

Original Article

Prevalence and genotyping of *Giardia duodenalis* among Arabian horses in Ahvaz, southwest of Iran

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ABSTRACT

Giardia duodenalis is globally recognized as an important zoonotic intestinal protozoan parasite. So far, eight assemblages of *G. duodenalis* (A-H) have been identified. Substantial evidence suggests the zoonotic potential of assemblages A, B, and E in livestock. In this study, the genotype of *Giardia duodenalis* isolates was genetically identified by determining the sequence of ssu-rRNA gene and performing polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) on glutamate dehydrogenase gene of the parasite in Arabian horses from Ahvaz, located in southwest of Iran. The results revealed that assemblages AI and E (livestock-associated *G. duodenalis*) were present in horse isolates. Also, based on the findings, prevalence of *G. duodenalis* infection among horses was estimated at 35.7%. The results indicated that *G. duodenalis* is highly prevalent among Arabian horses, posing a zoonotic risk for giardiasis in Ahvaz, Iran.

Keywords: *Giardia duodenalis*, Genotyping, ssu-rRNA, gdh, Arabian horse, Iran

Prévalence et génotypage de *Giardia duodenalis* chez les chevaux arabes d'Ahvaz, au sud-ouest de l'Iran

Résumé: *Giardia duodenalis* est reconnu mondialement comme un parasite intestinal protozoaire zoonotique. Jusqu'alors, huit assemblages (A-H) ont été identifiés pour l'espèce *G. duodenalis*. Des preuves substantielles laissent supposer un potentiel zoonotique chez les animaux d'élevages pour les assemblages A, B et E. Dans cette étude, les génotypes des isolats de *Giardia duodenalis* ont été génétiquement identifiés. Pour ceci, la séquence ssu-ARNr a été déterminée par polymorphisme de longueur des fragments de restriction (PCR-RFLP) sur le gène du glutamate déshydrogénase des parasites isolés à partir des chevaux arabes (Ahvaz, sud-ouest de l'Iran). Nos résultats révèlent que les assemblages AI et E (*G. duodenalis* associé à l'élevage) étaient présents dans les isolats des chevaux analysés. De plus, la prévalence de cette infection a été estimée à 35,7%. Ces données reflètent la forte prévalence du parasite *G. duodenalis* parmi les chevaux arabes, constituant de ce fait un risque zoonotique majeur de giardiase dans la région d'Ahvaz en Iran.

Mots clés: *Giardia duodenalis*, Génotypage, ssu-ARNr, gdh, Cheval arabe, Iran

INTRODUCTION

Giardia duodenalis, also known as *G. intestinalis* and *G. lamblia*, is one of the most common intestinal protozoan parasites with a worldwide distribution, infecting humans and a wide range of animals (Buret et

al., 1990; Yoder et al., 2010). This parasite produces resistant cysts, which are infective when excreted through the feces. Transmission of *G. duodenalis* occurs directly (fecal or oral transmission) by cyst-contaminated food or water (Porter et al., 1990; Adam, 2001; Coklin et al., 2007). The infected host may be

either asymptomatic (shedding the infective cysts in the environment) or present with a variety of clinical problems such as acute or chronic diseases (Gardner and Hill, 2001), probably due to host-related factors or strain variations (Sousa et al., 2006). Overall, diarrhea is recognized as the predominant symptom in *G. duodenalis* infection in host (Buret et al., 1990). According to the literature, horses infected with *G. duodenalis* are mostly asymptomatic, although subclinical signs such as reduced growth rate, impaired feed conversion efficiency, and persistent diarrhea are occasionally observed (Traub et al., 2005). The genetic characterization of *G. duodenalis* on the basis of conserved gene loci has identified eight assemblages (A-H) for this parasite (Feng and Xiao, 2011). Assemblages A and B have been detected in humans and a wide range of other mammalian hosts; therefore, these two genotypes pose the highest zoonotic risk to public health (Lalle et al., 2005; Xiao and Fayer, 2008). On the other hand, assemblages C and D have been only detected in canine isolates, assemblage E in livestock (cattle, horse, sheep and goats), assemblage F in feline isolates, assemblage G in rats, and assemblage H in seals (Caccio and Ryan, 2008; Feng and Xiao, 2011). According to the literature, specification of *G. duodenalis* genotypes was performed after substantial sequence differences were detected in genes, such as 18s small-subunit ribosomal RNA (18s rRNA), glutamate dehydrogenase (gdh), triose-phosphate isomerase (tpi), and β -giardin (bg) (Wielinga and Thompson, 2007; Plutzer et al., 2010). Although *G. duodenalis* infection has been reported in a number of livestock breeds in some regions of Iran, no molecular epidemiological data is available on the prevalence of this parasite among Arabian horses in this country. Therefore, the main objective of the current study was to determine the genotype of *G. duodenalis* and to specify the prevalence of this parasite in Arabian horses of Ahvaz, located in southwest of Iran.

MATERIALS AND METHODS

Fecal samples. During May 2011 and February 2012, fecal samples were collected from Arabian horses in farms of Ahvaz, southwest of Iran. Stool samples were collected from 42 Arabian horses and stored at 4 °C. After preparing the fecal smears on slides, *G. duodenalis* cysts were detected by light microscopy. Afterwards, the cysts from positive samples were purified by centrifugal flotation in sucrose solution. The supernatant was collected and stored at 20 °C after washing with phosphate-buffered saline (PBS, pH=7.4) before DNA extraction.

Extraction of genomic DNA. *G. duodenalis* cysts were re-suspended in 400 μ l of lysis buffer via stool DNA extraction kit (AccuPrep®) and lysed by six freezing–thawing cycles at -70 °C for 30 min and at +70 °C for 30 min, respectively. Genomic DNA was extracted, using the mentioned kit according to the manufacturer's instructions.

Polymerase chain reaction (PCR). The selected genes for the PCR included a 292-bp segment of ssu-rRNA (Appelbee et al., 2003) and a 432-bp gdh gene (Read et al., 2004). The applied approach in this study was modified accordingly. The ssu-rRNA sequence of genomic DNA was amplified in 50 μ l reaction volume, containing 1–5 μ l of DNA template, 2.0 mM of MgCl₂, 200 mM of each deoxynucleotide (dNTP), 5% dimethyl sulfoxide (DMSO), 12.5 pmol of each forward and reverse primer, and 0.5 U of Taq DNA polymerase. The cycling protocol included an initial denaturation hot start at 94 °C for 3 min, 40 PCR cycles at 94°C for 30s, at 58.8°C for 30 s, and at 72°C for 60 s, and a final extension at 72°C for 7 min. For the 432-bp gdh gene, 50 mL of the reaction volume, containing 5 μ l of DNA template, 2.0 mM of MgCl₂, 200 mM of each dNTP, 12.5 pmol of each oligonucleotide primer, and 1.25 U of Taq DNA polymerase, was used. Moreover, 12.5 pmol of GDHeF and GDHiR primers were used in the primary PCR reaction. Then, 1 μ l of the PCR product from the primary reaction was added to the secondary PCR, containing GDHiF and GDHiR primers. The cycling protocol included an initial denaturation cycle at 94 °C for 3 min, followed by 40

cycles at 94 °C for 30 s, at 54.4 °C for 30 s, and at 72 °C for 60 s and a final extension at 72 °C for 30 min. The size of the amplified DNA was determined by 1.5% agarose gel electrophoresis and ethidium bromide staining. The PCR products were identified by 50-bp ladder.

Sequencing. PCR products of ssu-rRNA gene were purified, using pfu DNA polymerase enzyme (Fermentas ®, USA). The purified products were sequenced with the same PCR primers, used for the original amplification in 10 µl reactions by Biosystems DNA Analyzer (Bioneer®, Korea). To determine the Giardia genotype in the samples, each sequence was independently compared with the GenBank database, using the BLAST tool (available on <http://www.ncbi.nlm.nih.gov/blast/>).

Restriction analysis of PCR products of *gdh* gene. Restriction digest was carried out directly on PCR products in 30 µl reactions, and 10 µl of the PCR product was added to 2X reaction buffer and 2 U of NlaIV gene (Fermentas®, USA); digestion took place at 37 °C for 3 h. Each digested DNA preparation was subjected to 12% polyacrylamide gel electrophoresis and ethidium bromide staining.

RESULTS

In this study, the overall prevalence of *G. duodenalis* in Arabian horses was 35.7% (15/42). Clinical findings were reported to be normal and the feces were soft and formed. Potentially zoonotic Giardia species were identified in the horses.

PCR amplification and sequence analysis of ssu-rRNA gene fragment. Among 15 tested samples by PCR, 13 (86.6%) showed positive results for *G. duodenalis*. Positive samples were successfully sequenced, confirming the infection with *G. duodenalis* assemblages. Sequence analysis showed that nine (69.2%) isolates belonged to assemblage E, while four (30.7%) were assigned to assemblage A.

PCR-restriction fragment length polymorphism (RFLP) of *gdh* gene. The 432-bp *gdh* gene segment was amplified from 12 (80%) out of 15 positive

isolates. According to the present results, nine (75%) out of 12 isolates were confirmed to belong to assemblage E, while three (25%) isolates were from assemblage AI; however, the mixture of assemblages AI and E was not detected in the isolates. The expected fragments after digesting by specific restriction NlaIV enzymes are presented in Table 1.

Table 1. Restriction fragment length polymorphism (RFLP) profiles of *G. duodenalis* assemblages after digesting with NlaIV in assemblages AI and E

Assemblages of <i>G. duodenalis</i>	Diagnostic genotyping profile
AI	90, 120, 90 bp
E	80, 100, 220 bp

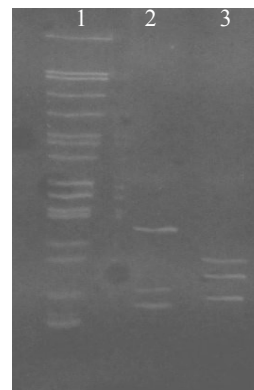


Figure 1. Restriction fragment length polymorphism (RFLP) products of *gdh* gene. Line 1: 50-bp ladder, Line 2: assemblage E (80, 100, and 220 bp), Line 3: assemblage AI (90, 120, and 90 bp).

DISCUSSION

Infection with *G. duodenalis* is widespread in both animals and humans. To understand the epidemiology of the infection or implement control measures, it is important to identify animals which can act as a source of human infection. In this study, *G. duodenalis* cysts were detected in the feces of Arabian horses in Ahvaz, southwest of Iran. The point prevalence of *G. duodenalis* in Arabian horses was estimated at 35.7%. In the present study the examined fecal samples from horses were normal, and none of the samples were diarrheal or formless. Overall, giardiasis in horses mostly presents without clinical signs and is not associated with mortality. The risk posed by animals to the general population can be better understood by the

molecular characterization of cysts for better genotypic identification. In the present study, ssu-rRNA and gdh genes were applied. The primers used in the PCR assay were specific for *G. duodenalis* in hoofed animals. The ssu-rRNA and gdh genes were successfully amplified from 13/15 (86.6%) and 12/15 (80%) Giardia-positive fecal samples, respectively. The ssu-rRNA gene is a commonly used marker for species and assemblage differentiation of Giardia (not sub-assemblages), while in the gdh locus, subtypes can be easily grouped into sub-assemblages of *G. duodenalis* (Plutzer et al., 2010). The sequencing of ssu-rRNA fragments detected in 69% of the positive samples of *G. duodenalis* in assemblage E and 30.7% of the positive samples in assemblage A. The sequence analysis of ssu-rRNA locus in this study was correlated with the RFLP findings of gdh gene. Overall, gdh- and ssu-rRNA-based genotyping tools are useful for the epidemiological investigation of giardiasis in hosts and are proper markers for genotypic analysis of *G. duodenalis* parasites. Traub et al. (2005) by evaluating 281-bp sequences of ssu-rDNA gene and 446-bp sequences of tpi gene demonstrated that horses in Ithaca, New York, and Perth were infected with assemblages AI, AII, and BIV, which pose the greatest zoonotic risks to humans. Moreover, Veronesi et al. (2010) reported that horses in Italy are infected with livestock-specific assemblage E. According to molecular epidemiological studies on giardiasis, assemblages A and B of *G. duodenalis* can infect humans. Also, it is commonly believed that assemblage E can mainly infect livestock, although unusual hosts including humans have been recently identified in Egypt (Foronda et al., 2008). In the current study, we performed the first definitive genotypic assessment of Giardia species, isolated from Arabian horses. Based on the findings, anthroponotic transmission of *Giardia* isolates is possible between humans and Arabian horses.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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