

**GENOTYPIC AND PHYLOGENETIC CHARACTERIZATION OF
GIARDIA INTESTINALIS FROM HUMAN AND DAIRY CATTLE
IN KAFR EL SHEIKH GOVERNORATE, EGYPT**

By

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Abstract

Giardiasis is a wide-spread intestinal disease of both humans and animals, with no morphological distinction between different assemblages and/or genotypes. The present study aimed to demonstrate the molecular identity of *Giardia intestinalis* parasites in clinical samples collected from infected cases screened at commercial laboratories and neonatal calves at dairy cattle farms, in Kafr El Sheikh. Molecular analysis based on PCR-sequence of triose phosphate isomerase (TPI) gene fragment. Results reported herein indicated that assemblage A was detected in 18/48 human isolates (37%) and 4/18 (22.2%) of the animal derived isolates, with subassemblage AII was the predominant in human samples and AI in the animal isolates. Whereas assemblage B was the predominant assemblage in the human infections (32/48: 66.7%), assemblage E was the major genotype in the animals samples (14/18: 77.8%). Genetic heterogeneity was pronounced feature in the generated sequences of all assemblages referring to wide diversity of *Giardia* population in Egypt. Also, results stressed the significance of neonatal calves as reservoir of zoonotic *Giardia* infections in human. Phylogenetic relationship of the analyzed isolates was inferred based on the reference sequences in the database.

Keywords: Egypt, *Giardia*, Assemblage A, Assemblage B, Assemblage E, Triose Phosphate Isomerase (TPI), Zoonosis.

Introduction

Giardia duodenalis (syn. *G. lamblia* & *G. intestinalis*) is a cosmopolitan enteric protozoan that infects a wide range of vertebrates, including humans, livestock, companion animals or wildlife (Thompson and Monis, 2004; Thompson *et al*, 2008). Giardiasis outbreaks occurred worldwide (Karanis *et al*, 2007), but often the original source

of contamination is not identified. Typically, infection is acquired after the ingestion of highly environmental resistant infective cysts (Feng and Xiao, 2011) by direct fecal-oral route or in contaminated food or water (Thompson, 2004; Tien and Earn, 2010; Plutzer *et al*, 2010). The parasite is recognized as a major etiologic agent of diarrhea in the infected hosts resulting in direct and indirect economic losses in

animals (Aloisio *et al*, 2006) and health consequences in human (Heresi and Cleary, 1997; Chin *et al*, 2002; Troeger *et al*, 2007; Buret, 2008; Cotton *et al*, 2011; Ignatius *et al*, 2012). In the developing countries, children are at most risk from the clinical consequences of *G. duodenalis* infection, (Savioli *et al*, 2006; Mehraj *et al*, 2008), and may be implicated for retarded growth and reduced cognitive function (Fraser *et al*, 2000; Thompson 2001; Thompson and Monis, 2004). Giardiasis was described as a typical poverty disease, whereas compromised hygiene, sociodemographic conditions, contaminated environment and/or direct and frequent contact with the animals may contribute in the high prevalence (~50%) reported in the developing countries versus ~5% in industrialized ones (Thompson, 2000; Thompson and Smith 2011; Santos *et al*. 2012). Studies concerned with the occurrence of *Giardia* in animals show pronounced differences from one to other (Becher *et al*, 2004; Trout *et al*, 2005; Abeywardena *et al*, 2012; Li *et al*, 2012).

Morphologically undistinguished *Giardia* parasites can be assigned to eight distinct assemblages (A to H) based on enzymatic and molecular studies (Monis *et al*, 2003; Wielinga and Thompson, 2007). Available data showed that many *Giardia* lineages are highly host-specific with *G. duodenalis*, assemblages A and B have the widest host ranges, infecting humans, domestic animals and livestock, and terrestrial wildlife (Thompson *et al*, 2000), and marine mammals and birds (Lasek-Nesselquist *et al*, 2008). Other *G. duodenalis* asse-

mbles have more restricted host ranges as assemblage E associated with hoofed livestock (O'Handley *et al*, 2000; Trout *et al*, 2005), assemblages C and D typically isolated from dogs, assemblages F and G infecting cats and rodents, respectively (Monis *et al*, 2003). Assemblage H was isolated from marine vertebrates (Feng and Xiao, 2011). Assemblage-associated differences in the pathogenesis and clinical outcome have been reported both in clinical cases (Homan and Mank, 2001; Read *et al*, 2002; Sahagun *et al*, 2008; Breathnach *et al*, 2010; Ignatius *et al*, 2012) and experimental models (Solaymani-Mohammadi and Singer, 2011; Koh *et al*, 2012).

Although giardiasis is very common in Egypt (Zaki *et al*, 1986; Bakr *et al*, 2009; Baiomy *et al*, 2010; Mousa *et al*, 2010; Badawy *et al*, 2012) limited studies have been done on the molecular characterization of the parasite so far (Foronda *et al*, 2008; Soliman *et al*, 2011). El-Shazly *et al*. (2004) determined *Giardia* genotypes by using sequence differences at triosephosphate isomerase (tip) gene locus divide *Giardia* into three groups. The groups 1, 2 & 3 were assigned by restriction fragment length polymorphism (RFLP) of PCR-amplified DNA extracted from the original stool samples of the parasitologic proved patients with *Giardia*. But, extensive farming practices in the Nile Delta address the public health problem concerns possible of zoonotic transmission.

The present study aimed at demonstration the molecular identity of *G. duodenalis* derived from human iso-

lates, at Kafr El Sheikh Governorate, based on PCR-sequence analysis of triose phosphate isomerase (TPI) gene. The study also extended to ascertain isolates from the nearby farms in order to assess the potential zoonotic transmission and possible implications to public health.

Materials and Methods

The approach of collecting sporadic clinical cases, from infected human and naturally infected calves was adopted in the present study to cover a wide range of possible heterogeneity rather than one point prevalence which might be biased to certain genotype circulate in certain group under study. A total of 48 positive isolates were collected during January-September 2010 from the commercial medical laboratories at Kafr El Sheikh (130 Km North West of Cairo). The samples were obtained after the consent of the patient. Also, 18 positive samples were obtained from neonatal calves (1-8 weeks old) from dairy farm cattle in the same study area. Positive samples were stored at 4 °C in potassium dichromate until molecularly analyzed.

DNA extraction: Positive samples were washed off dichromate with double distilled water. Washed samples were processed as previously described (Amer *et al*, 2009) and DNA was extracted using QIAamp[®] DNA Stool Kit (Qiagen, Maryland, USA), following the manufacturer's instructions.

PCR amplification: Genotyping was done by amplification of a fragment (~530 bp) from triphosphate isomerase (TPI) gene by nested PCR following

previous described protocols (Sulaiman *et al*, 2003). Briefly, PCR was done in 50µl reaction volume utilizing 1 µl of the extracted DNA (2µl primary PCR product for the 2nd PCR) as template. The reaction mixture contained 200 nM of the forward and reverse primers, 200 mM dNTPs, 3.0mM MgCl₂, 10% (v/v) PCR buffer, 0.5 U Taq polymerase and H₂O to a total volume of 50µl. PCR was done under the conditions of 94°C for 5 min to denature the template DNA, followed by 35 cycles of 45s at 95°C, 45s at 50°C (54°C for the 2nd PCR) and 1 min at 72°C. A final step of 10 min at 72°C was included. Secondary PCR products were electrophoresed on 1.5% agarose gel and visualized after staining with ethidium bromide.

DNA Sequencing: Secondary PCR products were purified using Exonuclease I/Shrimp Alkaline Phosphatase (Exo-SAP-IT[™]; USB, Cleveland, OH, USA). Purified products were sequenced using the 2nd PCR primers in 20µl reaction volume using Big Dye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Bio-systems Japan Ltd., Tokyo, Japan) on an automated sequencer (Applied Biosystems 3130xl Genetic Analyzer; Applied Biosystems Japan Ltd.). The accuracy of data was confirmed by two-directional sequencing.

For phylogenetic analysis sequences were aligned using Clustal X software, and phylogeny was inferred by Maximum Likelihood and Maximum Parsimony methods as implemented in the MEGA5 software (Tamura *et al*, 2011).

Nucleotide sequence accession numbers: Nucleotide sequence data report-

ed here will appear in DDBJ/EMBL/ Gene-Bank nucleotide sequence.

Results

Triphosphate isomerase (TPI) gene is a polymorphic gene providing a good target for analyzing and genotyping *Giardia* (Wielinga and Thompson, 2007; Caccio and Ryan, 2008). A total of 48 human-derived *Giardia* isolates and 18 animal-derived ones were successfully amplified (Fig. 1) and subsequently sequenced. Alignment of the generated sequences revealed noticeable intra-sequence variations in form of nucleotide substitutions and no deletions or insertions were observed. Results showed that sequences generated from human isolates belong to assemblage A in 16 sequences and assemblage B in 32 sequences. Intra-variation showed that isolates of assemblage A arranged in 3 different sequence types; whereas isolates of assemblage B generated 11 different sequences. Two genotypes were identified in calves-derived isolates, assemblage A in 4 sequences and assemblage E in 14 sequences. All calves' sequences of assemblage A were identical but different than those generated from human isolates, whereas assemblage E showed 4 different types of sequences.

Blast search revealed high similarity of the generated sequences with the corresponding ones in the Gene-bank (99-100%). Also, analysis of the phylogenetic trees inferred using the Maximum likelihood method were done to show the relationship of the generated sequences with reference ones of the corresponding assemblages (Fig. 2), as

well as with reference sequences belonging to other assemblages (Fig. 3).

The generated sequences with the corresponding references were separated into 3 monophyletic groups; each clade was subdivided into subclades referring to sequence variation (Fig. 2). The first category included all sequences belong to assemblage A, with sequences KF-H17 & KF-H19 clustered with other sequences belonging to sub-assemblage AII including AY368157 and EU518562 derived from waste water, USA and herring gull (*Larus argentatus*) from Canada, respectively. They showed high similarity with sequences AB569403 & AB516351 from human from Japan and GQ444449 from lamb in Australia (99-100% identity). AI sub-assemblage occupied a separate branch with high bootstrap value (88) sequence KF-C8 derived from neonatal calf and JF792423 from sheep in Spain and EU518565 from herring gull (*L. argentatus*) from Canada and GU564274 from human in China. Interestingly, human-derived sequence KF-H21 clearly separated from all other sequences (99) indicating the uniqueness of this sequence. In non-zoonotic assemblage E, 3 sister groups were displayed on the tree with sequence KF-C6 and KF-C7 cluster with EU189331 isolated from goat in Spain; sequence KF-C1 group with AY655705 and KF-C3 assembled with that AY655706 from neonatal calves in USA.

In the third clade, Assemblage B, sequence KF-H1, KF-H5, KF-H10 and KF-H12 assembled with sequence JF918463 derived from human in In-

dia, with KF-H5, KF-H10 more related to each other (bootstrap 50). The clustering pattern indicates that sequences KF-H3, KF-H6, KF-H4, KF-H13, KF-H9, KF-H8 and KF-H7 form a separate subclade with sequence JQ863254 derived from wastewater in China and AY368170 derived from wastewater in USA, with subsequent subdivision indicating intra-sequence variation.

In reference of relationship of obtained sequences with other *Giardia* assemblages (Fig. 3) ML was constructed using known reference assemblages' sequences in the database and *G. muris* as outgroup. The resultant tree formed 2 clear clades, the first one include assemblages A, E & F, whereas assemblages B, D, C & G are more related and clustered in one clade. Also, the tree revealed that assemblage E & F are more close and separate from assemblage A with high bootstrap value (bootstrap 88). Assemblages C & D are more related and assemblage G is clearly separated from assemblage B, in spite of the low bootstrap values. The generated sequences keep their relative positions in the corresponding assemblages with minor modifications than that described (Fig. 2). The five sequences generated from human isolates (one sequence type (KF-H21) belongs to assemblage A and 4 sequence types (KF-H6, KF-H7, KF-H10 & KF-H19) belong to assemblage B and the 4 sequence types belong to assemblage E proved to be novel sequences not reported in Gene-bank before.

Discussion

Molecularly, *G. intestinalis* has been

divided into several assemblages and sub-assemblages that differ in the virulence and clinical outcome (Solaymani-Mohammadi and Singer, 2011; Ignatius *et al*, 2012; Koh *et al*, 2012). In Egypt, giardiasis is very common disease (Zaki *et al*, 1986; Bakr *et al*, 2009; Baiomy *et al*, 2010; Mousa *et al*, 2010; Badawy *et al*, 2012), with little background on molecular level (Foronda *et al*, 2008; Soliman *et al*, 2011). Kafr El Sheikh Governorate is a rural province with extensive animal farm practices creating situation likely favor the transmission from human to animals or vice versa (Traub *et al*, 2004).

In the present study, analysis of 48 sporadic human cases generated 14 different sequence types assembled into 2 assemblages A and B. Assemblage A accounted for 16/48 (33.3%) and assemblage B accounted for 32/48 (66.7%) of the generated sequences. These results are corroborating with those reported by Boontanom *et al*. (2011) and Ferreira *et al*. (2012). On the other hand, Gelanew *et al*. (2007) reported predominance of assemblage A over B in clinical samples from Ethiopia, whereas Souza *et al*. (2007) and Yang *et al*. (2010) reported all analyzed isolates from Brazil and Australia were exclusively belonged to assemblage In Egypt, Foronda *et al*. (2008) and Soliman *et al*. (2011) reported 1/18 and 1/15 as assemblage A/B. The relatively high proportion of assemblage A in the present study might be attributed to rural nature of Kafr El Sheikh City and the frequent contact with farm animals which may favor zoonotic transmission (Traub *et al*, 2004). In this re-

gard, it is reported that the host distribution of assemblage B is predominantly human and to a much lesser extent to other animals (Winkworth *et al.*, 2008; Sprong *et al.*, 2009; Lim *et al.*, 2012). The factors responsible to the high occurrence of assemblage B over A lineage are still obscure. Also, Kohli *et al.* (2008) found that assemblage B has a high cyst excretion pattern that facilitated oral-fecal transmission and contributed to make genotype B the most prevalent with the highest dispersion.

In spite of intra-sequence variation, assemblage A sequences retrieved from clinical samples were assigned to sub-assemblage AI and AII. Results of previous studies in various areas have shown that humans are mostly infected with AII, although AI is also seen in some areas or studies (Sahagun *et al.*, 2008; Xiao and Fayer, 2008). However, there is still debate about the anthroponotic and zoonotic potential of assemblage A infections in dairy cattle (Mark-Carew *et al.*, 2012).

The present results revealed that assemblage E was the predominant genotype (14/18: 77.8%), whereas assemblage A showed low occurrence (4/18: 22.2%). These results are in agreement with those reported from dairy cattle (Thompson and Monis 2004; Trout *et al.* 2004; Abeywardena *et al.*, 2012; Mark-Carew *et al.*, 2012), in beef cattle (Santin *et al.*, 2012), sheep (Becher *et al.*, 2004; Hunter and Thompson 2005; Ryan *et al.*, 2005; Trout *et al.*, 2005; Robertson *et al.*, 2010), and goat (Ruiz *et al.*, 2008; Lim *et al.*, 2012). On the contrary, Santin *et al.* (2009) detected

only assemblage E in cattle to 18 weeks of age in USA. Besides, predominance of assemblage A over E was reported in lambs in Spain (Gomez-Munoz *et al.*, 2012). Assemblage A sequences derived from calves' isolates of the present study belongs to sub-assemblage AI. These findings agreed with Caccio and Ryan (2008), Xiao and Fayer (2008) and Sprong *et al.* (2009) that sub-assemblage AI is preferentially found in livestock and to lesser extent in other animals (Li *et al.*, 2012). However, this sub-assemblage still has the potential zoonotic transmission to human (Xiao and Fayer, 2008).

The present results showed high degree of heterogeneity in the form of nucleotide substitutions resulted in high number of different sequences. Similar findings were reported by Li *et al.* (2012), Zhang *et al.* (2012). In this regard, several explanations were in debate including recombination/genetic exchange between homologous loci from different assemblages was identified in at least three *G. duodenalis* isolates (Lasek-Nesselquist *et al.*, 2009) or the same assemblage (Takumi *et al.*, 2012). Also, heterozygosity played important role in the sequence variation (Ankarklev *et al.*, 2012).

Notably, it is well established that assemblage E is not zoonotic genotype of *G. intestinalis* (Thompson, 2003). But, Foronda *et al.* (2008) reported assemblage E in 15% of the clinical samples from Egypt, the observation not reported anywhere else till now. In conclusion, results reported herein indicate that pre-weaned calves still act as res-

ervoir for zoonotic infection of *G. intestinalis* assemblage A, and may have public health concerns.

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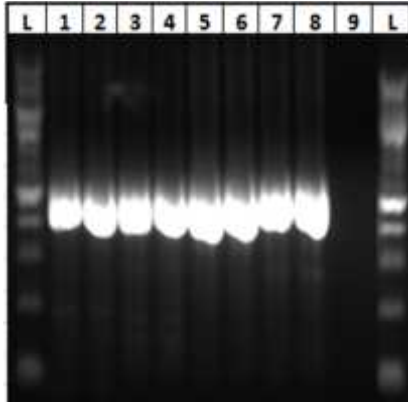


Fig. 1: PCR amplification of ~530 bp of TPI gene fragment. L, 100 bp ladder; numbers of 1-8 correspond to test samples; lane no 9 refer to negative control.

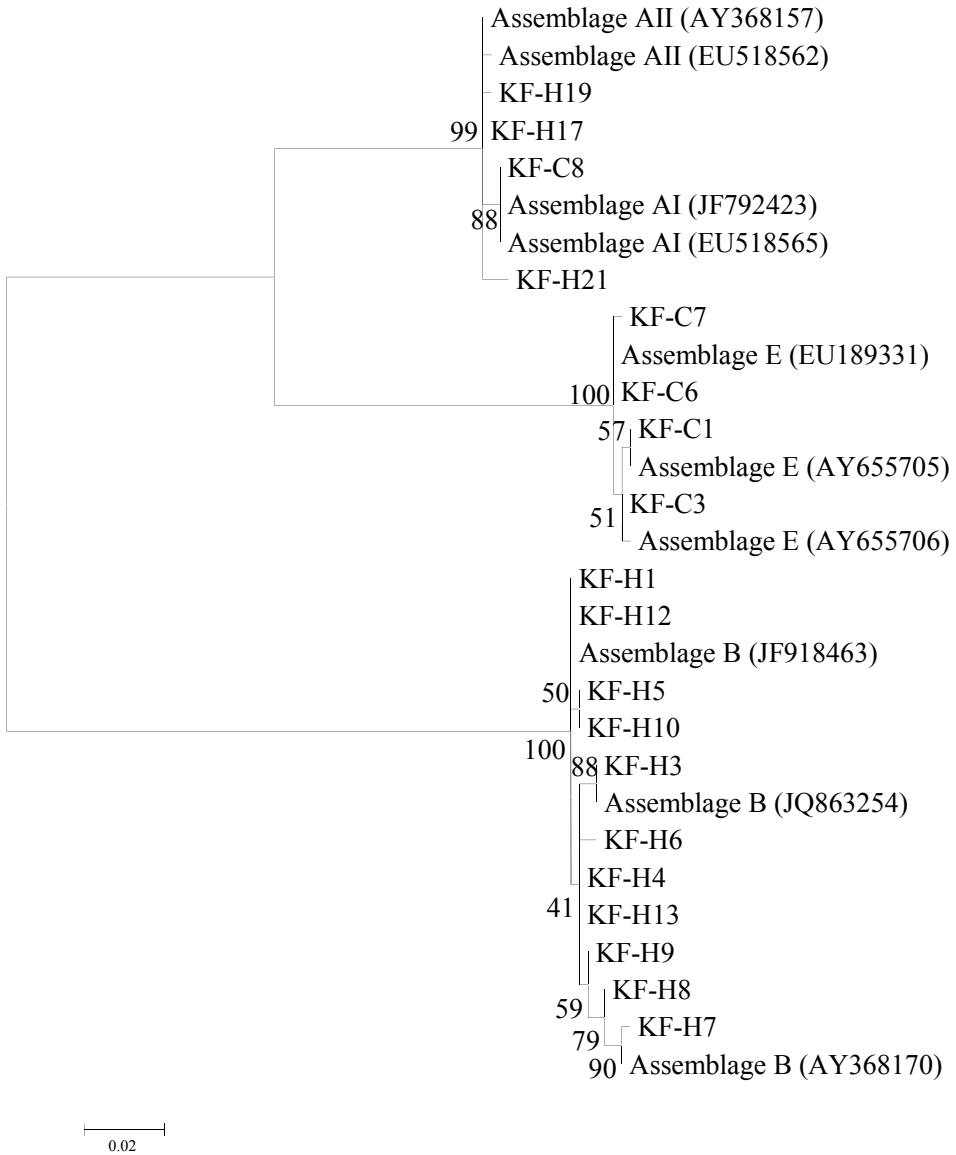


Fig. 2: Relationship of analyzed isolates to corresponding *Giardia intestinalis* assemblages based on sequences of the partial Triose Phosphate Isomerase (TPI) gene. Evolutionary relationships of 29 taxa inferred using Maximum Likelihood method (Tamura and Nei, 1993). Numbers at internodes correspond to percent bootstrap values from 2,000 replicates. Evolutionary distances computed using Maximum Composite Likelihood (MCL) method (Tamura et al., 2011). Tree unrooted.

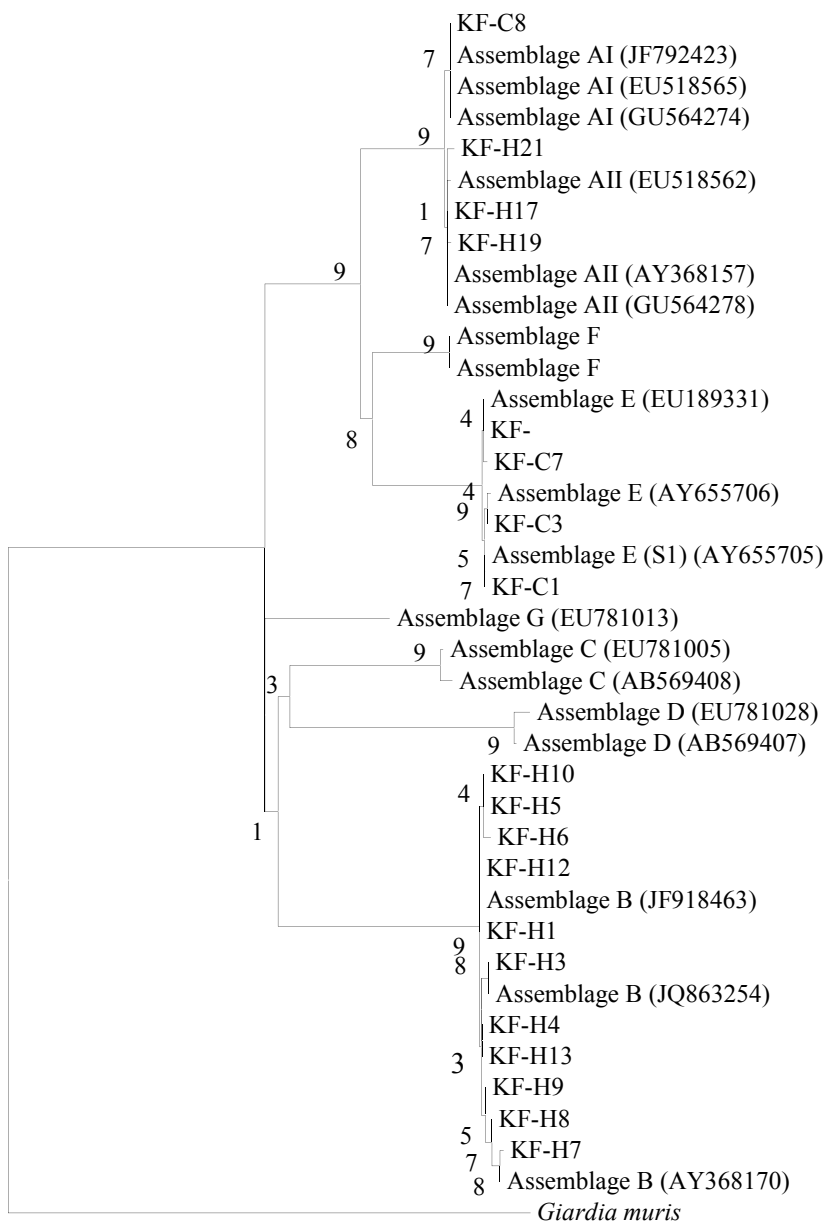


Fig. 3. Phylogenetic relationship *Giardia intestinalis* based on sequences of partial Triose Phosphate Isomerase (TPI) gene. Evolutionary relationships of 39 taxa were inferred using Maximum Likelihood method (Tamura and Nei, 1993). Numbers at internodes correspond to percent bootstrap values from 2,000 replicates. Evolutionary distances were computed using Maximum Composite Likelihood (MCL) method (Tamura et al, 2011). *Giardia muris* was used as outgroup.