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Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm

Original research http://dx.doi.org/10.1016/j.apjtm.2016.07.013

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

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ARTICLE INFO

Article history:
Received 17 May 2016
Received in revised form 16 Jun 2016
Accepted 1 Jul 2016
Available online 26 Jul 2016

Keywords:
Giardia lamblia
Ferredoxin oxidoreductase
Nitroreductase
Nitazoxanide
Metronidazole

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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Peer review under responsibility of Hainan Medical College.

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.

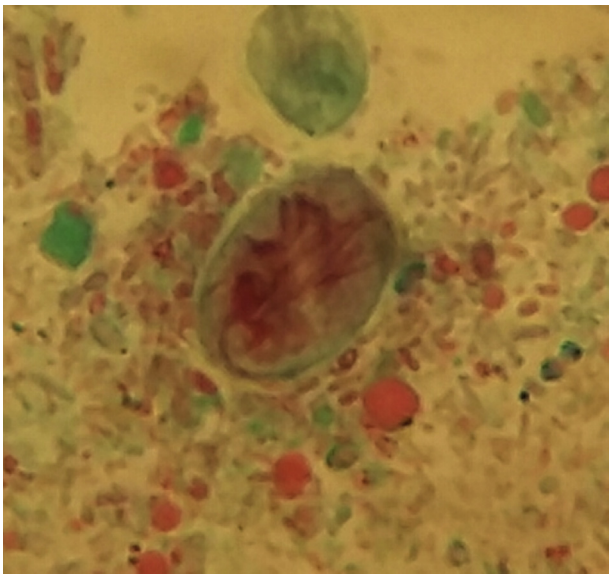

Figure 1. The stained *Giardia lamblia* cyst by trichrome staining (1 000× magnification).

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.

2.3. Sequencing, haplotype network and phylogenetic analysis

The all amplicons of *PFOR* and *G1NR* genes were purified using Gel Purification Kit (BiONEER, South Korea) according to manufacturer's instructions. The final DNA concentration was estimated by comparing with DNA Ladder Marker (Promega) in 1.5% agarose gel. Amplicons were directly sequenced by targeting ferredoxin and *GlNR* genes by ABIPRISMTM 3130 Genetic Analyzer automated sequencer (Applied Biosystems, USA). Ambiguous sites were coded using the standard IUPAC codes for combinations of two or more bases. Contigs (overlapped sequences) of all samples were aligned and edited visually at consensus positions compared to GenBank sequences using Sequencher™ v.4.1.4 and BioEdit softwares [23]. The number of novel haplotypes, diversity indices (Haplotype diversity; Hd and Nucleotide diversity: π) and neutrality indices (Tajima's D and Fu's Fs tests) were estimated by DnaSP software version 5.10 [24]. A haplotype network based on mitochondrial concatenate sequences of *PFOR* and *GlNR* genes was drawn by PopART software in order to distribute identified haplotypes [25]. Also, the phylogenetic tree was constructed by MEGA 5.05 software based on maximum likelihood algorithm and circle model to show topology of distinct isolates compared to same isolates [26]. The accuracy of phylogenetic tree was evaluated by 1000 bootstrap resampling.

3. Results

The *PFOR* (nearly 980 bp) and *GlNR* (nearly 552 bp) genes were successfully amplified from all 40 isolates (Figure 2). Only two haplotypes NR2 (accession number; KX181723) and NR3 (accession number; KX181724) (Transition model; Cytosin converted to thymine, F: 5%) were identified in metronidazole resistant isolates of GlNR (Figure 3) whilst, no point mutations was found in symptomatic isolates. Eight novel haplotypes (F: 20%) were identified by analyzing ferredoxin gene which among of them, two (PFOR2: accession no: KX181715 and PFOR3: KX181716) and six (PFOR4-PFOR9: accession nos: KX181717-KX181722) haplotypes were belonged to resistant and symptomatic isolates respectively (Figure 3). Various amino acid substitutions were characterized at different codons (76, 97, 116, 134, 172, 224, 238, 245, 248, 250 and 276) of resistant isolates (PFOR2 and PFOR3). Moreover, amino acid replacement identified at various codons (28, 29, 76, 97, 99, 116, 134, 172, 245, 250 and 276) of some symptomatic isolates (PFOR4, 5, 8 and 9) (Figure 4). However, no new mutation (haplotypes) was found in the rest sequences of ferredoxin gene compared to wild type (PFOR1, Ref L27221). According to the analysis of molecular variance (AMOVA) test, the high Hd value of ferredoxin (0.891) was shown the principal pattern of the total genetic variability within *Giardia* populations whilst nucleotide diversity was low in both genes (π; 0.00093 to 0.00679) (Table 1). Unlike *GlNR* gene, the neutrality indices of the ferredoxin oxidoreductase were shown negative values (−0.44013 for Tajima's D to −1.046 for Fu's Fs statistic) (Table 1). The status of metronidazole-resistant isolates and other sequences were shown in Figure 5 which isolates PFOR2, 3, 8, 9 and NR2, 3 have placed in distinct branches.

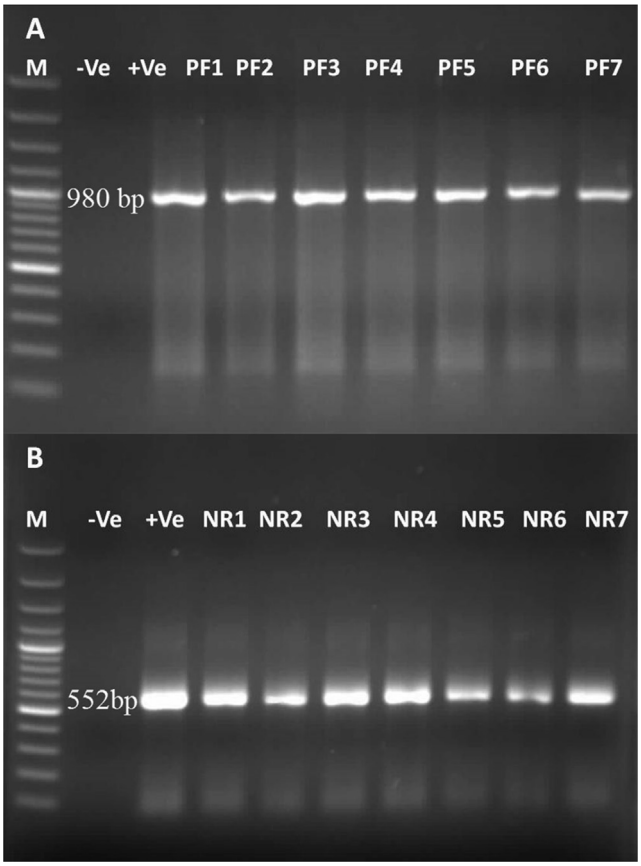

Figure 2. Agarose gel electrophoresis of PCR products.
A: PF1-PF7: *G. lamblia* isolates amplified based on ferredoxin gene (PF). B: NR1-NR7: *G. lamblia* isolates amplified based on Nitroreductase gene (NR) +Ve: Positive control, −Ve: Negative control, M: 100 bp DNA size marker.

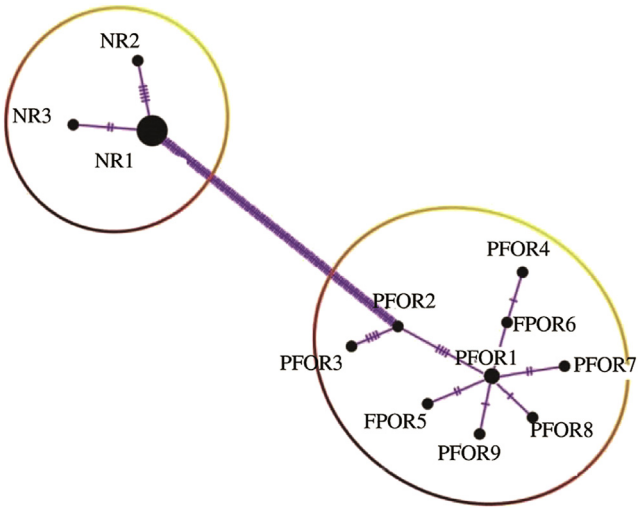

Figure 3. A haplotype network according on mitochondrial concatenate sequences of ferredoxin and *GlNR* genes in metronidazole resistant *Giardia* isolates (PFOR2, 3 and NR2, 3) and symptomatic specimens.

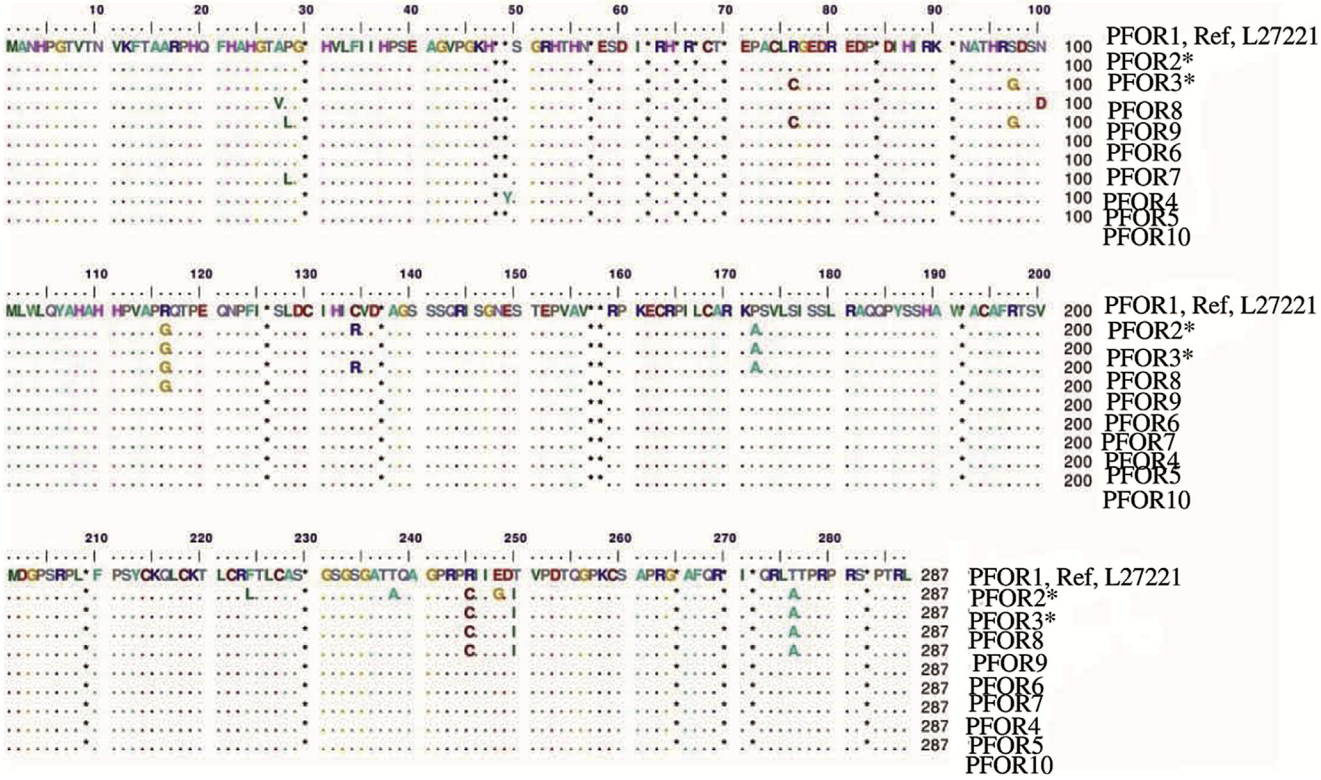

Figure 4. Comparison of protein sequences of ferredoxin oxidoreductase gene of *G. lamblia* in metronidazole resistant isolates (PFOR2**, PFOR3**) and symptomatic specimens compared to wild type (PFOR1).

Table 1

Diversity and neutrality indices of *Giardia lamblia* based on nucleotide sequences of Pyruvate ferredoxin oxidoreductase and Nitroreductase genes.

Gene	Diversity indices				Neutrality indices	
	N	Hn	Hd ± SD	Nd (π) ± SD	Tajima's D	Fu's Fs statistic
PFOR	40	8	0.891 ± 0.092	0.006 79 ± 0.001 73	−0.440 13	−1.046
NR	40	2	0.345 ± 0.172	0.000 93 ± 0.000 88	0.778 15	0.659

Hn: number of haplotypes; Hd: haplotype diversity; Nd: nucleotide diversity.

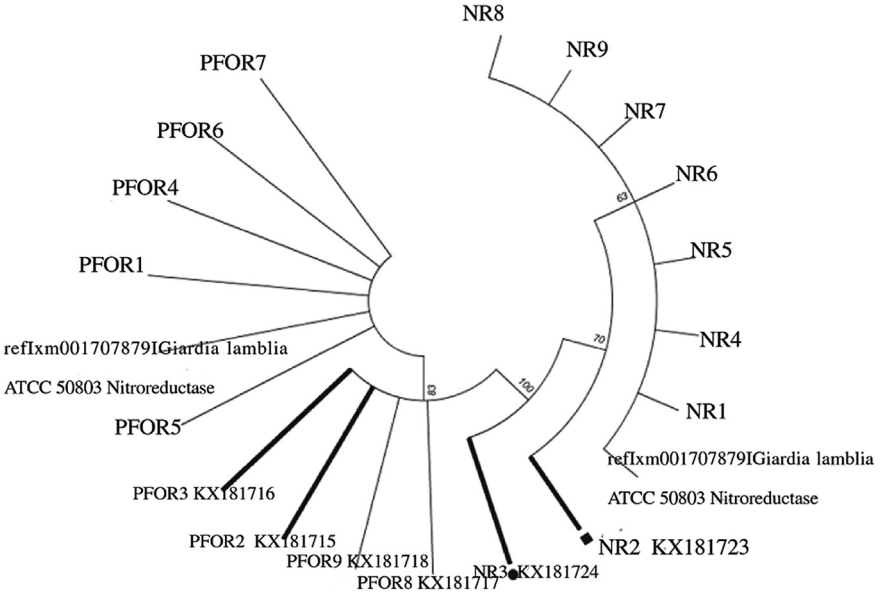

Figure 5. Phylogenetic tree of *G. lamblia* ferredoxin oxidoreductase and *GlNR* sequences in metronidazole resistant isolates (PFOR2, 3 and NR2, 3) and symptomatic specimens using maximum likelihood algorithm with kimura 2-parameter model and 1 000 bootstrap re-sampling.

4. Discussion

In this investigation, for the first time the various ranges of PFOR and GlNR mutations were amplified and identified in symptomatic to metronidazole resistant Iranian clinical isolates of *G. lamblia* by sequencing and AMOVA analysis. The mechanisms of metronidazole resistance have been extensively negotiated during the last 40 years, although the approaches are often contradictory to put into viewpoint.

The majority clinical investigations on chemotherapy of giardiasis reveal that, despite the drug used, cure rates are below 100%. Apart from drug refractory, other predisposing factors can also be accountable for treatment failure, covering sequestration of parasite in the gallbladder or the pancreas, re-infection, inadequate amounts of drug administered and immune deficiency [27].

It is noteworthy that *G. lamblia* isolated from patients with resistant giardiasis do not regularly show drug resistance *in vitro* [28]. It can be justified by some immune-physiological influences of the human host on the success of a given chemotherapy.

Several studies have been shown a strong association between down regulation of *PFOR* gene and drug resistance on cell lines (*in vitro*), bacteria and *Trichomonas vaginalis* in translation and mRNA expression levels [17,29–31]. Reduction of ferredoxin oxidoreductase translation in resistant strains enhances this assumption that by the decrease of the cells' ability to regenerate metronidazole into cytotoxic free radicals. In addition, it is hypothesized that mutations of ferredoxin gene reduce the activity of PFOR. However, there is no associated study between correlation of ferredoxin mutation and drug resistance on clinical isolates of *G. lamblia* in replication level.

In this study, 20% mutant strains of ferredoxin gene containing various codon substitution and high haplotype diversity (0.891) was unequivocally identified among all resistant isolates (n = 2) and six symptomatic specimens. This fact can be described by non-synonymous mutations of ferredoxin reduce translational regulatory protein's binding affinity which conclude 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 resistance of them to metronidazole.

On the other hand, 5% mutant strains of *GlNR* gene without any codon substitution and low haplotype diversity (0.345) was only recognized in resistant isolates (n = 2). This no to low synonymous mutations of *GlNR* exhibits that nitazoxanide can be potentially used as an alternative treatment in symptomatic to metronidazole-resistant Iranian clinical isolates. Unlike metronidazole drug, so far no evidence has been found for any teratogenic or mutagenic activity of nitazoxanide, in addition the last drug has more effectual cytotoxic activity on trophozoites and mature cysts in encystations process [8,22].

Up to now, nitazoxanide resistance has been merely observed in the laboratory [16]. In a study, a nitazoxanide-resistant cell line was robustly resistant to metronidazole, while a cell line with induced metronidazole resistance was susceptible to nitazoxanide [16]. In consistent with our findings, this indicates that cross resistance between both drugs is not essentially reciprocal. In a study on the nitazoxanide resistant cell line illustrated chaperone proteins Hsp 70 and Hsp 90 to be up-regulated in expression [17]. Several research papers have been revealed that NR1 and NR2 have crucial role in the metabolism of metronidazole in *G. lamblia*. Down-regulation of NR1 is lead to metronidazole-resistant laboratory cell line whereas NR2 was found to be up-regulated in metronidazole-resistant [18,21].

In conclusion, we showed that a high frequency range of ferredoxin mutations including amino acids/haplotypes are unambiguously circulating among resistant metronidazole patients and some symptomatic giardiasis in Iran. This is an alert that a number of symptomatic specimens are potentially converting to resistant isolates. No to low synonymous mutations of GlNR demonstrates that nitazoxanide as promising alternative drug can be prescribed to treatment symptomatic to metronidazole-resistant Iranian clinical isolates. Taken together, although treatment failures and drug resistance in *Giardia* can be problematic, but hopeful novel drugs and combination therapy may be affected in promising treatment strategies.

Conflicts of interest statement

The authors declare that there is no conflict of interests.

Acknowledgments

This study was financially supported by Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. This article is derived from the master's thesis of the first author (Grant No. 93/2-4/12).

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