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Introducing nitazoxanide as a promising alternative treatment for symptomatic to metronidazole-resistant giardiasis in clinical isolates

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ABSTRACT

Objective: To identify the frequencies (F) of ferredoxin and nitroreductase mutations on Iranian clinical isolates of *Giardia lamblia* in order to predict whether the nitazoxanide can be prescribed as suitable drug for symptomatic to metronidazole-resistant giardiasis.

Methods: Forty *Giardia lamblia* isolates as of 38 symptomatic and two metronidazole-resistant patients were collected from Iran. DNAs were extracted and amplified by targeting ferredoxin and *GlNR* genes. The amplicons were directly sequenced to determine gene mutations.

Results: The various amino acid substitutions (F: 20%, Haplotype diversity: 0.891, Tajima's D: −0.440 13) were identified by analyzing ferredoxin gene in four symptomatic and two resistant isolates. Only two haplotypes (F: 5%, HD: 0.345; Tajima's D: 0.778 15) characterized in metronidazole-resistant isolates of GlNR, however, no point mutations was found in symptomatic isolates.

Conclusions: Non-synonymous mutations of ferredoxin oxidoreductase gene reduce translational regulatory protein's binding affinity which concludes reduction of ferredoxin expression and its activity. This leads to decrease in metronidazole drug delivery into the cells. Mutations in these isolates may lead to their resistance to metronidazole. No to low synonymous mutations of *GlNR* demonstrates that nitazoxanide can be prescribed as promising alternative treatment for symptomatic to metronidazole-resistant giardiasis in Iranian clinical isolates.

1. Introduction

Giardia lamblia (*G. lamblia*) (syn. *Giardia intestinalis* or *Giardia duodenalis*) as a microaerophilic gastrointestinal parasitic protest is a causative agent of steatorrhea affecting one billion people worldwide with annual incidence of (200–300) million cases [1,2]. Approximately, 15% of *Giardia* infections are explicitly occur among children (0–24) months in the developing countries [3] and contributes to the second-leading cause of death in children under five years old [4,5]. Based on type of parasitic assemblage and its sensitivity/resistance to treatment, giardiasis may be manifested to chronic and acute symptoms including nausea, diarrhea, vomiting, bloating, dehydration, malabsorption syndrome and failure to thrive [1]. On the one hand *G. lamblia* has been also attributed in the etiology of type II diabetes, obesity and irritable bowel syndrome [6,7]. Giardiasis treatment is currently restricted to nitroheterocyclic (Metronidazole; efficacy 73%–100%, nitazoxanide and furazolidone) and benzimidazoles (Albendazole; efficacy 79%–100% and mebendazole) compounds [8,9]. A number of drugs such as paromomycin and quinacrine because of their low efficacy and high toxicity are infrequently prescribed [8]. However, one of the global difficulties (particularly in Iranian clinical isolates) concerning first line treatment of *G. lamblia* is

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initial treatment failure to metronidazole in symptomatic clinical isolates. The inactive form (oxide) of metronidazole enters the cell through passive diffusion into *G. lamblia* and is reduced by pyruvate-ferredoxin oxidoreductase (PFOR) in mitochondria organelle and nitrogen is converted to toxic radicals [10,11]. Ferredoxin oxidoreductase gene mutations of *G. lamblia* can reduce translational regulatory protein's binding affinity which leads to reduction of ferredoxin expression and its resistance to metronidazole. However, it is not easy to distinguish between cure followed by re-infection or other disorders such as lactose intolerance. A number of studies have been evaluated resistance to differential regulation of oxidoreductase enzymes in mRNA expression levels on nitroheterocyclic resistance laboratory lines (WB, 106, and 713 isolates) [12–19], however, no comprehensive study has conducted on nitroheterocyclic resistance of clinical isolates of *G. lamblia* in replication (DNA) level yet. One of the major problems in treatment of metronidazole-resistant clinical isolates is attributed to metronidazole permeability that does not affect on the respiration of encysting trophozoites and mature cysts, also it do not has significant changes in cyst production at lower concentrations [20]. On the other hand, the numerous side effects of metronidazole have been extensively proven among users. Therefore, using an effectual alternative drug with more cytotoxic activity on both trophozoites and mature cysts and lowest adverse effects should be noticed [21]. In 2004, the nitrothiazolide nitazoxanide (Alinia) as an alternative option has been approved for the pediatric treatment of giardiasis in the USA [22]. This drug compromises the integrity of the cell by causing lesions in the ventral cell membrane and inducing vacuolization [22]. *G. lamblia* nitroreductase (GlNR) has known as a nitazoxanide-binding protein in drug resistance. In this study, the frequencies (F) of ferredoxin and nitroreductase mutations were identified on Iranian clinical isolates of *G. lamblia* in order to predict whether the nitazoxanide can be prescribed as suitable treatment for symptomatic to metronidazole-resistant giardiasis.

2. Material and methods

2.1. Sampling, cyst purification and DNA extraction

During Feb 2014–March 2015, forty *Giardia* positive-microscopic human fecal samples were collected from north-west Iran (East Azerbaijan and Ardabil provinces). A total of 38 patients had various range of symptoms including nausea, bloating, dehydration and steatorrhea (fatty diarrhea). Also, based on tracking of clinical and microscopic investigations, two patients did not have any effective treatment to metronidazole in one last year. The collected specimens were preserved in 70% ethanol and stored at 4 °C until molecular analysis. Specimens, especially cysts, were purified and concentrated by flotation on sucrose with specific gravity of 0.85 M. The purified cyst was stained by trichrome staining (Figure 1). The genomic DNA of *G. lamblia* isolates was extracted through the freeze-thawing technique [10 cycles of freezing (10 min at liquid nitrogen) and thawing (10 min at 95 °C)]. Also, 200 μL of *Giardia* cyst suspension were combined with an equal volume of glass beads followed by vortexing for (8–10) min. After adding 30 μL of proteinase K, the suspension was incubated at 60 °C for one overnight. DNA extraction was done using Stool DNA Isolation mini kit (Yekta Tajhiz Azma, Iran) following the manufacturer's instructions with some modifications. All extracted DNA was stored at −20 °C.

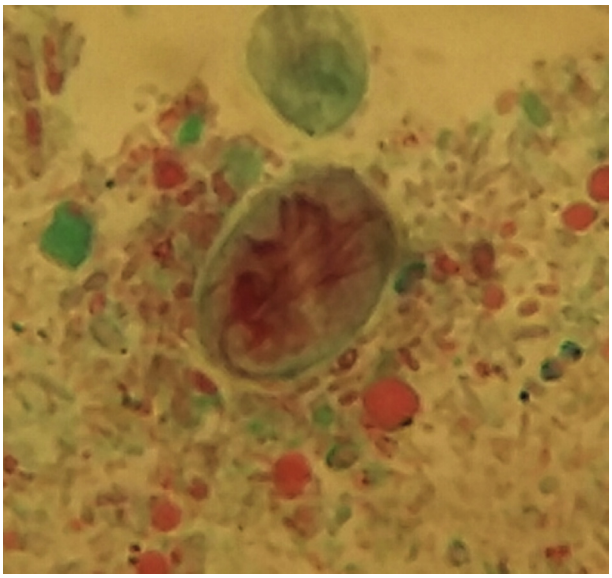

Figure 1. The stained *Giardia lamblia* cyst by trichrome staining (1 000× magnification).

2.2. Primer designing and PCR amplification

Given that the earlier used primers had a short length in order to identify unknown mutations (haplotypes), the target primers of *PFOR* and *G1NR* genes were designed by Oligo Analyzer 3.1 tool based on reference accession numbers of XM_001707879.1 (NR) and L27221 (PFOR). In the PCR reaction, ferredoxin gene was amplified using the forward primer (PFOR-F) 5′-GCCAGCTCCTTCTTGTCTT-3′ and the reverse primer (PFOR-R) 5′-CACGACGATCAGGTCTCTCG-3′. Also, *G1NR* gene was amplified using the forward primer (NR-F) 5′-CGAGACAAAGGTAGTGGCGT-3′ and the reverse primer (NR-R) 5′-GCCACAGCAGCTAAGAGGAA-3′. The efficacy of the designed primers was tested by standard positive control. The PCR amplification was carried out in 25 μL reaction volumes containing 0.3 μL (5 U/μL) of Taq DNA polymerase (Cinnagen, Iran), 2.5 μL of 10× PCR buffer (Cinnagen, Iran), 0.9 μL (50 mM) $MgCl_2$ (Cinnagen, Iran), 0.5 μL (10 mM) of dNTP Mix (Cinnagen, Iran), (10–13) μL deionized distilled water, 1 μL of each forward and reverse primers (10 pmol), (3–4) μL of bovine serum albumin 0.1% as enhancer, and 4 μL of DNA template. Reactions were performed in a thermal cycler PCR System (Eppendorf-Germany). The thermal cycling protocol for both *PFOR* and *GlNR* genes included an initial cycle of 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 45 s, 72 °C for 1 min and a final extension of 72 °C for 10 min. The PCR products were electrophoresized on 1% (W/V) agarose gel stained with DNA safe stain.