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Determination of *Giardia duodenalis* (Metamonada: Hexamitidae) genotypes in water buffalo

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ABSTRACT

Giardia duodenalis is considered as one of the important common protozoa between humans and animals in the intestine, which has eight groups (A-H) in different hosts. Studies have shown that the assemblages A, B, and E can infect livestock. In this study, the prevalence and genotype *of Giardia duodenalis* was determined by polymerase chain reaction of ssu-rRNA gene and by performing polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) on glutamate dehydrogenase gene of the parasite in water buffalo (n = 60) from Iraq. Based on the results, prevalence of *G. duodenalis* infection in water buffalo was estimated 25 % (n = 15), and according to age groups, higher infection group was 40% at ≤ 6 months, followed by > 6 -12 months, 1 -2 years, >2 years which showed 26.6%, 13.3% and 20.1%, respectively. The result of distribution of genotypes of *G. duodenalis* showed that assemblages AI and E were recorded in water buffalo isolates by 60 % and 40 %. Infection of assemblage AI was reported in buffaloes under 12 months. The present study determined that *G. duodenalis* is highly prevalent in water buffalo, and is involved in creating a zoonotic disease for giardiasis in Iraq. Due to the direct relationship between food and public health, as well as the influence of geographic and host conditions on the spread and pathogenicity, it is necessary to further investigate different genotypes and their common aspects between humans and livestock.

Keywords: *Giardia duodenalis*, Genotype, ssu-rRNA, glutamate dehydrogenase, water buffalo, Iraq.

Article type: Research Article.

INTRODUCTION

Giardia sp. is a flagellated protozoan that causes infection of the small intestine in a wide variety of hosts including domestic, wild, and human animals. The parasite is found in two forms: trophozoite and cyst. It is transmitted by cysts through water and food (Soulsby 1968; Dixon 2021). The most common symptom of giardiasis in young animals is loose faeces with mucus, acute diarrhoea, although diarrhoea in infection with this protozoan is chronic and mild (Geurden *et al.* 2008; Horton *et al.* 2019). In addition to diarrhea, there is a potential effect of giardiasis on animal products, in experimental contaminations in goats and lambs. The infection leads to the reduced feed

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efficiency and subsequent weight loss (Trout *et al.* 2007). Molecular analysis of *Giardia duodenalis* (*Giardia lamblia, Giardia instinalis*) and sequencing of glutamate dehydrogenase, thyrophosphate isomerase, β giardin and ssrRNA genes, 8 of which are known as assemblages A-H. Assemblages A and B have been observed in human and animal populations. Assemblages C, D, E, F and H have been isolated from a wide range of vertebrate and non-vertebrate hosts. Assemblages C and D were detected in dogs, foxes and coyotes. Assemblages E was isolated in a wide range of herbivores, cattle, buffaloes, sheep, goats, horses and pigs. Assemblages F were detected in cats, while assemblage G infected the rodents and assemblage H was found in marine vertebrates (Appelbee *et al.* 2003; Capewell *et al.* 2021). Studies on the prevalence of *Giardia* sp. in herbivores and the intraspecific diversity of this parasite have reported a wide range of contamination from assemblages A (I, II), B (III, IV) (Monis, *et al.* 2003; Plutzer *et al.* 2010). In the studies conducted, the relationship between infection with AII assemblage and symptomatic infections, as well as between assemblages B and asymptomatic infections in the young human infected were observed (Sahagun *et al.* 2008). Given the role of *G. duodenalis* in pathogenicity in animal and human populations and drug resistance under species and inadequate immunogenicity in re-infection with this protozoan, identification of genotypes of *G. duodenalis* isolates in disease control is important (Dixon 2021; Fu *et al.* 2022). Therefore, the aim of this study was to determine the genotypes of *G. duodenalis* isolates in water buffalo in Iraq and their possible role in human infection in this region.

MATERIALS AND METHODS

Sampling

The present study was performed on a stool sample of water buffalo in Iraq. Samples of stool samples were taken directly from the rectum of each animal and stored in a 4 °C-refrigerator after transferring to the laboratory. Samples were examined by the trichrome staining technique for the primary identification and samples infected with the *Giardia duodenalis* cyst were used for molecular study.

Isolation and preparation of *Giardia* cyst

The cysts of positive samples were collected by Sheather's flotation sugar, then during 6 stages of freezing-thawing process at -70 and +70 ºC (30 minutes / 30 minutes), the parasite cyst was broken. Then DNA extraction was performed using the AccuPrep® Stool DNA Extraction Kit made by BioNEER in Korea.

Amplification of 16 srRNA fragment and glutamate dehydrogenase

The positive stool samples were used to identify genotypes of *G. duodenalis*, ssu-rRNA gene (~ 292 bp) and the gdh gene (~ 430-bp) and were amplified using Semi-Nested PCR and PCR respectively (Table 1). Reaction mixtures for ssu-rRNA included 5 µL template DNA. Cycling conditions were consisted of an initial denaturation at 94 °C for 3 min, followed by 40 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and elongation at 74 °C for 60 s), and a final extension at 74 °C for 7 min.

For amplifying the gdh gene based in semi- nested PCR, reaction mixtures included 5 µL template DNA. The primary PCR was performed as follows: denaturation initial at 94 °C for 5 min, 40 cycles at 94 °C for 30 s, at 54 °C for 30 s, and at 74 °C for 60 s, with a final extension at 74 °C for 7 min. The secondary PCR cycling conditions included 1 µL of the product obtained by the same thermal program. The PCR and semi-nested products were analysed on 1.5% agarose gel.

Table 1. External and internal primer sequence of ssu-rRNA and glutamate dehydrogenase gene of *G. duodenalis.*

Sequence of primer	Primer name	Reference
ssu-rRNA (~ 292 bp)	RH11 (5_-CATCCGGTCGATCCTGCC-3_) RH4 (5_AGTCGAACCCTGATTCTCCGCCAGG-3-)	Appelbee 2003
glutamate dehydrogenase (~ 430-bp)	GDHeF: TCA ACG TYA AYC GYG GYT TCC GT , Internal Forward Primer GDHiF: CAG TAC AAC TCY GCT CTC GG and Reverse Primer GDHiR: GTT RTC CTT GCA CAT CTC C	Reada 2004

Enzymatic digestion of glutamate dehydrogenase product

NIa IV (Fementase®) enzyme was used to determine the genotypes of *G. duodenalis* with glutamate dehydrogenase gene by RFLP method. 10 µL of PCR product along with 2.5 µL Buffer (1X), 1 µL NIaIV enzyme

(10 U μL^{-1}) in a final volume of 25 μL for 16 h at 37 °C. Then the enzyme sections were electrophoresed and examined after staining gel 12% polyacrylamide with ethidium bromide.

Statistical methods

The data of this study were analysed according to the SPSS software statistical program (SPSS 18). Quantification of prevalence of *Giardia duodenalis* within the risk factor such as age, sex and genotype was statistically analysed by One-Way ANOVA. The difference in mean values was considered as significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

In the present study, 60 samples of water buffalo faeces collected from livestock farms in different regions of Iraq were examined for *G. duodenalis* infection. The PCR and nested-PCR of Giardia ssu-rRNA and glutamate dehydrogenase (gdh) gene detected 292 and 430 bp fragments in 15 out of 60 samples (25%) of the buffaloes. The study between sex distribution and prevalence of *G. duodenalis* infection showed that out of 25 males and 35 females, 7 (41.6%) and 9 (58.33%) were infected, respectively (Table 2). There was no statistically significant relationship between sex and the prevalence of *G. duodenalis* in buffaloes ($p > 0.05$).

Table 2. Sex distribution and prevalence of *G. duodenalis* infection in water buffaloes.

Sex	Positive *G. duodenalis*
Male	7 (41.6%)
Female	9 (58.33%)
Total	15 (25%)

Out of a total of 60 water buffalo in this study, water buffalo were in the age groups of ≤6 months, 6-12 months, 1-2 years and >2 years. In the four age groups, 40%, 26.6%, 13.3% and 20.1% were infected, respectively. Using Fisher's exact test, it was shown that giardiasis and age in water buffalo have a significant relationship (Table 3). The infection of buffaloes ≤ 6 months was significantly higher than those over one year ($p < 0.05$). The rate of infection and concentration of loose and normal faeces in infected buffaloes were 26.6% (4 individuals) and 73.3% (11 individuals) respectively. There was statistically significant relationship between loose stools and infection status ($p > 0.\ 05$).

Table 3. The giardiasis relationship with age in water buffalo based on Fisher's exact test.

Age	Positive *G. duodenalis*	Assemblage
≤6 months	(6) 40%	100% assemblage AI
6-12 months	(4) 26.6%	25%assemblage E, 75% assemblage AI
1-2 years	(2) 13.3%	100% assemblage E
>2 years	(3) 20.1%	100% assemblage E
Total	15 (25%)	

Giardia duodenalis genotype in 15 faecal samples of infected buffaloes, according to the obtained enzymatic digestion of PCR product of gdh gene, showed that 6 (40%) isolates related to E genotype; 9 (60%) isolates were A1 genotype and no mixed infection was founded (Table 3). The digestion pattern of assemblage E was 220, 100 and 80 bp bands, while assemblage A1 was 120 and 90 base pairs (Fig. 1).

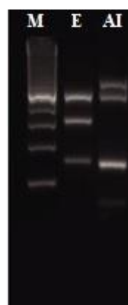

Fig. 1. Enzymatic digestion by NIa IV of nested PCR product of gdh gene of *Giardia duodenalis,* M (marker 100 bp, assemblage E and assemblage AI).

All AI assemblage in water buffalo samples in this study was isolated in those less than one-year olds, while the presence of this assemblage was not observed in those over one-year olds. The frequency distribution of Giardia assemblages was significantly different according to age groups ($p < 0.05$). Assembly E is specific to herbivores, but AI assembly is zoonosis type of giardiasis. In Portugal, the prevalence of *Giardia duodenalis* in cattle was 9%. Assemblages E, A, and B were identified using two genes of glutamate dehydrogenase and beta giardin. An important issue in this study was the presence of AII assembly in cattle. This is a human-specific assemblage and the first report of its occurrence in cattle (Mendonça *et al.* 2007). The presence of zoonotic genotype in the region is due to the close relationship between humans and animals and interference in the parasite life cycle between humans and animals. However, it is doubtful whether infected cows have the ability to spread A assemblage infection to other livestock and humans, or whether humans are the source of A assemblage infection in the area (Geurden *et al.* 2008). The molecular determination of *G. duodenalis* in buffalo calves from Brazil showed that 100% of claves infected with assemblage E (de Aquino *et al.* 2019). A study on cow diathesis in New Zealand has shown different results in the prevalence of infection. In 2000, Hunt *et al.* reported 40.6% of Giardia infections, 73% with assembly A and 36.4% with assembly B. In another study, the infection rate of dairy cows in the same area was 31% and assemblages A and B were 87% and 12.5%, respectively (Winkworth *et al.* 2008). In three studies, cows were infected with *G. duodenalis* with different prevalence, however, *Giardia* genotype was assembled A and B in all three studies. These two assemblages have the potential to infect a wide range of vertebrates, especially humans. There has been no report of the presence of E-assemblage, which is specific to herbivores. This suggests that manure management of livestock, especially in broiler farms that have access to pastures drained of human wastewater, can be effective in the type of pollution (Appelbee *et al.* 2003). In study of prevalence of genotype of *G. duodenalis* 42 % of dairy cows were infected in Canada, with 25 being infected with E and 35% with B. As the results of this study show, the contamination of dairy cows with zoonotic assemblage in this area is higher than the specific assemblage of herbivores. Infection prevalence in female cattle and calves in Canada were 38% and 51%, respectively (Uehlinger *et al.* 2011). Trout *et al*. (2007) based on their study, concluded that the incidence of zoonotic assemblages decreases by age. In addition, equal to other assemblages, they believe that long-term studies on the same domains are effective in answering this question. However, the role of cows as a source of *G. duodenalis* infection in the study area should not be overlooked (Trout *et al.* 2007). Molecular prevalence and genotypes of *G. duodenalis* in cattle, due to the wide prevalence of livestock-specific assemblages E and A, reflect the importance of cattle for zoonotic transmission of giardiasis in Turkey (Onder *et al.* 2020). Given that the studies in determining the contamination in buffaloes have been very limited, the result of the present study showed that the water buffalo seems to be an important source of *G. duodenalis* infection, and this infection is also important for zoonosis. In a study conducted in Iraq, water buffalos were infected with zoonotic assemblage (AI). The water buffalos less than one-year-olds exhibited the highest frequency, and in addition, infection with zoonotic assemblage was observed only at this age, while infection with specific herbivorous assemblage E was more common at older ages. As a result, infection of the water buffalo less than one-year-olds with *G. duodenalis* is of great health importance, since they are an important source of infection for humans and other livestock.

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