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Genetic heterogeneity at the β-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes☆

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Abstract

Human giardiasis, caused by the intestinal flagellate *Giardia duodenalis*, is considered a zoonotic infection, although the role of animals in the transmission to humans is still unclear. Molecular characterisation of cysts of human and animal origin represents an objective means to validate or reject this hypothesis. In the present work, cysts were collected in Italy from humans ($n=37$) and animals (dogs, one cat and calves, $n=46$), and were characterised by PCR amplification and sequencing of the β-giardin gene. As expected, only Assemblages A and B were identified among human isolates. The host-specific Assemblages C and D were found in the majority of dog isolates; however, 6 dog isolates were typed as Assemblage A. The cat-specific Assemblage F has been identified in the single feline isolate available. Among calf isolates, most were typed as Assemblages A ($n=12$) and B ($n=5$), whereas the host-specific Assemblage E was rarely found ($n=3$). Sequence heterogeneity in the β-giardin gene allowed a number of subgenotypes to be identified within Assemblage A (8 subgenotypes), B (6 subgenotypes), D (2 subgenotypes), and E (3 subgenotypes). Five of these subgenotypes, namely A1, A2, A3, A4 and B3, were found to be associated with infections of humans, of dogs and of calves; these data, therefore, supported the role of these animals as a source of infection for humans.

Keywords: *Giardia duodenalis*; Zoonotic transmission; Molecular typing; β-giardin; Human.

1. Introduction

Giardia is a genus of intestinal flagellates that infect a wide range of vertebrate hosts. The genus currently comprises six species, namely: *Giardia agilis*, *Giardia ardeae*, *Giardia duodenalis*, *Giardia microti*, *Giardia muris* and *Giardia psittaci*, which are distinguished on the basis of the morphology and ultrastructure of their trophozoites (Adam, 2001).

G. duodenalis (syn. *G. intestinalis*, *G. lamblia*) is the only species found in humans, although it is also found in other mammals, including pets and livestock (Thompson et al., 2000). This protozoan produces robust cysts, which are voided in the faeces and transmitted directly through faecal/oral contact, or by ingestion of contaminated water and food. Water has been increasingly recognised as an important vehicle, and many waterborne outbreaks have been reported in economically developed countries (Slifko et al., 2000). A considerable amount of data has shown that *G. duodenalis* should be considered as a species complex,

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whose members, albeit morphologically identical, can be assigned to at least seven distinct Assemblages based on genetic analyses (Monis et al., 2003). Only Assemblages A and B have been detected in humans and in a wide range of other mammalian hosts, whereas the remaining Assemblages (C to G) are likely to be host-specific (Monis et al., 1999, 2003; Sulaiman et al., 2003). However, the role played by animals in the transmission of the infection to humans is still uncertain (Monis and Thompson, 2003).

In the present study, *G. duodenalis* isolates from humans and animals (pets and livestock) from Italy were genetically characterised at the β-giardin locus in order to evaluate the potential zoonotic transmission of *Giardia* infection.

2. Materials and methods

2.1. Source of isolates

Human faecal samples ($n=37$) were collected at day hospitals in central and northern Italy (Rome, Padua, Pisa and Perugia), from March 2002 to February 2004. Samples were collected from 15 females and 22 males, who ranged from 8 months to 84 years in age. Reference strains WB and Nij5, previously typed as Assemblages A and B, respectively (Cacciò et al., 2002), were included for comparison. Dog faecal samples ($n=21$) were collected in kennels, pounds and from dogs of private owners in north-eastern Italy (Milan, Padua and Vicenza) in 2003. Two canine reference strains (dog7 and dog12) collected in Australia and typed as Assemblage C (Read et al., 2004), were included for comparison. A cat faecal sample was collected in north-eastern Italy (Rovigo) in 2004. Calf faecal samples ($n=24$) were collected in farms from central Italy (Lazio and Marche) in 2003 and 2004. The reference strain P15, of pig origin, was included as a representative of Assemblage E (Cacciò et al., 2002). Samples were stored for 1–3 months at 4 °C or at −20 °C before DNA extraction. An aliquot of faeces (about 5 g) was filtered through a mesh, washed with phosphate buffered saline (PBS), and resuspended in 20 ml of PBS. The presence of cysts was assessed by immunofluorescence (IF) microscopy using FITC-conjugated wall-specific antibodies (Merifluor, Meridian Bioscience, Cincinnati OH, USA).

2.2. DNA isolation and PCR amplification

DNA was extracted directly from concentrated faecal material containing a large number of cysts, according to the method of Da Silva et al. (1999). Briefly, an aliquot (0.4 ml) of concentrated faecal material was homogenised using the FastPrep 120 instrument (Savant, Thermo Electro Corporation, Woburn MA, USA). The DNA released from disrupted cysts was purified using the FastDNA kit (Qbiogene, Illkirch Cedex, France), and stored at 4 °C.

In the case of faecal samples containing a low numbers of cysts, a second concentration step was performed. Briefly, 1 ml of faecal material was diluted 1:10 with double distilled water, and the cysts were purified using immunomagnetic beads (Dynabeads GC-Combo kit, DYNAL, Norway), according to the manufacturer's instructions. Recovered cysts were resuspended in 25 µl of lysis buffer (10 mM TRIS–HCl pH 8.3; 50 mM KCl; 0.5%Tween 20), and lysed by ten freeze/thaw cycles. Cellular debris was pelleted, and the supernatant was used for PCR.

The amplification of the β-giardin gene was performed using a nested PCR protocol. In the primary PCR reaction, a 753 bp fragment was amplified using the forward primer G7 and the reverse primer G759, as previously described (Cacciò et al., 2002). In the sequential nested PCR reaction, a 511 bp fragment was amplified using the forward primer 5′-GAACGAACGAGATCGAGGTCCG-3′ and the reverse primer 5′-CTCGACGAGCTTCGTGTT-3′. The primers were chosen to match two conserved regions in a multiple alignment of β-giardin nucleotide sequences representing Assemblages A, B and E. In the analysis of dog and cat isolates, the identity of the *G. duodenalis* Assemblages was further assessed by the analysis of two genetic loci previously described. In particular, a 292 bp fragment of the small subunit ribosomal RNA (ssrRNA) gene was amplified using the conditions described by Hopkins et al. (1997), and a 220 bp fragment of the glutamate dehydrogenase (GDH) gene was amplified by the nested PCR assay described by Abe et al. (2003). In all cases, the PCR mix consisted of 1X buffer containing 1.5 mM $MgCl_2$, 200 µM of each dNTP, 10 pmol of each primer, 2.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Monza, Italy), and 1–5 µl of purified DNA in a final volume of 50 µl. PCR was performed as follows: after an initial denaturation step of 15 min at 95 °C, a set of 35 cycles was run, each consisting of 30 sec at 95 °C, 30 sec of annealing (65 °C for the primary β-giardin PCR, 55 °C for the nested β-giardin, 59 °C for the ssrRNA, 55 °C for the primary GDH, and 59 °C for the nested GDH) and 60 sec at 72 °C, followed by a final extension of 7 min at 72 °C. PCR products were electrophoresed on ethidium bromide-stained 1% agarose gels.

2.3. DNA sequencing and restriction analysis

PCR products were purified using the Qiaquick purification kit (Qiagen S.p.a., Milan, Italy) and fully sequenced using the ABI Prism BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems). Sequencing reactions were analysed on an ABI 310 automatic DNA sequencer (Applied Biosystems). Sequences were assembled by using the program SeqMan II (DNASTAR, Madison WI, USA). Multiple alignment of the nucleotide sequences was performed using Clustal X (Jeanmougin et al., 1998).

Aliquots (8–15 µl) of β-giardin PCR products were digested using 10 U of *Hae* III (New England Biolabs Inc., Beverly MA, USA) in a final volume of 20 µl for 4 h at