Molecular genotyping of *Giardia duodenalis* in municipal waste workers in Ahvaz, southwestern Iran

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Received 28 February 2018; received in revised form 7 July 2018; accepted 12 October 2018

**Abstract.** *Giardia duodenalis* is one of the most common intestinal parasites in a wide range of vertebrates, including humans and animals. It is estimated that there are approximately 200 million symptomatic giardiasis per year globally. The aim of the present study was to evaluate the prevalence and molecular diversity of *G. duodenalis* in municipal waste workers in Ahvaz County, southwestern Iran. This cross-sectional study was conducted among municipal waste workers aged 21 to 59 years in Ahvaz County, southwestern Iran in 2015. Stool samples collected from 400 workers were examined initially by microscopy and sucrose flotation methods, and then *G. duodenalis* isolates were confirmed by SSU rRNA and subsequently the genotypes were identified by triose phosphate isomerase (*tpi*) gene of the parasite. In total, a prevalence of 4.0% was found for *G. duodenalis* by microscopy and sucrose flotation methods. All microscopic-positive samples were successfully amplified at the SSU rRNA gene while *tpi* gene was amplified in 13 (81.25%) samples. Out of the 13 amplified isolates at *tpi* gene, 10 (76.9%) were typeable while the other three (23.1%) were untypeable. Assemblage A, sub-assemblage AII, was found in nine (69.2%) typeable isolates, and one (7.7%) was assemblage B, sub-assemblage BIII. The current study represents the first molecular epidemiological data on the occurrence and genotypic diversity of *G. duodenalis* in municipal waste workers in southwestern Iran. Assemblage A, sub-assemblage AII was the predominant assemblage of *G. duodenalis*. Since all infected subjects were asymptomatic, periodic examinations are recommended to prevent and control giardiasis.

**INTRODUCTION**

Giardiasis, one of the most common protozoan diseases with a worldwide distribution, is considered a neglected tropical disease (Savioli *et al.*, 2006). It is caused by *Giardia duodenalis*, an enteric protozoan of humans, and domestic and wild animals (Thompson, 2000). This cosmopolitan protozoan is one of the most common agents of diarrhea in developing countries (Ramírez *et al.*, 2015). In developed countries, *G. duodenalis* has been also reported from 2% to 7% in different populations (Fletcher *et al.*, 2012). Children, especially those living in developing countries and rural areas, are more at the risk of *Giardia* infection (Ramírez *et al.*, 2015). Approximately 200 million people in Asia, Africa, and Latin America experience symptomatic giardiasis, and according to the World Health Organization (WHO), about 500,000 new cases have been reported annually (Savioli *et al.*, 2006). *Giardia duodenalis* is mainly transmitted indirectly through the fecal-oral route by ingestion of contaminated food, water, and also by direct contact with domestic animals and infected individuals (Robertson *et al.*, 2010, Sprong *et al.*, 2009).

Currently, *G. duodenalis* has been classified into eight assemblages (A to H),
which differ in host range and specificity. Assemblages A and B are considered zoonotic. Other assemblages have strong animal host specificity; however, assemblages C–F are sporadically isolated from human infections. Based on isoenzyme and phylogenetic analyses, *G. duodenalis* is classified into at least five sub-assemblages (AI-III, BIII-IV) (Feng & Xiao, 2011).

In Iran, several studies have been carried out on giardiasis in recent years (Hazrati Tappeh et al., 2014; Hooshyar et al., 2017, Rayani et al., 2014). Based on socioeconomic status and geographical location, the prevalence rate of giardiasis was reported from 1.4% to 59.6% (Hatam-Nahavandi et al., 2017). However, data on the prevalence of *G. duodenalis* in human communities in Khuzestan Province are insufficient and available data on the molecular diversity of *G. duodenalis* is limited to a single study (Roointan et al., 2013).

Therefore, the current study aimed to evaluate the prevalence and genotyping of *G. duodenalis* among Ahvaz municipal waste workers, southwest Iran. Municipal solid waste (MSW), which contains household and commercial waste, could be contaminated with infectious agents such as bacteria, fungi, viruses, and parasites (Alvarado-Esquivel et al., 2008; Athanasiou et al., 2010). In Iran, collecting solid waste from households, hospitals, factories, or cleaning streets and green spaces is performed by municipal waste workers. Thus, workers who are involved in collecting solid waste are at risk of infection.

**MATERIALS AND METHODS**

**Ethics statement**
The protocol of this study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (Approval No IR.AJUMS.REC. 2015-244).

**Study area**
This cross-sectional study was conducted among municipal waste workers in Ahvaz County, Khuzestan Province, southwestern Iran in 2015. Ahvaz, the biggest city of Khuzestan Province, has an estimated population of 1,112,021 million people and extends over an area of 528 km². The average annual rainfall in Ahvaz is around 250 mm. It is located at 31°19’2 N 40°00’2 E and has an elevation of 20 meters above sea level. The climate is warm and dry, and occasionally humid in summer (Zarasvandi et al., 2011). Ahvaz is divided into seven municipal districts, and approximately 2419 workers are responsible for collecting solid waste in the county.

**Study population, fecal sample collection and processing**
Of the 2419 municipal waste workers aged 21 to 59 years in Ahvaz County, southwestern Iran, 400 workers were selected randomly. All participants were male (in Iran, all municipal waste workers must be male). Initially, information about the aims of the study was provided to the participants, and then they were asked to complete a questionnaire. The associated risk factors with *Giardia* transmission, such as age, educational status, and clinical symptoms were included in the questionnaire. A plastic stool container labelled with individual identification was given to each participant. They were asked to return the stool sample the following day. The collected fecal samples were transported to the Department of Parasitology, Ahvaz Jundishapur University of Medical Sciences. Samples were processed on the same day of collection.

**Microscopic examination and sucrose flotation method**
For the detection of intestinal parasites, all stool samples were examined microscopically using normal saline and lugol’s iodine stain at 100X and 400X magnification. Additionally, the isolation of *Giardia* cyst was performed on all samples by sucrose flotation (1 M) method. Approximately 5 grams of each fecal sample was suspended in 50 mL of tap water and mixed thoroughly. The fecal suspension was passed through a four-layer surgical gauze (10×10 cm) and centrifuged at 1000× g for 5 min. After discarding the supernatant, 30 mL distilled water was added to the pellet and completely homogenized.
with vortex. The suspension was gently added to 15 mL of sucrose solution and centrifuged for 10 min at 800× g. Thereafter, the superior layers (upper and middle layers) were transferred to a 50 mL clean tube, and the final volume was reached to 50 mL with distilled water and centrifuged for 5 min at 1000× g. The supernatant was discarded, and the sediment was washed three times with 50 mL distilled water and then centrifuged at 1000× g for 5 min. The obtained pellet was examined microscopically using normal saline and lugol’s iodine stain at 100X and 400X magnification. Approximately 200 µL of the sediment containing G. duodenalis cysts were transferred to a 2 ml microtube and stored at -20°C until further analyses.

DNA extraction
Genomic DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Before extraction, seven cycles of freeze/thaw (7 min in liquid nitrogen and 7 min in boiling bath each time) were performed to disrupt the cyst wall. DNA extracts were eluted in 200 µL elution buffer and stored at -20°C until further analyses.

Molecular detection
Nested PCR was conducted for amplification of the small subunit of ribosomal RNA (SSU rRNA) and tpi genes to identify G. duodenalis according to previous descriptions (Gillhuber et al., 2013; Sulaiman et al., 2003). A fragment of 130-bp of the SSU rRNA gene was amplified using the primer pairs RH11 (52-CATCCGGTGCATCCTGCC-32) and RH4 (52-AGTCCAACCTGATTCTCCGCAGG-32) in the primary reaction, and GiarF (52-GACGCTCTCCCCAAGGAC-32) and GiarR (52-CTGCGTCACGCTGCTCG-32) in the secondary reaction (Gillhuber et al., 2013). The PCR reaction was conducted in a 25 µL reaction using 12.5 µL of the Taq DNA Polymerase (Ampliqon-Biomol, Hamburg, Germany), 3 µL of template DNA for the primary and 1 µL for the secondary reactions, 1 µL of each forward and reverse primers (10 µM), and 7.5 and 9.5 µL of distilled water for the primary and secondary reactions, respectively (Gillhuber et al., 2013). A nested PCR for the amplification of a 530-bp fragment of tpi gene was performed using the primer pairs AL3543 (52-AAATIATCGCTGCTCGG-32) and AL3546 (52-CACCACTCCGCAAACC-32) in the primary reaction, and AL3544 (52-CCCTGCTGCTCGTCG-32) and AL3545 (52-GTGCCACCACCCCGTGCC-32) in the secondary reaction (Sulaiman et al., 2003). PCRs were carried out in 25 µL volume containing 12.5 µL Taq DNA Polymerase (Ampliqon-Biomol, Hamburg, Germany), 3 and 2.5 µL of template DNA for the primary and secondary reactions, 1 µL of forward and reverse primers (10 µM), and 7.5 and 8 µL of distilled water for the primary and secondary reactions, respectively (Sulaiman et al., 2003). The PCR conditions were identical to those described previously (Gillhuber et al., 2013; Sulaiman et al., 2003). PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide, and visualized using a UV transilluminator imaging system (Syngene, Cambridge, UK).

Sequencing
PCR products of tpi gene of G. duodenalis were directly sequenced at the Bioneer Co. (Daejeon, South Korea). Sequences were edited and aligned using ClustalW in MEGA software version 7.0. The phylogenetic tree was constructed using the neighbour-joining (NJ) method.

Statistical analysis
The statistical analyses were performed using SPSS software version 21 (SPSS Inc., Chicago, IL, USA).

RESULTS
The mean age of the participants was 35.6 (standard deviation: 7.8). The overall prevalence of intestinal parasites was 15.0% (60/400), of which Blastocystis hominis (45/400; 11.2%) was the most common parasite, followed by G. duodenalis (16/400; 4.0%) and Hymenolepis nana (3/400; 0.7%) infections.
The maximum and minimum infection rate belonged to 20-40 (12/400; 3.0%) and 51-60 (1/400; 0.25%) age groups, respectively. *Giardia* infection was not significantly associated with age among the participants ($p>0.05$). Educational status was another variable that was taken into consideration in this study. Contrary to expectation, workers with high school educational level showed the highest (8.6%) and those attending middle school showed the lowest rate (1.5%) of *Giardia* infection. Although a statistical difference was not found in terms of infection and district ($p=0.53$), the workers belonging to District 6 were more infected (9.3%) than others (Table 1). Among the 400 participated workers, 388 (97.0%) were asymptomatic and 12 (3.0%) showed abdominal pain. All *Giardia* positive workers were asymptomatic.

The 16 positive isolates by microscopy were successfully amplified at the SSU rRNA gene while *tpi* gene was amplified in 13 (81.25%) samples. Of the 13 isolates successfully sequenced at *tpi* gene, 10 (76.9%) were typeable while the other three (23.1%) were untypable. Assemblage A, sub-assemblage AII, was found in nine (69.2%) isolates, and one (7.7%) isolate was assemblage B, sub-assemblage BIII (Fig. 1). Of the nine assemblage AII, six showed 100% identity with reference sequence U57897 (Baruch *et al.*, 1996) while the remaining three differed by two to four single-nucleotide polymorphisms (SNP) at positions 643 (A/T), 662 (A/G), 729 (A/G), and 770 (T/G). The only one isolate recognized as assemblage B, sub-assemblage BIII differed by a SNP at position of 57 (C/T) of reference sequence AF069561 (Monis *et al.*, 1999). Representative sequences were deposited in the GenBank under accession numbers LC329320 and LC329331.

**Table 1. Prevalence of *Giardia duodenalis* based on microscopy examination among municipal waste workers (n=400) Ahvaz county, southwestern Iran**

<table>
<thead>
<tr>
<th>Category</th>
<th>G. duodenalis</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. examined (%)</td>
<td>No. Negative (%)</td>
</tr>
<tr>
<td>Age groups (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20–30</td>
<td>121 (30.2)</td>
<td>115 (95.0)</td>
</tr>
<tr>
<td>31–40</td>
<td>175 (43.8)</td>
<td>169 (96.6)</td>
</tr>
<tr>
<td>41–50</td>
<td>88 (22.0)</td>
<td>85 (96.6)</td>
</tr>
<tr>
<td>51–60</td>
<td>16 (4.0)</td>
<td>15 (93.8)</td>
</tr>
<tr>
<td>Educational status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary school</td>
<td>39 (9.7)</td>
<td>37 (94.9)</td>
</tr>
<tr>
<td>Middle school</td>
<td>196 (49.0)</td>
<td>193 (98.5)</td>
</tr>
<tr>
<td>High school</td>
<td>105 (26.3)</td>
<td>96 (91.4)</td>
</tr>
<tr>
<td>Diploma</td>
<td>60 (15.0)</td>
<td>58 (96.7)</td>
</tr>
<tr>
<td>Clinical symptoms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>12 (3.0)</td>
<td>12 (100.0)</td>
</tr>
<tr>
<td>No</td>
<td>388 (97.0)</td>
<td>372 (95.9)</td>
</tr>
<tr>
<td>District</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>84 (21.0)</td>
<td>82 (97.6)</td>
</tr>
<tr>
<td>2</td>
<td>44 (11.0)</td>
<td>42 (95.5)</td>
</tr>
<tr>
<td>3</td>
<td>46 (11.5)</td>
<td>45 (97.8)</td>
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<tr>
<td>4</td>
<td>65 (16.3)</td>
<td>63 (96.9)</td>
</tr>
<tr>
<td>5</td>
<td>58 (14.5)</td>
<td>56 (96.6)</td>
</tr>
<tr>
<td>6</td>
<td>54 (13.5)</td>
<td>49 (90.7)</td>
</tr>
<tr>
<td>7</td>
<td>49 (12.3)</td>
<td>47 (95.9)</td>
</tr>
</tbody>
</table>
Figure 1. Phylogenetic tree for *Giardia duodenalis* based on *tpi* gene sequences. The evolutionary history was inferred using the Neighbor-Joining method. For each sequence used, GenBank accession numbers are provided. The bootstrap consensus tree was inferred from 1,000 replicates. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site.

A cluster was obtained from our All isolates with Iranian human and environmental All sequences. Assemblage B formed a cluster with AF069561 (Monis et al., 1999) obtained reference sequences from the GenBank.

**DISCUSSION**

Waste management, which includes the processes of collecting, transporting, and disposing of solid waste, plays an important role in public and environmental health status (Porta et al., 2009). It is estimated that annually more than 2 billion tons of waste are generated globally (Giusti, 2009). In a majority of developing countries, lack of political will and poor education have made waste management a low priority (Giusti, 2009). Furthermore, workers might be infected through ingestion of infective agents by dirty hands during eating at work (Alvarado-Esquivel et al., 2008).
Although several studies have investigated the viral diseases in waste solid workers (Ansari-Moghaddam et al., 2016; Mol et al., 2015; Rachiotis et al., 2012), available data on parasitic diseases have been restricted to a few studies (Alvarado-Esquivel et al., 2008; Eassa et al., 2016; Mba, 2004).

In Iran, like other developing countries, intestinal parasitic diseases are considered a public health problem. In a study by Sharif et al. in 2015, a prevalence of 53.9% was reported for G. duodenalis in food handlers in Sari, northern Iran (Sharif et al., 2015). In another study from western Iran, G. duodenalis was observed in 2.9% of food handlers (Kheirandish et al., 2014). Previous microscopic studies have been focused only on a special worker group, food handlers; and the only study on municipal waste workers in Iran has been conducted by microscopy methods (Molavi et al., 2007). In Khuzestan Province, no comprehensive data are available on the prevalence of G. duodenalis, and only a few studies have been conducted in recent years (Kasaei et al., 2018; Rafiei et al., 2013).

In the present study, the positive samples were confirmed by the SSU rRNA gene, and then typing was performed at the tpi gene. Because of the multi-copy nature of the SSU rRNA gene, it often only used for confirmation of microscopic positive samples, while the single-copy genes, such as tpi, gdh, and β-giardin genes are used to differentiate species and sub-genotypes of G. duodenalis (Thompson & Ash, 2016). In addition, associated risk factors such as age, educational status, and clinical symptoms were investigated. In the present study, 16 (4.0%) of the municipal waste workers were infected with G. duodenalis by microscopy showing a significant decrease in the Giardia frequency compared to a previous study carried out in Esfahan (Molavi et al., 2007). The difference in the prevalence might be attributed to several factors, including the sample size, studies conducted in different periods, diagnostic methods, and geographic and climatic conditions of the regions. In a similar study carried out in Egypt, 346 municipal waste workers were investigated microscopically for intestinal parasitic infections. The results indicated that 2.9% of the workers were infected with G. duodenalis (Eassa et al., 2016). Although age is an important factor in intestinal parasitic infections (Faria et al., 2017), no significant association was found between age and Giardia infection.

Educational status and awareness are the other effective factors associated with intestinal parasitic infections, which can reduce the chance of infection (Ostan et al., 2007). However, our findings revealed a higher infection among workers with high school educational level. It seems that, the educational status did not have an effect on the prevention of intestinal parasites infection among workers. Regarding Giardia infection in different districts of the county, a notable point is that approximately one third (5/16; 31.2%) of the infections was observed in District 6, which includes northern and west northern parts of Ahvaz. Compared to other districts, people living in this district are in poor hygiene conditions. Moreover, due to keeping domestic animals at home, they could be more at risk of zoonotic infections (Table 1).

A noteworthy finding in our study was that all infected workers (100.0%) were asymptomatic and unaware about their disease. Since asymptomatic patients are cyst passer, therefore, they can play an important role in the transmission of Giardia. Clinical symptoms in giardiasis vary from mild diarrhea to severe malabsorption syndrome. It seems that in giardiasis, symptoms are associated with various host and parasite related factors (Homan and Mank, 2001). The possible correlation of clinical symptoms with parasite strain is not completely clear. However, according to previous studies, assemblage A is more frequent in symptomatic subjects (Cardona et al., 2011; Haque et al., 2009; Sahagun et al., 2008).

Interestingly, in contrast with previous studies (Cardona et al., 2011; Haque et al., 2009; Sahagun et al., 2008), our findings revealed the predominance of assemblage A in asymptomatic subjects. Assemblage A, sub-assemblage AII was the most common identified assemblage with a frequency of
56.2% (9/16), and assemblage B, sub-assemblage BIII was found in 6.2% (1/16) of the isolates. However, the worrying thing is that asymptomatic workers could be a potential source of infection to others (Cardona et al., 2011; Haque et al., 2009; Sahagun et al., 2008). Our findings are in line with a recent study from central Iran in which assemblage A, sub-assemblage AII was reported in 24 (54.5%), and assemblage B, sub-assemblages BII and BIV were identified in seven (15.9%) and two (4.6%) examined isolates, respectively. In the study, co-infections of assemblages A and B were also observed in 11 (25.0%) isolates (Hooshyar et al., 2017). In contrast to our study, Rafiei et al. observed sub-assemblages BIII and AII in 74.0% and 36.0% of the asymptomatic subjects, respectively (Rafiei et al., 2013). When looking at the studies available, it can be claimed that assemblage AII is the most common sub-assemblage in Iranian population.

CONCLUSIONS

The current study provided new data on the prevalence and genotypic diversity of G. duodenalis among municipal waste workers in Ahvaz, southwestern Iran. Our findings revealed that Assemblage A, sub-assemblage AII was the predominant assemblage. Furthermore, we found that transmission of human giardiasis in Ahvaz County is mainly of anthroponotic origin. Given that all infected participants were asymptomatic, it is suggested that health educational programs along with periodic health examinations be implemented to prevent and control intestinal parasitic diseases.

Acknowledgements. We would like to thank Waste Management Organization, Ahvaz Municipality for their invaluable collaboration, in particular, to Mr. Ahmadi. Thanks also are indebted to the municipal waste workers for their participation in this study.

CONFLICT OF INTEREST

The authors have no conict of interest.

FINANCIAL SUPPORT

This project was supported by the Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (Grant No. OG 94136).

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