very wide host range, including domestic and wild animal species as well as human beings. With the advent of molecular tools, it has been shown that *Giardia* is a species complex, made up of morphologically indistinguishable isolates that are genetically distinct [2]. Isolates of *Giardia* can be grouped into a number of genotypes or Assemblages, at least seven, recognisable by isoenzyme and DNA sequence analysis [2]. These Assemblages include A and B, which are potentially zoonotic, and C, D, E, F and G, which appear to be host restricted [2]. The two major Assemblages A and B have been recovered from a broad range of hosts, including humans, livestock, cats, dogs, beavers and guinea pigs. Though the genetic distance between both Assemblages is larger than the genetic distance between species in other protozoa, there are no phenotypic characteristics supporting differentiation into two species [2]. Assemblage C and D only contain isolates from dogs and cats, Assemblage E contains isolates from cattle, sheep, and goats, Assemblage F contains isolates from cats, and Assemblage G contains isolates from rats [2]. All human isolates characterized today have been grouped into either Assemblage A or B, whereas cats, dogs, and livestock are susceptible to infection with isolates from either their own host specific Assemblage or from the potentially zoonotic Assemblages A and B [2].

Diarrhoea in shelter dogs is one of the most common diseases facing the veterinary clinicians and managers of canine shelters. In particular, a recent survey identified *Giardia* as one of the most common enteric parasites and frequent causes of diarrhoea in shelter dogs [14], and thus as one of the main concerns of veterinarians who treat dogs living in shelter settings. Clinical signs of canine giardiasis can range in severity from enteritis with diarrhoea to depression, anorexia, weight loss, poor condition and vomiting [4]. Diarrhoea may be intermittent or continuous and faeces are usually soft, pale, very foul smelling and steatorrhoeic [4]. The disease is usually acute and self limiting. However, chronic infections can occur with or without an acute phase, may

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result in recurrent symptoms and are often associated with treatment failure. The chronic form may persist for weeks or months, or may last even for years, and is more common in immune-compromised dogs [4]. How to explain this clinical heterogeneity is unclear, since factors determining the variability of clinical symptoms in giardiasis are still poorly known even in humans. Host immune status, nutritional state, and age, as well as different virulence and pathogenicity of Giardia strains are recognised as important factors to determine the outcome and severity of the infection [7]. In addition, in some studies in humans, some authors have suggested that the parasite Assemblage may contribute to the development of symptoms [6, 7, 12]. A number of canine isolates from various countries have been genotyped [1, 3, 5, 8-10, 17, 18]; however, to the best of our knowledge, there has been no reports so far aiming to investigate the possibility of an Assemblage dependent genetic basis for the variability of clinical manifestations in canine giardiasis. Giardia has highly been detected in symptomatic and asymptomatic shelter dogs in Italy but the Assemblage of isolates remained unknown because infections were identified by a coproantigen ELISA test [11]. Therefore, in an extension of the original survey [11], the present study was carried out where faecal specimens were collected from a sub-sample of 20 out of 101 shelter dogs testing positive for Giardia antigen, and then examined by DNA extraction, triose phosphate isomerase (TPI) gene amplification, and DNA sequence analysis. Our aim was first to identify the Assemblages of Giardia isolates harboured by shelter dogs, and second to investigate their association with presence or absence of diarrhoea in this cohort of dogs.

Materials and Methods

SOURCE OF SAMPLES

Faecal samples were collected from a cohort of 20 shelter dogs, as representative of 101 shelter dogs for which a diagnosis of Giardia infection had been made by coproantigen ELISA test prior to the initiation of the present study [11]. The 101 dogs were living in 3 shelters located in the metropolitan area of Rome and managed as previously described [11]. The 20 enrolled dogs were 13 males and 7 females, with an age ranging from 2 months to 6 years (median = 2.5 years). None of the dogs had been given antiparasitic treatments within 15 days before sampling. They included both diarrhoeic (n = 10) and asymptomatic (n = 10) dogs. The majority of the dogs were cross-bred (n = 12). Pure breed dogs included 2 Maremma sheepdogs, 2 Pitt-bull, 1 German sheepdog, 1 Doberman, 1 Setter, and 1 Great Dane. After collection, the samples were stored at +5°C for approximately 12-24 hours, then were kept at room temperature (20-25°C) and centrifuged using sucrose gradient centrifugation to concentrate Giardia cysts prior to DNA extraction [13]. All samples were then frozen at -20°C until the use for the molecular study.

DNA EXTRACTION

Genomic DNA was extracted from 200µl of concentrated suspension of Giardia cysts using the QIAamp® DNA Stool Mini Kit (QIAGen, Hilden, Germany) after three freeze/thaw cycles (liquid nitrogen for 5 min, at 95°C for 5 min). All the DNA extracts were frozen until the molecular analyses were performed.

PCR AND SEQUENCING

A 250 bp fragment of the gene encoding for the TPI was amplified by a semi-nested PCR approach. In the first step the degenerated primer set AL3544 (Forward: 5’-CCC TTC ATC GGI GGT AAC TT-3’) and AL3545 (Reverse: 5’-GTG GCC ACC ACI CCC GTG CC- 3’) was used [15], while in the second step the degenerated primers AL3544 and TPRI (Reverse: 5’-CCC ATG TTC IGI AGC ATC TC- 3’) were used [PAOLETTI et al., unpublished data]. All the primers were designed on the basis of the entire sequence of the gene encoding for the TPI (GS/M isolate – GenBank Accession number LO2116).

The PCR was performed in a 50 µl PCR mixture containing 2.5-5 µl of DNA, 100 pM of each primer, 200 µM of each dNTPs, 1X buffer (100 mM Tris-HCl pH 8.3, and 500 mM KCl), 0.5 µg of bovine serum albumin, 1.5mM of MgCl2 and 2.5 U of Taq Gold polymerase (Applied Biosystems, Foster City, CA 944404, USA). PCR reactions of both steps were performed under the following conditions: 94°C for 12 min (Taq Gold activation temperature), 35 cycles of amplification: 45 sec at 94°C, 45 sec at 50°C, 60 sec at 72°C, with a final 7 min elongation step at 72°C. Samples containing DNA of Giardia duodenalis and samples without DNA (i.e. distilled water as template) were included in all the PCR reactions (to act as positive and negative controls, respectively). In particular, as a positive control, G. duodenalis DNA was extracted from cultured G. duodenalis WB strain provided by Prof. Tai-Soon Yong (Department of Parasitology and Institute of Tropical Medicine, Yonsei University, College of Medicine, Seoul 120-752, South Korea).

PCR amplicons were electrophoresed in a 2% TAE-agarose gel, visualized after ethidium bromide staining and photographed with the Gel Doc 2000 (BioRad Laboratories, Hercules, CA 94547, USA) documentation system. PCR products were purified by spin columns (ultra free –DA PRISM 377) and directly sequenced with the kit Byg Dye Terminator Cycle Sequencing (Applied Biosystem). Sequences were assembled according to THOMPSON et al. [16] by using the program ClustalX and compared with the Giardia sequences available in the GenBankTM database.

Analysis of results

Fisher’s Exact Test was chosen to determine if there was non-random association between dogs with or without diarrhoea and Giardia Assemblages or subgenotypes. This statistical test is used to calculate an exact probability value for the
relationship between two dichotomous variables where sample sizes are small, as found in a two by two cross table. A probability value of $P<0.05$ was considered significant.

**Results**

All the 20 samples produced amplicons of about 250 bp (Figure 1). Overall, 2 distinct Assemblages were identified, namely A and C, with genetic identity of 100%. The comparison with the *G. duodenalis* sequences available in the GenBankTM database revealed that Assemblage C was the more common in these dogs: 17 of the infections were due to Assemblage C and 3 to Assemblage A, subgenotype A1 in particular (Genbank accession number: AY228647). Sequencing of the amplification products allowed 2 subgenotypes to be identified within Assemblage C, namely C1 and C2. Subgenotype C1 accounted for most of the Assemblage C infections observed in this study: 13 of 17 Assemblage C infections were with subgenotype C1 (Genbank accession number: AY228641) and 4 with C2 (Genbank accession number: AY228642), as shown in Table I. Assemblage A, subgenotype A1, was associated with 3 cases of asymptomatic infections. Assemblage C was found both in symptomatic (n = 10) and asymptomatic dogs (n = 7). Nine of 13 infections with subgenotype C1 were symptomatic, whereas 3 of 4 infections with subgenotype C2 were asymptomatic.

However, despite this trends, no statistically significant correlation was found between the presence or absence of diarrhoea and the Assemblage ($P = 0.105$) or subgenotype ($P = 0.670$) of *Giardia*. Details are shown in Table I.

![Figure 1](image)

**Table 1**: Identification of *Giardia* Assemblages (A and C) and sub-genotypes (A1, C1 and C2) in symptomatic (n = 10) and asymptomatic (n = 10) shelter dogs.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Assemblage</th>
<th>Subgenotype</th>
<th>Sex</th>
<th>Age</th>
<th>Breed</th>
<th>Faeces</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>A1</td>
<td>M</td>
<td>4 years</td>
<td>Cross-bred</td>
<td>Normal</td>
</tr>
<tr>
<td>9</td>
<td>A</td>
<td>A1</td>
<td>F</td>
<td>6 years</td>
<td>Maremma sheepdog</td>
<td>Normal</td>
</tr>
<tr>
<td>13</td>
<td>A</td>
<td>A1</td>
<td>M</td>
<td>4 years</td>
<td>German sheepdog</td>
<td>Normal</td>
</tr>
<tr>
<td>15</td>
<td>C</td>
<td>C1</td>
<td>M</td>
<td>1 year</td>
<td>Maremma sheepdog</td>
<td>Normal</td>
</tr>
<tr>
<td>16</td>
<td>C</td>
<td>C1</td>
<td>M</td>
<td>4 years</td>
<td>Pitt-bull</td>
<td>Diarrhoeic</td>
</tr>
<tr>
<td>20</td>
<td>C</td>
<td>C1</td>
<td>M</td>
<td>1 year</td>
<td>Cross-bred</td>
<td>Diarrhoeic</td>
</tr>
<tr>
<td>22</td>
<td>C</td>
<td>C1</td>
<td>M</td>
<td>1 year</td>
<td>Cross-bred</td>
<td>Normal</td>
</tr>
<tr>
<td>23</td>
<td>C</td>
<td>C1</td>
<td>F</td>
<td>3 years</td>
<td>Cross-bred</td>
<td>Normal</td>
</tr>
<tr>
<td>32</td>
<td>C</td>
<td>C1</td>
<td>M</td>
<td>10 months</td>
<td>Cross-bred</td>
<td>Diarrhoeic</td>
</tr>
<tr>
<td>35</td>
<td>C</td>
<td>C1</td>
<td>M</td>
<td>6 months</td>
<td>Doberman</td>
<td>Diarrhoeic</td>
</tr>
<tr>
<td>39</td>
<td>C</td>
<td>C1</td>
<td>F</td>
<td>8 months</td>
<td>Cross-bred</td>
<td>Diarrhoeic</td>
</tr>
<tr>
<td>44</td>
<td>C</td>
<td>C1</td>
<td>M</td>
<td>3 years</td>
<td>Cross-bred</td>
<td>Normal</td>
</tr>
<tr>
<td>46</td>
<td>C</td>
<td>C1</td>
<td>M</td>
<td>5 months</td>
<td>Cross-bred</td>
<td>Diarrhoeic</td>
</tr>
<tr>
<td>50</td>
<td>C</td>
<td>C1</td>
<td>F</td>
<td>1 year</td>
<td>Cross-bred</td>
<td>Diarrhoeic</td>
</tr>
<tr>
<td>52</td>
<td>C</td>
<td>C1</td>
<td>M</td>
<td>4 years</td>
<td>Pitt-bull</td>
<td>Diarrhoeic</td>
</tr>
<tr>
<td>59</td>
<td>C</td>
<td>C1</td>
<td>F</td>
<td>2 months</td>
<td>Cross-bred</td>
<td>Diarrhoeic</td>
</tr>
<tr>
<td>60</td>
<td>C</td>
<td>C2</td>
<td>M</td>
<td>3 years</td>
<td>Setter</td>
<td>Normal</td>
</tr>
<tr>
<td>78</td>
<td>C</td>
<td>C2</td>
<td>F</td>
<td>4 months</td>
<td>Cross-bred</td>
<td>Normal</td>
</tr>
<tr>
<td>85</td>
<td>C</td>
<td>C2</td>
<td>F</td>
<td>2 years</td>
<td>Great Dane</td>
<td>Normal</td>
</tr>
<tr>
<td>91</td>
<td>C</td>
<td>C2</td>
<td>M</td>
<td>3 years</td>
<td>Cross-bred</td>
<td>Diarrhoeic</td>
</tr>
</tbody>
</table>

M = male ; F = female.

**Table 1**: Identification of *Giardia* Assemblages (A and C) and sub-genotypes (A1, C1 and C2) in symptomatic (n = 10) and asymptomatic (n = 10) shelter dogs.

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Discussion

At present, isolates from dogs fall into zoonotic Assemblages (A or B) or into dog specific Assemblages (C or D) [2]. In the present study, sequence analysis of 20 *Giardia* isolates at the TPI locus identified the great majority of Assemblages as belonging to the dog specific Assemblage C (17 isolates) with only three isolates which exhibited genetic identity with Assemblage A. The findings of cysts of Assemblage C and A in our samples is consistent with previous results [3, 8, 10]. Although Assemblage B and D were not detected in the present work, they have been documented in other studies [1, 3, 10, 17, 18]. The absence of Assemblage B was expected, since Assemblage B isolates appear to be less widespread and restricted to localised endemic foci [12], whereas the absence of Assemblage D was rather unexpected. Probably a larger number of samples would have been needed to find Assemblage D. The occurrence of intragenotypic variations within *Giardia* Assemblages is already known [1, 10] and it has been confirmed by the present study, where subgenotypes A1, C1 and C2 were found. The number of subgenotypes A found in dogs is increasing and includes subgenotype A1, A2, A3 and A4 [9, 10]. Subgenotype A1 is considered to be zoonotic and the presence in the dog isolates studied is consistent with previous results [9, 10]. The samples belonging to *Giardia* Assemblage C had two distinct subgenotypes, C1 and C2. However, the subgenotype C1 accounted for most of the Assemblage C samples. The significance of a predominant subgenotype is unclear. It is possible that a common source of infection was responsible for the higher occurrence of subgenotype C1 in this cohort of shelter dogs.

The present data support the notion that the dog specific Assemblage C may be more prevalent in shelter settings, probably because of a better host adaptation. In fact, there is increasing evidence that infection with the dog specific Assemblages prevail in areas where the main pattern of transmission is dog to dog [3, 10], as it may occur in shelter settings. Therefore, the high presence of Assemblage C in isolates from shelter dogs can probably be considered as usual [3, 10]. This Assemblage is commonly found in dogs and is not considered to be zoonotic, and thus is not thought to be of public health concern [2].

From the human health perspective, of importance is the finding that cysts of Assemblage A also occurred in shelter dogs in addition to Assemblage C. Assemblage A is geographically the most widespread and is thought to be zoonotic, since it has been identified previously in both dogs and humans [5, 9]. Thus the significance for public health of Assemblage A should be taken into account. Assemblage A is distributed worldwide. However, its distribution in dogs differs according to the studies and geographic locations. For example, in a study in Mexico using a beta-giardin nested PCR assay, 3 samples from pet dogs belonged to the subgenotype A1, 1 to the subgenotype A3, and 1 to a mixed A1 / B3 infections, with a complete lack of Assemblages B, C and D [9]. Another study, conducted in Mexico by sequence analysis of the small subunit 16S rRNA gene, also showed that all 11 isolates from puppies belonged to Assemblage A [5]. Whereas, 14, 1 and 6 isolates from household and kennel dogs in Japan, characterized using glutamate dehydrogenase gene sequencing, belonged to Assemblages A, C and D, respectively [8]. However, two similar studies carried out in Italy, showed that Assemblage A was responsible for less dog infections than Assemblages C and D: 4/17 and 7/21 by small subunit16S rRNA and beta giardin gene sequencing, respectively [3, 10]. It is unclear if these differences are due to the geographical distribution of Assemblages or to environmental factors. Otherwise, it is possible that these differences are due to variations in assay methodology. In our study, the sequence corresponding to the TPI gene was chosen because the use of a typing system based on sequence analysis of a single locus with high sequence heterogeneity, such as TPI, can provide a resolution as high as multilocus sequence analysis, as pointed out by SULAIMAN et al. [15]. In any case, the present finding of a higher prevalence of the non zoonotic dog specific Assemblage C and similar results from other recent molecular studies [3, 10] suggest a limited role of shelter dogs as reservoirs of zoonotic Assemblage A, at least in Italy. Managing *Giardia* infections in shelter dogs should decrease the risk of exposure to zoonotic Assemblage A for other shelter animals, personnel, and people adopting dogs from shelters. In addition to the present report, very few data exist in literature to date on the association between *Giardia* Assemblage and clinical signs in dogs. BERRILLI et al. [3] stated manifest diarrhoea in the majority of positive samples where Assemblages A, A/C, C, D or C/D were detected. Whereas, ABE et al. [1] reported that no clinical sings were detectable in dogs shedding isolates identified as the dog specific Assemblage D. Therefore, to the best of our knowledge, this is the first study conducted to date on the possible relationship between *Giardia* isolates and presence or absence of diarrhoea in dogs. In humans, in one study in symptomatic patients, a correlation between intermittent or persistent diarrhoeal complaints and Assemblage A or B was observed, respectively [7]. In two other studies, a correlation was found between the presence of Assemblage A and the occurrence of diarrhoea [6, 12]. In the present study, Assemblage A was found in three asymptomatic dogs, while Assemblage C was found in both symptomatic and asymptomatic dogs but no statistical correlation was found between the presence or absence of diarrhoea and Assemblages or subgenotypes of *Giardia*. Therefore, this suggests that host factors play an important, but not determinant, role in determining the clinical outcome of *Giardia* infections in dogs. However, the number of isolates examined was not large enough to allow definitive conclusion and also we can not exclude that the asymptomatic dogs had recovered from diarrhoea when faecal samples were collected. In addition, it was not possible to determine if the diarrhoea could be attributed entirely to *Giardia*, as the presence of viruses, bacteria or other intestinal parasitosis were not investigated. Any firm conclusion would need to be based on the analysis of larger data sets that include multiple samples of all the Assemblages that are known to infect dogs.

To conclude, these data provide genetic and clinical information which may be of value particularly with regard to dog population management in shelter settings. This study also...
presents an integrated approach, bringing together clinical and molecular data with regard to the investigation of the cases of diarrhoea associated with various Assemblages and subgenotypes of *Giardia* present in shelter dogs. Therefore, it is believed that the results reported from this current study contribute to our knowledge base. In future, similar studies may be applied in other animal population and may give further insight to understand the population genetic structure of *Giardia* in animals and their zoonotic potential.

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**References**


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