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Genetic variability and transcontinental sharing of *Giardia duodenalis* infrapopulations determined by glutamate dehydrogenase gene

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ABSTRACT

Microevolutionary data of *Giardia duodenalis* sub-assemblages is a prerequisite for determining the invasion zoonotic patterns of the parasite. To infer transmission patterns that could not be differentiated by the phenotypic features, a population genetic investigation is crucial for the elucidation of the genetic structure of *G. duodenalis* among the continents. Forty *G. duodenalis* positive fecal samples were collected from different foci of Northwest Iran. The specimens were subjected to Trichrome staining and sucrose gradient flotation. DNA samples were extracted, amplified, and sequenced by targeting glutamate dehydrogenase (gdh) gene. The global *gdh* sequences of sub-assemblages AII and BIV retrieved from NCBI GenBank were analyzed to estimate diversity indices, neutrality indices, and gene migration tests. Sequencing analyses indicated various levels of genetic variability of sub-assemblages AII and BIV among the five continents. Sub-assemblage BIV had greater genetic variability (haplotype diversity: 0.975; nucleotide diversity: 0.04246) than sub-assemblage AII. The statistical *Fst* value demonstrated that the genetic structure of sub-assemblages AII and BIV are moderately differentiated between European-American populations (*Fst*: 0.05352–0.15182), whereas a significant differentiation was not seen among other geographical population pairs. We conclude that a high gene flow of *G. duodenalis* sub-assemblages AII and BIV is unequivocally sharing among the continents. The current findings strengthen our knowledge to assess the evolutionary patterns of *G. duodenalis* in endemic foci of the world and it will become the basis of public health policy to control human giardiasis.

1. Introduction

Giardia duodenalis (syn. *G. lamblia*, *G. intestinalis*; Diplomonadida) is a ubiquitous enteropathogen protozoan parasite that can cause steatorrhea diarrhea and gastroenteritis disorders in humans in both developing (incidence 15–55%) and developed (incidence 5%) countries (Adam, 1991; Feng and Xiao, 2011).

To date, at least eight assemblages (A–H) and various sub-assemblages (AI-AIII, BIII, and BIV) have been identified regarding the analysis of genetic variability (Feng and Xiao, 2011; Lasek-Nesselquist et al., 2009; Thompson et al., 2000). In order of etio-parasitological importance, the zoonotic assemblages A and B are known to be the principal causative agents of human giardiasis. However, infrequent human infections by the assemblages C, D, and E of *G. duodenalis* have been globally reported, particularly in immunocompromised individuals and children (Feng and Xiao, 2011). Domestic animals and wildlife are frequently affected by various *Giardia* subtypes including the assemblages C and D (dog), E (hoofed livestock), F (cat), G (rat), and H (marine animals) (Thompson, 2004).

In recent years, DNA-based genotyping strategies have been broadly used in the epizootiology, transmission dynamic, evolutionary patterns, and distribution of drug-resistance alleles (Criscione et al., 2005; Mahami-Oskouei et al., 2016; Mohammadzadeh et al., 2017; Spotin et al., 2017). The evolutionary potential of parasites originates from gene migration (gene flow), genetic diversity range, and the extent of diversity recombination among genomes (Andras and Ebert, 2013).

Although, an increasing effort has been undertaken to illustrate the patterns of genetic diversity and inter–intra divergence levels of *Giardia* assemblages, there are no more works on population structure and expansion patterns of *Giardia* assemblages among the continents (Choy et al., 2015; Cooper et al., 2007; Lasek-Nesselquist et al., 2010; Lasek-Nesselquist et al., 2009; Takumi et al., 2012).

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The aim of the present study was to assess the genetic variability and population structure of *G. duodenalis* from global sequence data based on glutamate dehydrogenase gene (*gdh*) to recognize how zoonotic sub-assemblages AII and BIV are shared among the continents.

2. Materials and methods

2.1. Ethical approval, sample collection, and cyst purification

All patients were fulfilled an informed consent form. The ethics consent was approved by Ethical Review Committee of Tabriz University of Medical Sciences (No: 5/4/9264). Forty *G. duodenalis* positive fecal samples were collected from patients suffering gastrointestinal complications and fatty diarrhea (steatorrhea) in Northwest Iran (East Azerbaijan and Ardabil Provinces) in period of January 2014 to December 2016. The *G. duodenalis* cysts were purified, and concentrated by sucrose gradient flotation (Galeh et al., 2016). The purified cysts were stained by the Trichrome method.

2.2. Total genomic DNA extraction and polymerase chain reaction (PCR)

Total genomic DNA of *G. duodenalis* was extracted through the freeze-thawing method (Galeh et al., 2016). 50 μL of purified cysts were mixed with an equal volume of glass beads. After adding 25 μL of proteinase K, the suspension was incubated at 60 °C overnight. DNA extraction was performed by Stool DNA Isolation mini kit (Yekta Tajhiz Azma, Iran). A single-round PCR was developed to recognize the *Giardia* infection by targeting *gdh* gene. The PCR amplification was done by using forward primer of 5′ TCAACGTCAACCGCGGCTTCCGT3′, and reverse primer of 5′GTTGTCCTTGCACATCTCC3′ as described previously (Read et al., 2004). The PCR amplification was done in 25 μL reaction volumes including 1 μL of each forward and reverse primers (15 pmol), 0.3 μL (5u/μL) of Taq DNA polymerase (Cinacolon, Iran), 0.9 μL of MgCl2 (50 mM; Cinacolon, Iran), 0.5 μL of dNTP Mix (10 mM; Cinacolon, Iran), 2.5 μL of 10 × PCR buffer (Cinacolon, Iran), 4 μL of DNA template, 10–13 μL of deionized distilled water, and 3 μL of bovine serum albumin (0.1%; New England Biolabs). The amplicons (PCR products) were electrophoresized on 1.5% agarose gel stained with DNA safe stain (Yekta Tajhiz Azma, Iran).

2.3. Sequencing, retrieving sequence, and phylogenetic analyses

PCR products were purified and sequenced by targeting *gdh* gene (Pouya Gostar gene Company, Iran). The ambiguity sites were edited using the standard IUPAC codes. Contigs (overlapped sequences) were analyzed at consensus position using Sequencher Tmv.4.1.4 software. The population structure and genetic diversity of *G. duodenalis* were investigated in 167 (sub-assemblage AII) and 461 (sub-assemblage BIV) sequences generated at the *gdh* of the parasite retrieved from the GenBank database (Abe et al., 2005; Ankarklev et al., 2012; Babaei et al., 2008; Cacciò et al., 2008; Choy et al., 2015; Colli et al., 2015; De Liberato et al., 2015; De Lucio et al., 2016; De Lucio et al., 2015; Debenham et al., 2017; Flecha et al., 2015; Garcia-R et al., 2017; Geurden et al., 2009; Gil et al., 2017; Haramoto et al., 2012; Hatam-Nahavandi et al., 2017; Helmy et al., 2014; Hijjawi et al., 2016; Hogan et al., 2014; Hussein et al., 2009; Itagaki et al., 2005; Lasek-Nesselquist, 2010; Lasek-Nesselquist et al., 2009; Lebbad et al., 2011; Levecke et al., 2009; Martínez-Díaz et al., 2011; Oates et al., 2012; Oliveira-Arbex et al., 2016; Pelayo et al., 2008; Prystajecky et al., 2015; Robertson et al., 2007; Roellig et al., 2015; Santín et al., 2013; Souza et al., 2007; Vermeulen et al., 2015; Wang et al., 2013; Wegayehu et al., 2016). The value of gene flow for *G. duodenalis* populations was assessed using a pairwise fixation index (*F*st: F-statistics) and a number of migrants per generation (Nm) (Rozas et al., 2003). The retrieved sequences of *G. duodenalis* sub-assemblages from various hosts/sources are presented in Supplementary Table 1.

To authenticate genetic relationships among *G. duodenalis* assemblages (A, B, C, D, and E) provided by the *gdh* gene, a phylogenetic tree was drawn using the program Splits Tree 4.0 based on the Neighbor-Net method and Median Joining character (Huson and Bryant, 2006). *Giardia ardeae* was considered as an out-group branch (Accession no: AF069060).

To reveal the genealogical relationships at intra-genetic diversity of *G. duodenalis* haplotypes, a network was constructed by PopART software using the Median Joining algorithm (Bandelt et al., 1999). The distance scale was estimated 0.01. According to the analysis of molecular variance (AMOVA), the diversity indices (Nucleotide diversity (π) and Haplotype diversity (Hd), neutrality indices (Fu's Fs statistic and Tajima's D), and *F*st index (as a scale of gene migration) were calculated using DnaSP software version 5.10 (Rozas et al., 2003). The level of genetic differentiation from metapopulations (regional population) to infrapopulations presented by *F*st index with ranging; 0–1. *F*st < 0.05 (insignificant differentiation), 0.05–0.15 (moderate differentiation), 0.15–0.25 (large differentiation) and *F*st > 0.25 (immense differentiation). The pairwise distances (percent identity and divergence) among the aligned sequences were built using the DNASTAR's MegAlign program (Table 1).

3. Result

3.1. PCR, nucleotide sequence analysis, and sequences pairwise distances

The fragment of the *gdh* gene (nearly 458-bp) was successfully amplified and sequenced for all *Giardia* isolates. Based on the multiple sequence alignment analyses, 24 (14.4%; sub-assemblage AII) and 249 (54%; sub-assemblage BIV) new haplotypes were explicitly identified in 628 geographic isolates occurred at the Asian, Australian, European, African, and American populations (Tables 2 and 3). The lowest and highest number of haplotypes of sub-assemblage BIV belong to the Americas (n = 35) and Australia (n = 73) continents (Table 2). No deletions/insertion (*I*ndel) mutations characterized in sub-assemblages AII and BIV, whereas Transition and Transversion substitutions occurred among the consensus sequences. Within the 380-bp consensus position of sub-assemblage BIV, 109 variable (polymorphic) sites were detected. Of these, 66 were parsimony-informative sites, and 43 of these were singleton variable sites. Furthermore, 17 singleton variable sites and 7 parsimony-informative sites corresponded to sub-assemblage AII.

3.2. Neutrality, diversity indices, and Fst

The haplotype numbers (Hn), the number of isolates, diversity indices, neutrality indices of *G. duodenalis* sub-assemblages BIV and AII are given in Tables 2 and 3. DNA sequencing analyses of sub-assemblages AII and BIV indicated various ranges of genetic diversity among the five continents. Current findings show that the sub-assemblage BIV has greater genetic variation (Hd: 0.975; π: 0.04246) than sub-assemblage AII (Hd: 0.378; π: 0.00326) (Tables 2 and 3). Regarding the infrapopulation geography of *G. duodenalis* sub-assemblage BIV, the highest haplotype diversity appeared to the Australia population (Hd: 0.975), while the lowest haplotype diversity occurred in the Americas populations (Hd: 0.832) (Fig. 1).

Tajima's D (-2.18344) and Fu's Fs (-50.137) indices of sub-assemblages BIV and AII demonstrated negative values in all *G. duodenalis* geographical populations, suggesting a considerable divergence from neutrality (Tables 2 and 3). The sequence pairwise distances of sub-assemblage BIV provided a divergence of 0.00–3.3% and a high percent identity of 96.5–100% among the continents (Supplementary Fig. 1). The observed and expected mismatch distribution including Raggedness r (0.0106–0.2768), R2 statistic (0.0198–0.0305), and the estimate of *Tau*: 0.000 (τ; as a moment estimator in population expansion) are shown in Fig. 2 (Supplementary information).