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ORIGINAL PAPER

Identification of genotypes of *Giardia intestinalis* of human isolates in Egypt

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Abstract In Egypt, the genotyping study of *Giardia intestinalis* in human is limited. To determine the prevalence of *G. intestinalis*, faecal samples were collected from Egypt. Samples were concentrated using density gradient centrifugation. The samples were subjected to PCR and DNA sequence analysis for TPI gene. Prevalence of *Giardia* infection was 34.6% of 52 examined. DNA sequence showed that the Assemblage B was the most prevalent (80%) genotype, the 15% of the positive samples belonged to Assemblage E, and the 5% of them belonged to Assemblage A. Certainly, both genotypes A and B are highly common in human worldwide. However, up to now, Assemblage E had not been known to be infectious for humans. Therefore, this is the first time that Assemblage E is reported in human. However, further analyses of a second locus are required to confirm this result. The extent to which *Giardia*-infected cattle in Egypt might pose a risk of human infection is unknown.

Introduction

Giardiasis is a common cause of diarrhoeal disease in almost all vertebrates, including humans. In developing countries in Asia, Africa and Latin America, approximately 200 million people have symptomatic giardiasis (Thompson et al. 2000; Yason and Rivera 2007).

Based on morphology, six species of this genus are considered valid, one of them is *Giardia intestinalis* (syn. *G. lamblia* or *G. duodenalis*) in a wide range of mammals, including humans, livestock, and companion animals (Sulaiman et al. 2003). However, Thompson and Monis (2004), based on phenotypic and genetic studies, proposed that there are eleven species that should be recognised in the genus *Giardia*.

A number of molecular genetic techniques have been employed successfully to characterize *Giardia* isolates (Thompson and Monis 2004). In the case of *G. intestinalis*, molecular studies have demonstrated that comprises at least eight major genotypes or assemblages (Thompson and Monis 2004), some of which appear to have restricted host ranges (Monis et al. 1999, 2003). Only two of these Assemblages, A and B, have genotypes that have been isolated from humans (Thompson and Monis 2004).

Nucleotide sequence of the triosephosphate isomerase has been used to genotype *G. intestinalis* (Sulaiman et al. 2003) because of the high genetic heterogeneity displayed by *Giardia* spp. at this locus (Monis et al. 1999). Sulaiman et al. (2003) confirmed, basing on TPI sequences, that some animal isolates of *G. intestinalis* are of zoonotic potential, and they suggest that TPI should be useful in the detection, differentiation, and taxonomy of *Giardia* spp.

The current study was conducted to assess the prevalence of giardiasis in humans in Egypt and to identify the genotypes that were present. The isolates have been genotyped basing on the TPI nucleotide sequence to elucidate the prevalence of each genotype, and to evaluate the potential transmission of *G. intestinalis* from animals to humans in this area.

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Materials and methods

In September 2005, a total of 52 samples of faeces conserved in pottasic dichromate from Egypt were obtained. Thirteen samples were from Ambria (Ba), 27 from Gharbia (D, Ma), and 12 from Kafr El Sheik (Ds, Qu).

The faeces were processed for the study. In the first side, they were mixed with PBS solution and passed through a 45 μm screen. Then, they were concentrated by a PBS-ether sedimentation method (Peeters and Villacorta 1995).

From that point, a molecular study was carried out in order to determine the presence of several protozoa groups. DNA extraction was performed using the Fast DNA Spin (BIO 101 Systems) kit, following the manufacturer's instructions. Extracted DNA was stored at 4°C.

PCR amplifications were carried out using specific primers to amplify a fragment of both *Cryptosporidium* and Microsporidies ssU rRNA gene, and *Giardia* sp. triosephosphate isomerase (TPI) gene, as was previously described by Abreu-Acosta et al. (2005; 2007) and Sulaiman et al. (2003), respectively. The amplifications were carried out in a MyCycler thermocycler (Biorad, Hercules, CA). PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining.

PCR products were purified by using QIAX II Gel Extraction (150; Qiagen, Hilden, Germany) and cloned using the pGEM-T Easy vector (Promega, Wisconsin), following the manufacturers' instructions, and then transformed into *Escherichia coli* XLB-2 competent cells (Invitrogen, Groningen, The Netherlands). The clones were selected by picking seven colonies and were checked for correct insertion size by direct PCR amplification with T7 and sp6 primers. Correct plasmids were purified by QIAprep Spin Miniprep kit (Qiagen) according to the manufacturers' instruction and sequence accuracy was confirmed by two-directional sequencing and by sequencing of a new clone if necessary.

To elucidate any homologies or similarities previously published in GenBank, a BLASTA search (WUBLAST) was carried out. New and published sequences were aligned with the multiple alignment program ClustalW in Mega 3.1 (Kumar et al. 2004) and minor corrections were made by hand in order to increase the aligned sequence similarity and improve inferences on positional homology. *Giardia muris* (AF069565) was used as the outgroup. Positions corresponding to regions of uncertain alignment were always excluded from the analysis.

Neighbor-joining distance (NJ) method was used to estimate phylogenetic relationships, using Mega 3.1 software program. The evolutionary distances were calculated by Kimura two-parameter model (Kimura 1980) for nucleotide substitution. Relative support for clades was assessed by bootstrap resampling using 1,000 replicate data sets. Supplemental TPI sequences were obtained from GenBank: A4368164, A4368167, AY368157, AY655706, LO2116 and DQ157270.

Results

All the samples were negative for *Cryptosporidium* and Microsporidia. However, 34.6% (18 of 52) of the samples were positive for *Giardia* sp. showing an amplification of around 500 bp. The samples named Ma1 and Ds2 showed mixed genotype infection, with two different assemblages each.

TPI sequences were obtained and submitted to GenBank under the following accession numbers: EU272151–EU272170.

After performing a BLAST search with the obtained sequences, high similarities with *G. intestinalis* (GenBank AY368164, and AY368167 obtained from wastewater of Milwaukee, Wisconsin; AY655706 from *Bos taurus* from USA; LO2116; and DQ157270 from cattle from China) were revealed. Obtained sequences were identified as A, B and E by comparing to GenBank sequences of *Giardia* genotypes.

The bootstrap consensus tree obtained by NJ method yielded 3 monophyletic groups clearly separated from the outgroup (Fig. 1). Each node includes sequences from this study and sequences from GenBank data base corresponding with three different genotypes, assemblages A, B and E. The first node is formed by sequences that belong to assemblage B in accordance to Sulaiman et al. (2004). The phylogenetic tree shows that assemblage B was the most frequent one, corresponding with 16 cases (80%; Ba3, Ba4, Ba7, Ba12, Ba13, D2, D7, D13, Ds2, Ds2′, Ma1, Ma4, Ma6, Ma11, Ma13 and Qu4), the monophyly of this node is supported by strong bootstrap (99%).

The second more frequent genotype was the Assemblage E with three samples (D1, D6 and Ma1′; 15%). In this case, the monophyly of the node is also well supported (73% bootstrap). The Assemblage E clade consists of two principal subclades. The first one is formed by D6 sample sequence. The other one includes four sequences, two belong to sequences from GenBank that are more related between them (70%) than with another, being one of them previously identified as Assemblage E by Trout et al. (2004), and the other is an isolate from China not genotyped previously. These latest two are sister groups with D1 with a bootstrap of 66%, and all these three form a node with Ma1′ with a strong bootstrap (99%).

Finally, Assemblage A was found only in one (5%) of the studied samples, named D12. Furthermore, this node has a high bootstrap (99%) that supports the monophyly of this sequence and the sequence from GenBank genotyped as Assemblage A by Sulaiman et al. (2004).

The highest prevalence of *G. intestinalis* was found in Gharbia, with 44.4% of the faeces positive, following by

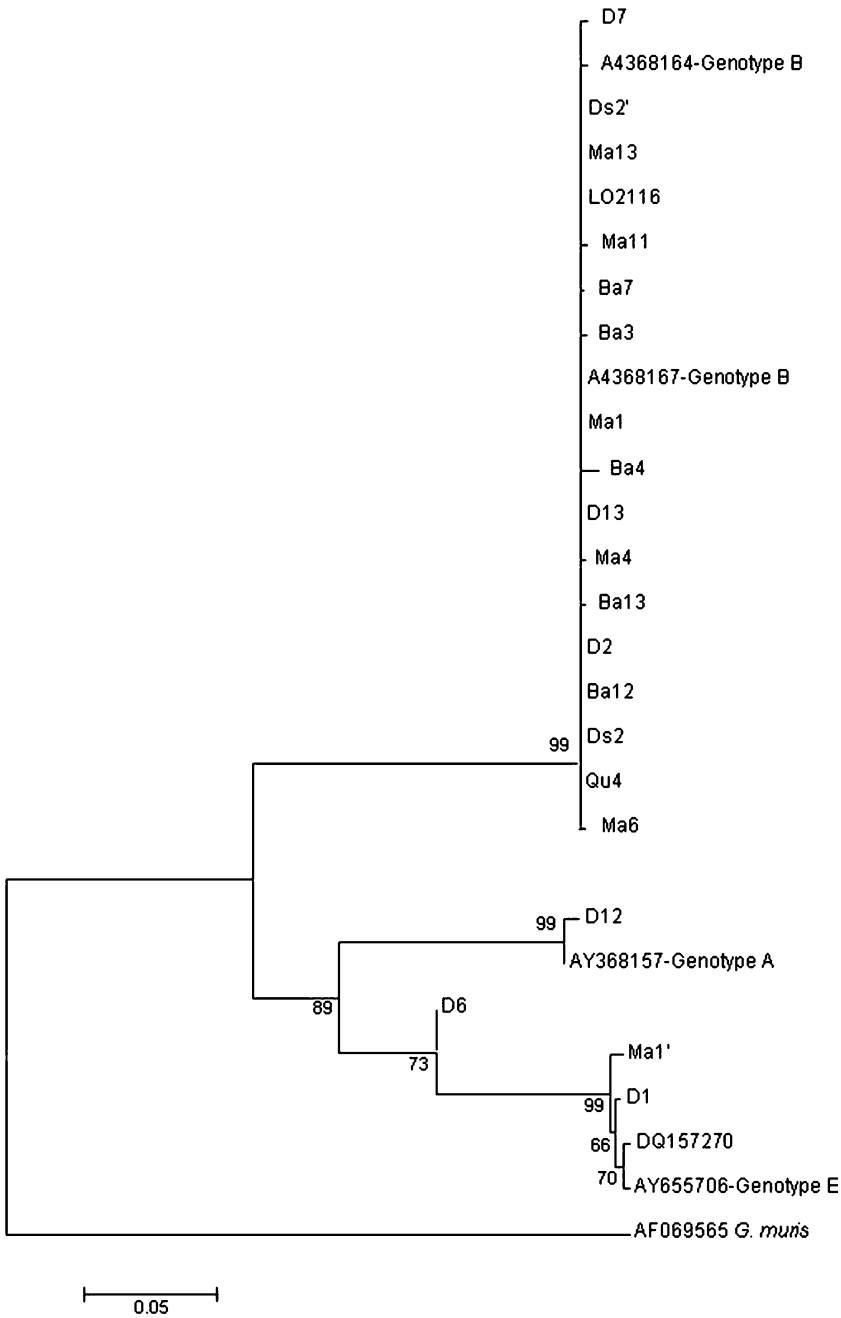

Fig. 1 Phylogenetic relationship of the human isolates of *G. intestinalis* genotypes from Egypt as inferred by neighbor—joining analysis, based on the nucleotide sequences of TPI. Numbers on branches are bootstrap values of 1,000 replicates

Ambria 38.5%, and Kafr El Sheik 25%. In the last two places, 100% of the positive samples were Assemblage B. However, in Gharbia, only the 66.7% of the samples with *G. intestinalis* were Assemblage B, the rest belonged to Assemblage E, 11.1%, and Assemblage A, 3.7%.

Discussion

The percentage of people expelling *Giardia* cysts was 34.6%, slightly higher than previous studies in Africa, where prevalences of 11.7% in Morocco (El Kettani et al. 2006), 29% in Sierra Leone (Gbakima et al. 2007), 17.3% in South Africa (Adams et al. 2005) were reported, and to other countries where, for example, prevalences of 23–48% in Albania (Berrilli et al. 2006), and around 20% in USA (Yoder and Beach 2007) were cited. However, this fact could be explained based on the employed detection method. Authors have detected differences between the prevalences of protozoa when they use morphological and molecular methods, obtaining higher prevalences with the molecular one. Therefore, prevalence data based on morphological detection most certainly underestimate true prevalence because of its low sensibility (Abreu-Acosta et al. 2007).

On the other hand, the most frequent genotype in human studies is Assemblage B, as in this study. Amar et al. (2002) studied human clinical samples in UK, and found that 64% were Assemblage B, 27% were Assemblage A, and the remainder was a mixture of Assemblage B and Assemblage A. Similarly, an institutional survey in Australia found that infections with Assemblage B were more prevalent (70%) than Assemblage A (30%; Read et al. 2002). The Assemblage B genotype was found to be responsible for an outbreak in a nursery in the UK where 88% of the cases were infected with this genotype (Amar et al. 2002). In India, the proportion of Assemblage B and A infections in people was 61% and 39%, respectively (Traub et al. 2004).

In Egypt, there are previous epidemiological data about *G. intestinalis* in humans. On one hand, there are several works about people with diarrhoea, in this case, Zaki et al. (1986) found *G. intestinalis* in 44% of the population studied in rural zones and Shukry et al. (1986) in 33% of the people in El Cairo. Secondly, Curtale et al. (1998) and Fawzi et al. (2004) studied faecal samples and detected *G. intestinalis* in 24.7% of the samples in Behera Governorate, and in 10.4% in El-Prince, Alexandria, respectively. Finally, Azab et al. (1991) found antibodies against *G. lamblia* in samples from women from Egypt and Mahmud et al. (2001) in infants in Bilbeis.

However, to our knowledge, despite the work of El-Shazly et al. (2004), there has been no previous prevalence studies of *Giardia* conducted in Egypt that includes genotyping analysis of isolates in humans. All the works cited above, only determine it to specific level.

El-Shazly et al. (2004) detected genotypes I and II that belong to Assemblage A, as well as Genotype III that belongs to Assemblage B, in humans. In the present study, the same assemblages were found. However, there are no previous data about the presence of Assemblage E. Therefore, this is the first report of Assemblage E in humans in Egypt. However, these must be considered as preliminary results, since the analysis based on another locus is necessary to confirm the *Giardia* genotyping.

Molecular characterisation of *Giardia* isolated from different host species has revealed the existence of a number of distinct genotypic assemblages, some of which appear to have distinct host preferences or have a limited host range. Only two of these assemblages A and B, have genotypes that have been isolated from humans (Thompson and Monis 2004). Assemblage B genotypes appear to be largely human specific although there are related genotypes within Assemblage B that have been isolated from animals. According to Castro-Hermida et al. (2006), Genotype A has been detected in humans and domestic ruminants over the widest geographical range. However, similar to Assemblage B, isolates within genetic group II of Assemblage A also appear to be human specific while other genotypes within Assemblage A appear to be restricted to animal hosts. Only one genotype of *G. intestinalis*, genetic group I from Assemblage A, has been isolated from both human and animal hosts (Thompson and Monis 2004).

In the case of Assemblage E, it is common in bovine livestock, particularly, cattle (Thompson and Monis 2004; Trout et al. 2004) and in other domestic ruminants including ovine livestock (Becher et al. 2004; Hunter and Thompson 2005; Ryan et al. 2005; Trout et al. 2005) and goats (Ruiz et al. 2008). Up to now, there are no epidemiological or genetic data showing that genotype E infects humans and therefore it is not considered to be zoonotic (Thompson 2003). However, in this study the presence of Assemblage E in human faecal samples is demonstrated. Furthermore, the fact that this assemblage was found only in the area of Gharbia is interesting. Gharbia is located in the centre of the Nile Delta and two thirds of the inhabitants belong to a rural community, in which people commonly live in close contact with their livestock (Ministry of Health and Population 2002). Therefore, livestock should be considered as a potential source of human infectious cysts in the environment. Although several authors (Hoar et al. 2001; O'Handley et al. 2000) indicate that the risk to public health from calves infected by *G. intestinalis* may be minimal, others (Thompson and Monis 2004; Trout et al. 2005) have cited that if cattle can harbour Assemblages A and E of *G. intestinalis*, they must be considered as a potential source of human infective cysts.

Further studies based on other locus for genotyping are required to confirm these results. In this case, the analyses of samples from livestock from the Gharbia area to confirm whether genotype E predominates in it, which would demonstrate the importance of livestock in the transmission of Giardiasis in humans and the possible risk that these animals present to public health, would be of interest. Thus, domestic, farm and wild animals, and environmental samples should be studied to demonstrate the source of the transmission.

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