Gut Pathogens

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Occurrence and assemblage distribution of *Giardia Duodenalis* in symptomatic and asymptomatic patients in southeastern Iran (2019–2022)

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Abstract

Background The ubiquitous protozoan parasite *Giardia duodenalis* is a major contributor to the global burden of diarrhoea, particularly in young children living in poor-resource regions. Although rarely mortal, giardiasis is associated with growth retardation and cognitive impairment in early childhood. Here we investigate the epidemiology of human giardiasis in Iranshahr (south-eastern Iran), a region where this information was previously lacking.

Methods Stool samples were collected from 17,455 outpatients and inpatients attended at three major hospital settings during April 2020 and March 2022. Microscopy was used as a screening method for the presence of *Giardia* cysts, and the identification of *G. duodenalis* assemblages was carried out using PCR and Sanger sequencing.

Results The overall prevalence of giardiasis was 1.87 (326/17,455; 95% CI: 1.7–2.1). Being female was positively associated with higher odds of giardiasis ($p=0.014$). Individuals without diarrhoea were less likely to have giardiasis ($p=0.022$). Individuals attending the Iran Hospital were more likely to harbour *G. duodenalis* infections compared to those attending at the Khatam Hospital and the Clinical Reference Laboratory ($p=0.001$). Our sequence analyses revealed the presence of assemblages A (56.5%, 13/23), B (39.1%, 9/23), and A + B (4.4%, 1/23). No association was observed between the occurrence of a given assemblage and the occurrence of diarhroea.

Conclusions *Giardia* infections were found at relatively low prevalence rates in both symptomatic and asymptomatic individuals seeking medical attention. Being female, having diarrhoea, and being sampled during 2020–21 were predictors of giardiasis. Although limited, our molecular data indicate that some *Giardia* infections may be zoonotic in nature. These data should be corroborated and expanded in future epidemiological studies targeting simultaneously

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human, animal, and environmental (water) samples to improve our understanding of the epidemiology of giardiasis in Iran.

Keywords *Giardia Duodenalis*, Assemblage, Risk factors, Iranshahr, Epidemiology, Genotyping, Infection outcome

Background

Giardia duodenalis is a cosmopolitan enteric protozoan parasite which can cause gastroenteritis in a wide range of mammalian host species globally [1, 2]. In humans, *G. duodenalis* infections are estimated to cause 1.84×10^8 symptomatic cases annually and 171,100 disability adjusted life years [3]. The prevalence of human giardiasis has been reported to be 2–5% and 20–30% in developed and developing countries, respectively [4]. Young children in low-resource settings with little or no access to safe drinking water and sanitation are at higher risk of infection, particularly those living in tropical and subtropical regions [5, 6]. Early childhood giardiasis has been linked with a wide range of disorders and clinical manifestations including malnutrition, persistent diarrhoea, cognitive deficits, and stunting [7]. Other symptoms reported include nausea, anorexia, bloating, belching, abdominal pain, malaise, anaemia, steatorrhea, and weight loss [8]. Because of its close association with conditions of poverty, giardiasis (together with cryptosporidiosis) joined the 'Neglected Diseases Initiative' launched by the World Health Organization in 2004 [9]. Intriguingly, recent prospective, large cohort studies in endemic areas have found *Giardia* significantly more often in non-diarrhoeal than diarrhoeal infected children [10, 11]. These findings strongly suggest that *G. duodenalis* infection outcomes are likely the result of a multifactorial process involving nutritional, microbial, metabolic, and pathogen-strain variables [12–14].

Transmission of giardiasis occurs via the faecal-oral route, either by direct contact with an infected host or indirectly through ingestion of infective cysts in faecal-contaminated water or food [15]. *Giardia duodenalis* infections usually occur sporadically, although waterborne [16] and foodborne [17] outbreaks of gastrointestinal giardiasis are well documented globally. Based on the genetic variability of the small subunit ribosomal RNA (*ssu* rRNA) gene, *G. duodenalis* comprises eight (A to H) distinct genotypes (the so-called assemblages) with marked differences in host specificity and range [18, 19]. Humans are primarily infected by zoonotic assemblages A and B, although infections by canine- (C and D), ungulate- E, and feline-adapted (F) assemblages are also sporadically documented globally [2]. No human infections by *G. duodenalis* assemblage H have been reported to date. Assemblages A and B (particularly the latter) exhibit large genetic variations, allowing the categorization into sub-assemblages AI-AIII and BIII-BIV, respectively. AI and AIII are mostly restricted to infect non-human animal species, whereas AII, BIII, and BIV are regarded as zoonotic genetic variants [2, 20, 21]. AII within assemblage A and BIV within assemblage B are the predominant genetic variants circulating in humans [22, 23]. The most common genotyping tools for assessing intra-assemblage genetic variability are based on sequence analyses of the glutamate dehydrogenase (*gdh*), beta-giardin (*bg*) and triosephosphate isomerase (*tpi*) genes [2], although this multilocus sequence typing (MLST) scheme has limitations to resolve the molecular diversity within assemblage B isolates [24]. The *G. duodenalis* assemblage causing the infection has been investigated as a potential determinant of virulence/pathogenicity in large epidemiological studies, including matched case-control surveys, with uncertain results [25–27].

In Iran, the pooled prevalence of *G. duodenalis* infection at the community level has been estimated at 10.6% (95% CI: 9.6–11.5%) in a recent systematic review [28]. In symptomatic populations, *G. duodenalis* infections have been documented at variable rates including 2–19% in patients with diarrhoea [29–31], 39% in immunocompromised individuals [32, 33], and in 1–7% in HIV-positive patients [34–36]. Molecular-based studies aiming at investigating potential associations between the genetic variant of the parasite and diarrhoea generated contradictory data: assemblage A was more prevalent than assemblage B in patients with diarrhoea in some surveys [37], whereas the opposite [38] or inconclusive [39, 40] results were obtained in other studies.

In this study, we investigated *G. duodenalis* infection rate in symptomatic and asymptomatic patients attended at two large public hospitals in Iranshahr, Sistan and Baluchestan Province in southeastern Iran, a region where information on the epidemiology and public health relevance of the parasite was limited. Additionally, we investigated the molecular diversity of *G. duodenalis* in a subset of positive samples and assessed associations between genotypes and potential risk factors with the outcome of the infection.

Methods

Study design and population

This study was carried out in Iranshahr County, in the central area of the Sistan and Baluchestan province in southeastern Iran [41]. The latest census in 2016 showed a population of 113,750 people [42]. Iranshahr is one of the most socio-economically deprived areas of Iran. This is an observational cross-sectional study among outpatients and inpatients with and without gastrointestinal

manifestations referred to three different health centers (Iran Hospital, Khatam Hospital, and Clinical Reference Laboratory) affiliated to Iranshahr University of Medical Sciences (IRSHUMS) in Iranshahr (Sistan and Baluchestan Province) for coproparasitological examination. The participants' demographic and clinical data from Khatam and Iran Hospitals were gathered via the hospital information system. However, at the Clinical Reference Laboratory, due to the lack of a health information system, a short questionnaire was designed to collect similar information from participants referred to this health center. Individuals with no age restrictions with or without diarrhoea were included in the study. Patients who took standard intestinal parasite treatment were excluded from it. The study was conducted from April 2020 to March 2022 during the COVID-19 pandemic and included individuals with or without COVID-19. Volunteer asymptomatic individuals (mostly requesting a health card to access health care services) were also included in the study.

Sample size estimation

According to the formula ($n = Z2\ P\ (1 - P)\ /\ d2$) [43], the minimum sample size needed for this study was estimated at ~16,000 individuals, considering a 95% confidence level (Z), a precision (d) < 0.0012, and a predicted prevalence (*P*) of human giardiasis of 0.6% [44].

Stool sample collection

A single stool sample was collected from each participant in a sterile plastic screw-topped specimen container labelled with a unique identifier code. Samples were transported in refrigerated containers to the Department of Parasitology and Mycology at IRSHUMS within 24–48 h of collection. Stool specimens improperly labelled or mixed with urine were discarded. Eventually, 17,455 stool specimens meeting the inclusion criteria were collected during the study period.

Data collection

At the time of specimen collection, the participant's demographic (e.g., age, gender, health centre of origin) and clinical (e.g., diarrhoea) data were recorded together with the sampling date and the unique identifier code. *Giardia* infection was considered as the dependent variable, whereas age, gender, health centre of origin, occurrence of clinical manifestations, and sampling year were considered as the independent variables.

Macroscopic and microscopic examination

Stool samples were assessed macroscopically in terms of colour and consistency (1: formed, 2: soft, 3: loose, 4: liquid) at reception. Direct wet mount microscopy was used for the detection of *G. duodenalis* trophozoites and cysts in fresh stools. Briefly, one drop of saline (0.85% NaCl) was placed on a clean glass slide, mixed with a small amount of faecal material, and covered with a coverslip. Slides were immediately examined under light microscopy at 100× and 400× magnifications using an Eclipse E100 microscope (Nikon, Tokyo, Japan). Identification of cysts and trophozoites of *Giardia* was made according to standard bench aids [45]. Negative samples were not retested with additional conventional techniques, such as concentration methods or staining. *Giardia*-positive samples at microscopy examination were shipped to the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences (Tehran) for downstream molecular testing. Stool samples were stored at 4 °C without preservatives until molecular analysis, for a maximum period of 20 weeks between sample collection and DNA extraction and purification.

DNA extraction and purification

Faecal DNA was isolated from about 250 mg of 28 randomly selected stool samples that were positive by microscopy using the FavorPrep™ Stool DNA Extraction Mini Kit (Favorgen, Ping Tung, Taiwan) according to the manufacturer's instructions, except that a preliminary freeze/thaw cycle was added to help disrupting the cyst wall [46, 47]. Extracted and purified DNA samples were eluted in 60 µl of AE buffer and kept at −20 °C until further PCR analyses.

Molecular characterization of *G. duodenalis* isolates

For assessing the molecular diversity of *G. duodenalis* isolates, we adopted a sequence-based multilocus genotyping (MLST) scheme targeting the genes encoding for the glutamate dehydrogenase (*gdh*), beta-giardin (*bg*), and triose phosphate isomerase (*tpi*) proteins of the parasite. Nested PCR protocols were used to amplify a 432-bp fragment of the *gdh* gene [48], a 511-bp fragment of the *bg* gene [49, 50], and a 530-bp fragments of the *tpi* gene [51]. Detailed information on the PCR cycling conditions and oligonucleotides used in these PCR protocols is presented in Additional file 1: Table S1 and Additional file 2 Table S2, respectively.

All PCR mixes (final volume: 25 µl) consisted of 12 µl of Taq 2× Master Mix RED (Ampliqon, Odense, Denmark), 10 pmol of each forward and reverse primer, and 2–5 µl of template DNA. PCR reactions were run on a Mastercycler Gradient 5331 machine (Eppendorf AG, Hamburg, Germany). Nuclease-free distilled water and laboratory-confirmed positive DNA samples of human origin were used as negative and positive control in all PCR runs, respectively. Separation of PCR products was done by electrophoresis on 1.5% agarose gels (Invitrogen™, Life Technologies GmbH, Leipzig, Germany) stained using

SYBR® Safe DNA gel stain (Thermo Fisher Scientific, Waltham, MA, USA).

Sequence and phylogenetic analyses

PCR amplicons of the expected size were purified using the AccuPrep® PCR Purification Kit (Bioneer, Daejeon, South Korea) and send for sequencing in both directions using appropriate internal primer sets (Additional file 2 Table S2) to Bioneer Company (Daejeon, South Korea). DNA sequencing was conducted by capillary electrophoresis on an ABI 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA USA). Obtained sequences were assembled and edited using Bioedit software version 7.2.6.1.

Generated nucleotide consensus sequences were compared with homologous sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) to determine *G. duodenalis* assemblages and sub-assemblages. To confirm BLAST search results, multiple nucleotide sequence alignments were also carried out using the Clustal W algorithm in MEGA XI software [52].

Nucleotide sequences at the *gdh*, *bg*, and *tpi* markers generated in the present study and appropriate *G. duodenalis* reference nucleotide sequences obtained from GenBank were included to generate phylogenetic trees. Nucleotide sequences were aligned with the Clustal W algorithm using MEGA XI [52]. Phylogenetic analyses were performed using the Maximum Likelihood (ML) method and pairwise distances were calculated with the Tamura 3-parameter model using MEGA XI [52]. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Bootstrapping with 1,000 replicates was used to determine support for the clades generated. Representative nucleotide sequences obtained in the present study were deposited in GenBank under the accession numbers OQ202298–OQ202307 (*gdh* marker), OQ202284–OQ202297 (*bg* marker), and OQ202308–OQ202323 (*tpi* marker).

Statistical analysis

Statistical analysis was performed with SPSS® Statistics for Windows software, version 25 (IBM Corp., Armonk, N.Y., USA). The participants' baseline data were summarized as frequencies or percentage into categorical variables. Binomial logistic regression analysed the crude and adjusted associations between *G. duodenalis* infection and determinants [53]. The association strength between outcome and risk factors was evaluated using the adjusted odds ratio (aOR) and the corresponding 95% confidence interval (95% CI). A probability value <0.05 was regarded as statistically significant.

Results

Demographic profile

Table 1 summarizes the main demographic and clinical features of the population under study. A total of 17,455 stool samples were collected from diarrhoeal (29.7%, 5,187/17,455) and non-diarrhoeal (70.3%, 12,268/17,455) patients seeking medical attention in three clinical settings affiliated with IRSHUMS: Iran Hospital ($n=5,720$), Khatam Hospital ($n=628$), and Clinical Reference Laboratory ($n=11,107$). The male/female ratio was 3.7. The age of the recruited patients ranged from two days to 100 years (median: 60.0 months; standard deviation: 150.3 months). Young children under seven years of age accounted for 32.4% (5,663/17,455) of the investigated clinical population. Most stool samples (36.9%, 6,446/17,455) were collected during the winter months of 2020–21 and 2021–22.

Prevalence of *G. duodenalis* infection

The overall, microscopy-based prevalence of *G. duodenalis* infection was 1.87% [326 of 17,455; 95% Confidence Interval (CI): 1.7–2.1]. The higher infection rate was observed in patients from Iran Hospital (3.44%, 197 of 5,720; 95% CI: 3.0–4.0) followed by patients from Khatam Hospital (1.59%, 10 of 628; 95% CI: 0.77–2.91) and Clinical Reference Laboratory (1.07%, 119 of 11,107; 95% CI: 0.89–1.28).

Giardia duodenalis infections were more frequently detected in female (3.0%, 113 of 3,738) than in male (1.6%, 213 of 13,717) patients, regardless of the clinical setting of origin (Table 2), even though females accounted for nearly one out of four patients participating in the survey. Overall, children under the age of seven harboured the highest *G. duodenalis* infection rates (3.2%, 180 of 5,663). This trend was true for all hospital settings except for Iran Hospital, where the parasite was more common in individuals over seven years of age (5.0%, 26/525) than in children younger than that age (3.3%, 171 of 5,195) (Table 2). *Giardia duodenalis* was more frequently detected in patients presenting with diarrhoea (2.8%, 143 of 5,187) than in those without it (1.5%, 183 of 12,268), although the opposite trend (2.8% vs. 5.3%) was observed in patients recruited from Iran Hospital (Table 2). The occurrence of the parasite was similar in the two sampling periods considered, being higher in the 2020–2021 campaign (2.1%, 163 of 7,870) than in the 2021–2022 campaign (1.7%, 163 of 9,585). The opposite trend was observed (3.1% vs. 3.7%) for participating patients from Iran Hospital (Table 2).

Detailed information on the temporal distribution of *G. duodenalis* cases according to gender and age group or gender and presence/absence of diarrhoea can be found in Additional file 3 Table S3 and Additional file 4 Table S4, respectively. The average positive rate was

Table 1 Main demographic and clinical features of the surveyed symptomatic and asymptomatic populations investigated in the present study

	Iran Hospital (*n* = 5,720)		Khatam Hospital (*n* = 628)		Clinical Reference Laboratory (*n* = 11,107)		All three (*n* = 17,455)	
Variable	***n***	**%**	***n***	**%**	***n***	**%**	***n***	**%**
Gender								
Female	2,296	40.1	281	44.7	1,161	10.5	3,738	21.4
Male	3,424	59.9	347	55.3	9,946	89.5	13,717	78.6
Age group (years)								
≤ 7	5,195	90.8	26	4.1	442	4.0	5,663	32.4
> 7	525	9.2	602	95.9	10,665	96.0	11,792	67.6
Symptom								
Diarrheic[a]	4,297	75.1	340	54.1	550	5.0	5,187	29.7
Non-diarrheic[b]	1,423	24.9	288	45.9	10,557	95.0	12,268	70.3
Season (2020–2021)								
Spring	358	6.3	ND	–	634	5.7	992	5.7
Summer	396	6.9	37	5.9	1,280	11.5	1,713	9.8
Autumn	1,025	17.9	57	9.1	1,302	11.7	2,384	13.7
Winter	725	12.7	68	10.8	1,988	17.9	2,781	15.9
Sub-total	2,504	43.8	162	25.8	5,204	46.9	7,870	45.1
Season (2021–2022)								
Spring	214	3.7	89	14.2	923	8.3	1,226	7.0
Summer	541	9.5	51	8.1	1,371	12.3	1,963	11.3
Autumn	1,183	20.7	171	27.2	1,377	12.4	2,731	15.6
Winter	1,278	22.3	155	24.7	2,232	20.1	3,665	21.0
Sub-total	3,216	56.2	466	74.2	5,903	53.1	9,585	54.9

ND: No data

[a] Stool samples with a loose or watery consistency

[b] Stool samples with s formed or soft consistency

higher during the spring-summer months (2.1%, 124 of 5,894) than in the autumn-winter months (1.7%, 202 of 11,561). The highest monthly positive rate of infection was recorded in June 2020 (3.7%, 8 of 219), while the lowest positive rate was recorded in November 2021 (0.7%, 6 of 813).

Risk association analysis: univariate analysis

Results of the univariate analysis are summarised in Table 3. Being female increased the odds of *G. duodenalis* infection (OR: 1.97; 95% CI: 1.55–2.50). Children younger than seven years of age had significantly higher odds of being infected with the parasite (OR: 2.61; 95% CI: 2.09–3.26). Individuals without diarrhoea were less likely to have giardiasis (OR: 0.53; 95% CI: 0.42–0.67). In contrast, the occurrence of the protozoon was similarly distributed through the whole period of the study irrespectively of the sampling year and season. Finally, patients attended at the Iran Hospital were more likely to harbour *G. duodenalis* infections (OR: 3.21; 95% CI: 2.55–4.05).

Risk association analysis: multivariate analysis

Results of the multivariate analysis are summarised in Table 4. In the multivariate model, factors that significantly increased the odds of *G. duodenalis* infection were being female (aOR: 1.36; 95% CI: 1.06–1.74), and being attended at the Iran Hospital (aOR: 3.26; 95% CI: 2.11–5.03). The odds of infection among people without diarrhoea were approximately 33% lower than those with diarrhoea (aOR: 0.67; 95% CI: 0.48–0.94).

Molecular characterization of *G. duodenalis*

We assessed the genetic diversity of 28 randomly selected *Giardia*-positive samples by conventional microscopy using a MLST scheme based on the amplification of three (*gdh*, *bg*, and *tpi*) genetic markers. Successful PCR amplifications and sequencing data were generated for 35.7% (10/28, *gdh*), 50.0% (14/28, *bg*), and 57.1% (16/28, *tpi*) of the samples investigated at the three loci (Table 5). Overall, 82.1% (23/28) of the *Giardia*-positive samples were successfully genotyped at one locus at least. MLST data at the three assessed loci was available for six samples (21.4%, 6/28). Subtyping data at a single locus and two loci were available for 42.9% (12/28) and 17.9% (5/28) of samples, respectively. No genotyping data could be obtained for 17.9% (5/28) of the *Giardia*-positive samples. Assemblage A (56.5%, 13/23) was more prevalent than assemblage B (39.1%, 9/23), while A + B mixed infections were detected in a single isolate (4.4%, 1/23). No host-adapted assemblages of canine (C, D), feline (F), or

Table 2 Frequency distribution of *Giardia duodenalis* infection by hospital setting according to gender, age group, presence/absence of diarrhoea, and sampling period of the cohort of patients investigated in the present study. 95% confidence intervals (CI) are indicated

	Iran Hospital (*n* = 5,720)			Khatam Hospital (*n* = 628)			Clinical Reference Laboratory (*n* = 11,107)			All three (*n* = 17,455)		
Variable	**Pos.**	**Total**	**% (95% CI)**	**Pos.**	**Total**	**% (95% CI)**	**Pos.**	**Total**	**% (95% CI)**	**Pos.**	**Total**	**% (95% CI)**
Gender												
Female	85	2,296	3.7 (3.0–4.6)	8	281	2.9 (1.2–5.5)	20	1,161	1.7 (1.1–2.7)	113	3,738	3.0 (2.5–3.6)
Male	112	3,424	3.3 (2.7–3.9)	2	347	0.6 (0.1–2.1)	99	9,946	1.0 (0.8–1.2)	213	13,717	1.6 (1.4–1.8)
Age group (yrs.)												
≤ 7	171	5,195	3.3 (2.8–3.8)	1	26	3.9 (0.1–19.6)	8	442	1.8 (0.8–3.5)	180	5,663	3.2 (2.7–3.7)
> 7	26	525	5.0 (3.3–7.2)	9	602	1.5 (0.7–2.8)	111	10,665	1.0 (0.9–1.3)	146	11,792	1.2 (1.1–1.5)
Diarrhoea[a]												
Yes	121	4,297	2.8 (2.3–3.4)	8	340	2.4 (1.0–4.6)	14	550	2.6 (1.4–4.2)	143	5,187	2.8 (2.3–3.2)
No	76	1,423	5.3 (4.2–6.6)	2	288	0.7 (0.1–2.5)	105	10,557	1.0 (0.8–1.2)	183	12,268	1.5 (1.3–1.7)
Sampling period												
2020–2021	77	2,504	3.1 (2.4–3.8)	3	162	1.9 (0.4–5.3)	83	5,204	1.6 (1.3–2.0)	163	7,870	2.1 (1.8–2.4)
2021–2022	120	3,216	3.7 (3.1–4.5)	7	466	1.5 (0.6–3.1)	36	5,903	0.6 (0.4–0.8)	163	9,585	1.7 (1.5–2.0)

[a] Defined as presenting stool samples with a loose or watery consistency

livestock (E) origin were identified circulating in the surveyed clinical population (Table 5). Overall, 53.9% (7/13) of the patients infected by assemblage A had diarrhoea, while this figure was 44.4% (4/9) for patients infected with assemblage B (OR: 1.46; 95% CI: 0.26–8.05).

Table 6 shows the frequency and molecular diversity of *G. duodenalis* at the *gdh*, *bg*, and *tpi* loci. Out of the 10 *gdh* sequences, five (50.0%) were assigned to the sub-assemblage AII, all of them showing 100% identity with reference sequence L40510. One isolate was identified as sub-assemblage BIII and showed 100% identity with reference sequence AF069059. The remaining four sequences belonged to sub-assemblage BIV and differed by 4–8 single nucleotide polymorphisms (SNPs) from reference sequence L40508. No ambiguous (double peak) positions were observed during chromatogram inspection.

Out of the 14 *bg* sequences generated in the present study, one was identified as sub-assemblage AII, being identical to reference sequence AY072723. A total of six sequences were assigned to the sub-assemblage AIII of the parasite. Of them, five showed 100% identity with reference sequence AY072724, while the remaining one differed from it by a single SNP. The seven sequences identified as assemblage B differed by 1–3 SNPs from reference sequence AY072727. As in the case of the *gdh* locus, all SNPs detected corresponded to clear mutations without evidence of double peaks at chromatogram inspection. All seven assemblage B sequences have a distinctive nucleotide (A) deletion in position 137 of reference sequence AY072727.

Out of the 16 sequences successfully analysed at the *tpi* locus, 11 were identified as sub-assemblage AII and differed by a single SNP (always a C to A mutation at position 794) of reference sequence U57897. Two isolates were assigned to sub-assemblage BIII. One of them being identical to reference sequence AF069561 and the other one differing form it by a single SNP. Two isolates belonged to sub-assemblage BIV and differed from reference sequence AF069560 by 2–3 SNPs. None of the SNPs detected corresponded to ambiguous double peak) positions. An additional isolate was identified as assemblage B due to the impossibility of clearly assign it to sub-assemblages BIII or BIV.

Phylogenetic analyses conducted at the *gdh* (Fig. 1), *bg* (Fig. 2) and *tpi* (Fig. 3) loci clearly showed that our *G. duodenalis* sequences formed well-supported clades with appropriate reference sequences retrieved from GenBank.

Discussion

Despite sustained effort to improve the performance indicators of health assistance and quality of life in Iran, shortcomings in sanitation infrastructures (i.e., public

Table 3 Univariate analysis of factors associated with *Giardia duodenalis* infection in the cohort of patients investigated in the present study. Crude odds ratios (OR) and 95% confidence intervals (95% CI) are indicated. Statistically significant values are bolded

Variables	No. Examined	No. Infected	% Infected	OR	95% CI	*P*-value
Gender						
Female	3,738	113	3.0	1.97	1.55–2.50	**< 0.001**
Male	13,717	213	1.6	1	Ref.	
Age (years)						
≤ 7	5,663	180	3.2	2.61	2.09–3.26	**< 0.001**
> 7	11,792	146	1.2	1	Ref.	
Symptom						
Diarrheic[a]	5,187	143	2.8	1	Ref.	
Non-diarrheic[b]	12,268	183	1.5	0.53	0.42–0.67	**< 0.001**
Year						
2020–2021	7,870	163	2.1	1.22	0.98–1.52	0.072
2021–2022	9,585	163	1.7	1	Ref.	
Location						
Iran Hospital	5,720	197	3.4	3.21	2.55–4.05	**< 0.001**
Kh. Hospital/Clin. Ref. Lab.	11,735	129	1.6	1	Ref.	

[a] Stool samples with a loose or watery consistency

[b] Stool samples with s formed or soft consistency

Table 4 Multivariate analysis of factors associated with *Giardia duodenalis* infection in the cohort of patients investigated in the present study. Statistically significant values are bolded

Variables	Regression coefficient	Standard error	Adjusted odds ratio (aOR)	95% Confidence interval (CI)	*P*-value
Gender	0.308	0.126	1.36	1.06–1.74	**0.014**
Being aged ≤ 7 yrs.	0.175	0.254	1.19	0.72–1.96	0.490
Without diarrhoea	–0.395	0.173	0.67	0.48–0.94	**0.022**
Hospital setting	1.182	0.222	3.26	2.11–5.03	**0.001**

toilets, wastewater treatment facilities) remain common particularly in the southeastern regions of the country. Although overall prevalence rates of parasitosis in Iran have declined significantly over the last few decades, giardiasis by *G. duodenalis* remains among the most common infections diagnosed [54]. The overall prevalence of giardiasis in Iran was estimated at 14.7% in the last systematic review available [55]. However, this figure should be interpreted with caution, as most of the studies considered in the associated meta-analysis were based on relatively small sample sizes and focused on symptomatic populations, where the prevalence differs between asymptomatic and symptomatic patients. To overcome this limitation, we present here giardiasis prevalence data among outpatients and inpatients with and without gastrointestinal manifestations referred to medical centres in Iranshahr (Sistan and Baluchestan Province). No previous studies have evaluated the presence of human giardiasis in this Iranian region.

We found a microscopy-based *Giardia* infection rate of 1.87%. This figure is very likely an underestimation of the true prevalence value for several reasons including (i) the limited diagnostic sensitivity of microscopy due to discontinuous cyst shedding in the stools of infected individuals and the low parasite burdens typically found in asymptomatic carriers, (ii) motile trophozoites may go undetected if wet mounts are not examined quickly after preparation [56], and (iii) misdiagnosis due to the limited skill of laboratory personnel and microscopists [57]. The prevalence of infection in this study was very close to the results of some other large-scale studies conducted on symptomatic patients visiting medical centers in Ahvaz (southwestern Iran) (1.77%, 887/50,000) [58], Karaj (north-central Iran) (3.84%, 534/13,915) [59], Shiraz (southwestern Iran) (0.53%, 73/13,686) [44], Rasht (north-central Iran) (2.47%, 206/8,356) [60], and Yazd (0.93%, 376/40,351) [61]. In addition, our result was in contrast with those obtained from previous small-scale studies conducted in Mazandaran (north-central Iran) (10.18%, 87/855) [62], Khuzestan (southwestern Iran) (10.9%, 163/1,494) [63], Shiraz (southwestern Iran) (10.7%, 107/1,000) [54], Kerman (southeastern Iran) (10.6%, 92/861) [64], Yasuj (southwestern Iran) (17.46%, 179/1,025) [65], Kamyaran (northwestern Iran) (11.0%, 22/245) [66], and Shushtar County (southwestern Iran) (7.74%, 90/1,163) [67]. The discrepancies in *Giardia* infection rates reported in the above-mentioned studies are likely associated with differences in (i) techniques used for detection purposes, (ii) sources of infection (i.e., drinking water quality, contact with domestic animals or

Table 5 Multilocus sequence typing results of the 28 *Giardia duodenalis*-positive samples randomly selected for genotyping purposes in the present survey. Main demographic (gender, age) and clinical (hospital of origin, diarrhoea) data of the patients are indicated. GenBank accession number are provided

					Genetic loci			
Sample Id.	**Gender**	**Age (yrs.)**	**Clinical setting**	**Diarrhoea**	**gdh**	**bg**	**tpi**	**Assigned genotype**
IRSUM1	Male	2	IH	Yes	BIII (OQ202298)	B (OQ202284)	BIV (OQ202308)	BIII/BIV
IRSUM2	Female	2	IH	Yes	–	AIII (OQ202297)	AII (OQ202319)	AII/AIII
IRSUM3	Female	1	IH	Yes	BIV (OQ202299)	B (OQ202285)	BIV (OQ202309)	BIV
IRSUM4	Female	2	IH	No	BIV (OQ202300)	B (OQ202286)	–	BIV
IRSUM5	Female	7	IH	No	–	–	AII (OQ202320)	AII
IRSUM6	Male	2	IH	Yes	BIV (OQ202307)	–	AII (OQ202321)	AII + BIV
IRSUM7	Male	23	CRL	No	–	–	–	Unknown
IRSUM8	Female	28	KH	No	–	–	AII (OQ202322)	AII
IRSUM9	Female	7	KH	Yes	–	AIII (OQ202296)	–	AIII
IRSUM10	Female	4	IH	No	AII (OQ202301)	AIII (OQ202287)	AII (OQ202310)	AII/AIII
IRSUM11	Male	4	IH	Yes	AII (OQ202302)	–	–	AII
IRSUM12	Male	2	IH	No	–	B (OQ202288)	–	B
IRSUM13	Female	2	IH	Yes	BIV (OQ202303)	B (OQ202289)	BIII (OQ202311)	BIII/BIV
IRSUM14	Male	2	IH	No	AII (OQ202304)	AII (OQ202290)	AII (OQ202312)	AII
IRSUM15	Female	8	IH	Yes	–	AIII (OQ202291)	AII (OQ202313)	AII/AIII
IRSUM16	Male	6	IH	Yes	–	–	–	Unknown
IRSUM17	Female	2	IH	No	–	–	–	Unknown
IRSUM18	Male	6	IH	No	–	–	–	Unknown
IRSUM19	Male	2	IH	Yes	AII (OQ202305)	AIII (OQ202292)	AII (OQ202314)	AII/AIII
IRSUM20	Female	6	IH	Yes	–	AIII (OQ202293)	AII (OQ202315)	AII/AIII
IRSUM21	Female	9	IH	No	AII (OQ202306)	–	–	AII
IRSUM22	Female	1	IH	No	–	–	B (OQ202316)	B
IRSUM23	Female	5	IH	Yes	–	B (OQ202294)	–	B
IRSUM24	Male	7	IH	No	–	B (OQ202295)	–	B
IRSUM25	Female	5	IH	No	–	–	BIII (OQ202317)	BIII
IRSUM26	Female	4	IH	No	–	–	–	Unknown
IRSUM27	Female	5	IH	Yes	–	–	AII (OQ202323)	AII
IRSUM28	Male	8	IH	No	–	–	AII (OQ202318)	AII

CRL: Clinical Reference Laboratory; IH: Iran Hospital; KH: Khatam Hospital

individuals with diarhroea), (iii) personal hygiene practices (i.e., hand washing after defecation), (iv) access to sanitation infrastructures (i.e., toilets), and (v) the socio-economic status of the investigated communities. In the only previously report conducted in southeast Iran, a *G. duodenalis* infection rate of 10.12% (158/1,562) was observed in patients with gastrointestinal complaints in Zahedan, the northern part of Sistan and Baluchestan Province [68]. This figure is five-fold higher than that (1.87%) found in the present study. The difference may be attributed to the fact that, despite Zahedan City being larger and having more urban facilities than Iranshahr, it is less ethnically homogeneous due to the presence of many unauthorized foreign nationals with poor hygiene lifestyles.

Individuals who attended Iran Hospital had a higher rate of infection compared to those at the Khatam Hospital or the Clinical Reference Laboratory. This result can be explained considering that 90.82% (5,195/5,720) of the patients attended at the Iran Hospital were children ≤ 7 years of age, the age group more vulnerable to giardiasis. This result was confirmed by univariate statistical analyses (OR: 2.61; 95% CI: 2.09–3.26) and is in line with previous studies published in Iran [64, 65, 69, 70] and other Asian countries including Malaysia [71] and Pakistan [72] showing that young age is a predisposing factor to giardiasis. Increased exposure to sources of infection and poor personal hygiene have been suggested as the main factors explaining higher prevalence of *Giardia* infections in young children [71, 73–75].

Univariate statistical analyses also demonstrated a significant association between *G. duodenalis* infection and diarrhoea (OR: 0.53; 95% CI: 0.42–0.67). Similar findings have been reported in other surveys conducted in Cuba [74], Iran [29], Malaysia [71], and South Africa [76]. Remarkably, some studies have shown an inverse correlation between *Giardia* infection in children and diarrhoea [77–79]. This may be the result of an acquired protective immunity in response to regular infection and re-infection events in endemic areas or of a protective effect of

Table 6 Frequency and molecular diversity of *G. Duodenalis* isolates identified at the *Bg*, *gdh*, and *Tpi* loci in the clinical population under study. GenBank accession numbers are provided

Marker	Assemblage	Sub-assemblage	No. isolates	Reference sequence	Stretch	Single nucleotide polymorphisms	GenBank ID
gdh	A	AII	5	L40510	307–690	None	OQ202305
		BIII	1	AF069059	313–628	None	OQ202298
		BIV	1	L40508	313–690	T387C, C396T, A476G, T549C, C610A, G651A, C660T, A666G	OQ202307
			1	L40508	310–690	T387C, C396T, T549C, C610A, C660T, A666G	OQ202299
			1	L40508	331–690	T387C, C396T, C610A, A666G	OQ202300
			1	L40508	307–690	T387C, T549C, C610A, G651A, A666G	OQ202303
bg	A	AII	1	AY072723	130–603	None	OQ202290
		AIII	5	AY072724	133–603	None	OQ202287
			1	AY072724	133–603	C321T	OQ202293
		B	3	AY072727	132–603	137DelA	OQ202286
			1	AY072727	132–603	137DelA, C165T	OQ202294
			2	AY072727	132–603	137DelA, C309T	OQ202285
			1	AY072727	132–603	137DelA, G315A, C450T	OQ202284
tpi	A	AII	11	U57897	324–805	C794A	OQ202314
		B	1	AF069560	30–479	T57C, A176G, C237T, A246G, A263G, T299C, A395G, A437C	OQ202316
		BIII	1	AF069561	10–456	None	OQ202317
			1	AF069561	10–456	C34T	OQ202311
		BIV	1	AF069560	100–479	G229A, A395G	OQ202308
			1	AF069560	30–479	A176G, C347T, A395G	OQ202309

bg, Beta-giardin; *gdh*, Glutamate dehydrogenase; *tpi*, Triose phosphate isomerase

Giardia colonization against other diarrhoea-causing intestinal pathogens [80]. Indeed, findings from prospective longitudinal cohort studies conducted in sub-Saharan African countries concluded that *Giardia* was not an independent risk factor for diarrhoea in children [10, 11, 81].

Being female was a significant risk for *Giardia* infection both in the present univariate (OR: 1.97; 95% CI: 1.55–2.50) and multivariate (aOR: 1.36; 95% CI: 1.06–1.74) analyses. This finding is in line with earlier studies conducted in Iran [82], Ethiopia [83], and South Africa [76], which reported a higher prevalence of *Giardia* infection in females compared to males. In contrast, males were more likely to have giardiasis in previous studies conducted in Iran [37, 65, 84, 85], Malaysia [75], and India [86]. However, *Giardia* distribution was gender-independent in most studies conducted in Iran [29, 59, 60, 62, 63, 68–70, 87] and other Asian countries including Malaysia [71], Pakistan [72], and Tajikistan [88]. This variation of exposure among the different sex groups may be attributed to differences in exposure levels across various communities and study areas.

A seasonal pattern of *Giardia* infections was identified, with a peak occurring from July to September. Similar trends have also been reported in other studies [60, 89, 90], coinciding with increased outdoor activities, particularly at the beginning of the school year.

The molecular diversity of *G. duodenalis* was investigated in 28 microscopy-positive samples at three markers (*gdh*, *bg*, and *tpi*) commonly used for genotyping and sub-genotyping purposes. Assemblage A was found to be more prevalent than assemblage B (56.5% vs. 39.1%) in the subset of samples investigated, with mixed infections A+B also detected at lower rates (4.4%). The predominance of assemblage A over assemblage B is the norm in most molecular studies conducted previously in Iran in patients with intestinal disorders (68.1% vs. 31.9%) [91], patients with and without gastrointestinal symptoms (92.6% vs. 7.4%) [60], food handlers (65.0% vs. 35.0%) [92], individuals with HIV or cancer (81.8% vs. 18.2%) [93], and samples submitted for laboratory testing (59.7% vs. 37.3%) [37], among others. In our recent study on 200 domestic animals in Iranshahr [10], we found that 19.3% (17/88) of cattle and 6.7% (2/30) of camels tested positive by qPCR, with one cattle isolate successfully genotyped as *G. duodenalis* assemblage B (accession no. PQ139658), displaying 99.46% sequence identity with a human isolate (LC184469) from Tehran (unpublished data). This suggests that an unknown proportion of human giardiasis cases detected in the present survey may be zoonotic in nature. Of note, assemblages A (37.5%) and E (58.3%) have been identified in slaughterhouse wastewaters in Tehran [94], strongly suggesting that untreated waters can be an important source of environmental contamination with *Giardia* cysts. Regarding pathogenicity, we

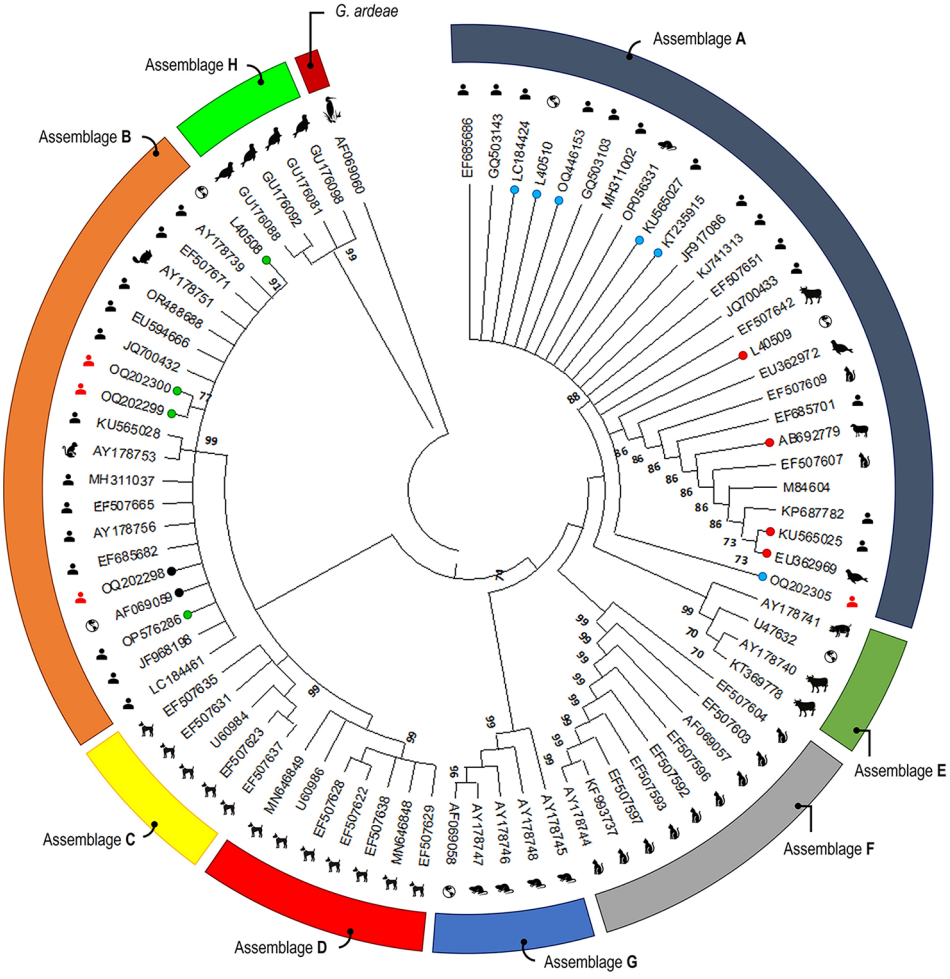

Fig. 1 Phylogenetic relationship among *Giardia duodenalis* assemblages and sub-assemblages revealed by a maximum likelihood analysis of the partial *gdh* rDNA gene. Substitution rates were calculated by using the general time reversible model. Numbers on branches are percent bootstrapping values over 70% using 1,000 replicates. The red human silhouettes indicate the nucleotide sequences generated in the present study. The filled red, blue, black, and green circles indicate the nucleotide sequences of sub-assemblages AI, AII, BIII, and BIV, respectively. The world map logos indicate reference sequences. Human, animal, and environmental sequences retrieved from GenBank were included in the analysis for comparative purposes

did not find a clear association between the presence of a given *Giardia* assemblage and the occurrence of clinical manifestations. Information from large epidemiological surveys investigating children with and without diarrhoea and matched by age and sex revealed unclear results. Whereas diarrhoea was not associated to a given *G. duodenalis* assemblage in Mozambique [26], assemblage A was more prevalently found in children with diarrhoea in Bangladesh [25].

The main strengths of this study include (i) the reporting of epidemiological data on human giardiasis from a geographical region in Iran where this information was previously lacking, (ii) the large sample size used and the coverage of three main medical centres in the Southeast of Iran, and (iii) the assessment of the impact of the COVID-19 restrictions on the epidemiology of *G. duodenalis*. The study has also limitations that might have compromised the accuracy of some of the results obtained. First, the limited diagnostic sensitivity of microscopy (together with the analysis of a single stool sample per patient recruited) suggest that the prevalence data presented here is an underestimation of the true one. Second, negative samples examined with direct smear were not subjected to further testing using conventional techniques (e.g., concentration and staining methods). Third, the associated metadata available was restricted to basic variables, so we were unable to assess the impact of important factors (i.e., drinking water source, sanitary and hygiene habits, latrine availability) that could be predictors of *Giardia* infection. Fourth, long-term storage of faecal samples (up to 12 weeks) at 4 °C might have affected (i) the morphology of *Giardia* cysts, impacting the diagnostic performance of conventional microscopy, and (ii) the quality and quantity of parasitic DNA,

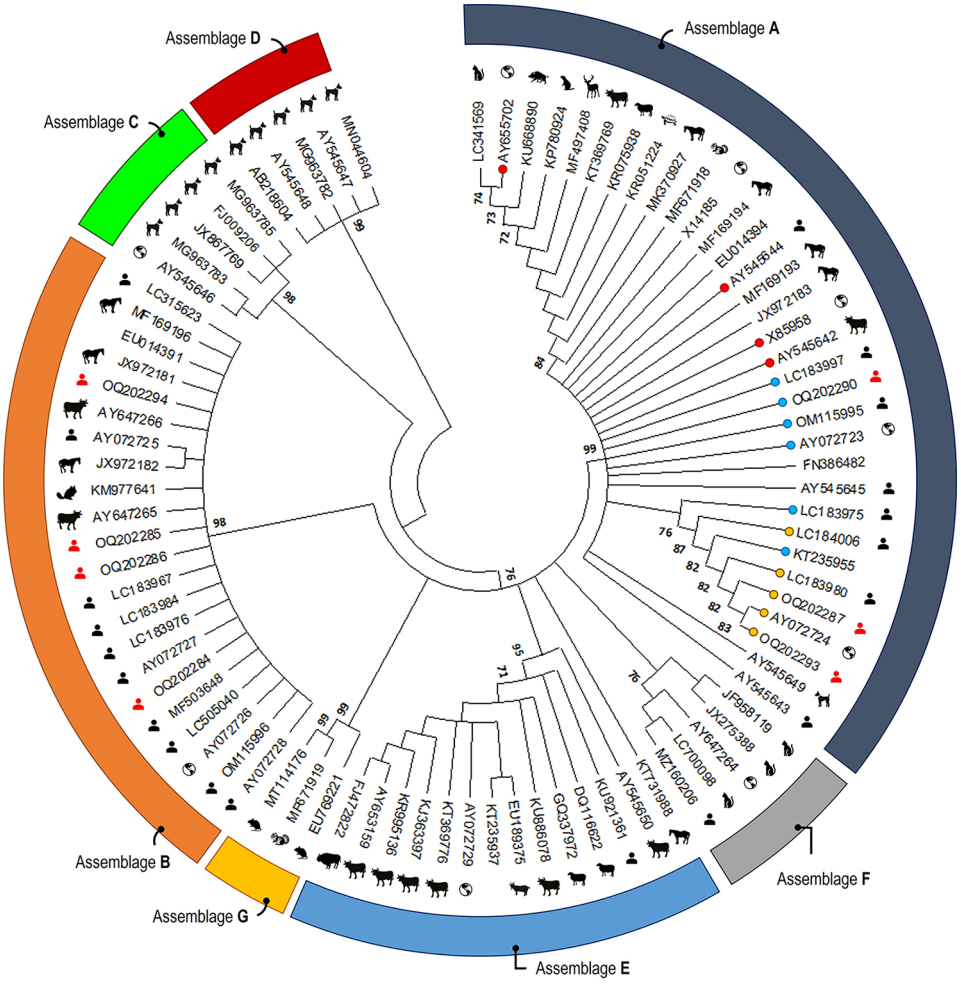

Fig. 2 Phylogenetic relationship among *Giardia duodenalis* assemblages and sub-assemblages revealed by a maximum likelihood analysis of the partial *bg* rDNA gene. Substitution rates were calculated by using the general time reversible model. Numbers on branches are percent bootstrapping values over 70% using 1,000 replicates. The red human silhouettes indicate the nucleotide sequences generated in the present study. The filled red, blue, and yellow circles indicate the nucleotide sequences of sub-assemblages AI, AII, and AIII, respectively. The world map logos indicate reference sequences. Human, animal, and environmental sequences retrieved from GenBank were included in the analysis for comparative purposes

compromising the performance of the genotyping PCR tools. Lastly, due to financial constraints, molecular analyses were only feasible for a limited number of samples, so the observed frequency and diversity of assemblages/sub-assemblages might not be representative of the one present at the whole population level.

Conclusion

This is the first study (i) investigating the epidemiology of giardiasis in south-eastern Iran, and (ii) systematically assessing risk factors potentially associated with higher odds of *Giardia* infection in Iran. *Giardia* infections were found at relatively low prevalence rates in both symptomatic and asymptomatic individuals seeking medical attention in Iranshahr. Being female and having diarrhoea were predictors of giardiasis. Individuals attended the Iran Children's Hospital were more likely to have giardiasis. Infections were caused by both assemblages A and B, with a higher frequency of A than B. Although limited, our molecular data indicate that an unknown proportion of *Giardia* infections may be zoonotic. These data should be corroborated and expanded in future epidemiological studies targeting simultaneously human, animal, and environmental (water) samples to improve our understanding of the epidemiology of giardiasis in Iran.

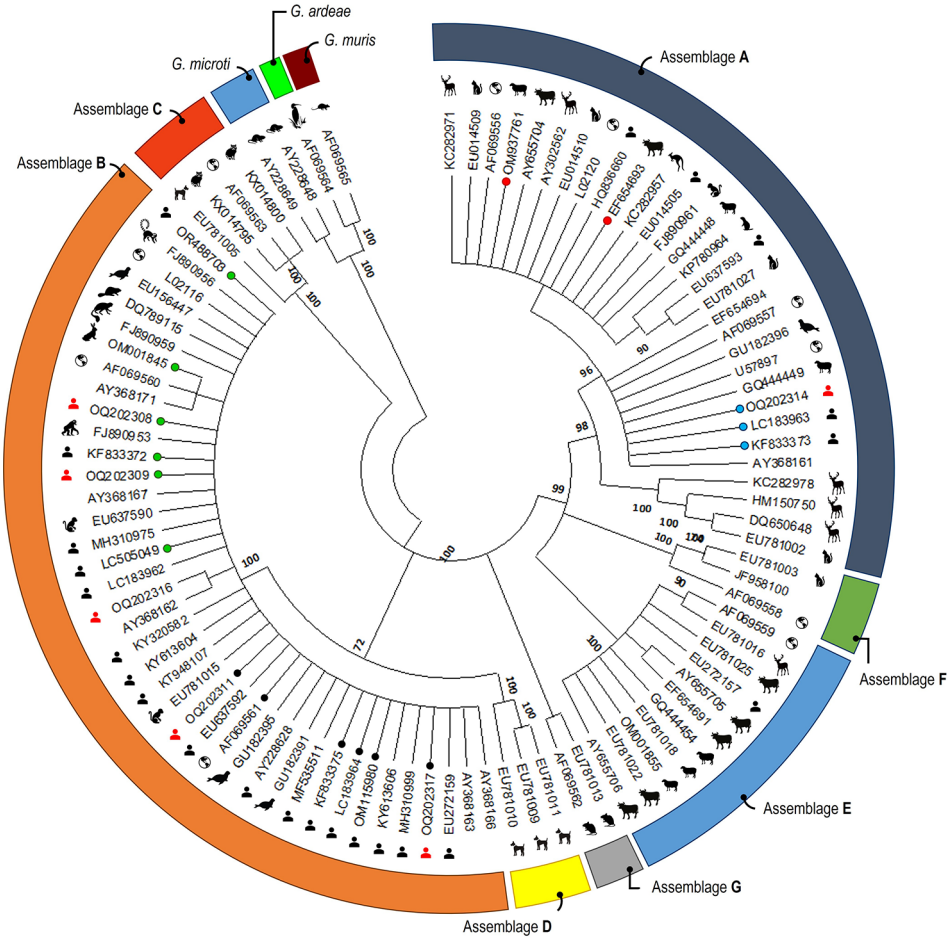

Fig. 3 Phylogenetic relationship among *Giardia duodenalis* assemblages and sub-assemblages revealed by a maximum likelihood analysis of the partial *tpi* rDNA gene. Substitution rates were calculated by using the general time reversible model. Numbers on branches are percent bootstrapping values over 70% using 1,000 replicates. The red human silhouettes indicate the nucleotide sequences generated in the present study. The filled red, blue, black, and green circles indicate the nucleotide sequences of sub-assemblages AI, AII, BIII, and BIV, respectively. The world map logos indicate reference sequences. Human, animal, and environmental sequences retrieved from GenBank were included in the analysis for comparative purposes

Abbreviations

aOR	Adjusted odds ratio
bg	Beta-giardin
BLAST	Basic local alignment search tool
COVID-19	Coronavirus Disease 2019
DNA	Deoxyribonucleic acid
gdh	Glutamate dehydrogenase
HIV	Human immunodeficiency virus
IRSHUMS	Iranshahr University of Medical Sciences
ML	Maximum likelihood
MLST	Multilocus sequence typing
PCR	Polymerase chain reaction
ssu	rRNA Small subunit ribosomal RNA
tpi	Triosephosphate isomerase
95% CI	95% Confidence interval

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13099-024-00666-0.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

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Author contributions

Study concept and design: KHN, EA, MR, DC, AM. Sample collection and acquisition of data: KHN, HMR, SS, EA, HM. Statistical analysis and interpretation of data: KHN, HMR, DC, EA. Writing-original draft preparation: KHN, MB, AVE, MB. Writing-review and editing: KHN, DC, EA, AVE, MB. Critical revision of the manuscript for important intellectual content: DC. Supervision: KHN. Project administration: KHN, EA, HAR. Funding acquisition: KHN. Final approval of the version: all authors.

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Data availability
The data that support the findings of this study are available from the First author upon reasonable request.

Declarations

Ethics approval and consent to participate
This study was carried out according to the principles of the Declaration of Helsinki, the ARRIVE guidelines and the 3Rs. The research protocol was approved by the Research Ethics Committee of IRSHUMS (protocol code IR.IRSHUMS.REC.1400.002, 06.03.2021). All human isolates were collected as part of a standard clinical investigation of anonymous patients with clinical manifestations. Signed and informed consent forms were not required.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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