

Original Article

## Determination of PFOR gene expression in strains of *G. intestinalis* with different inhibitory concentrations of metronidazole

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### Abstract

**Introduction:** *Giardia intestinalis* is the most important and common diarrhea-causing parasitic protozoa worldwide with growing clinical relevance in public health. There are many documented cases of *G. intestinalis* resistance to metronidazole (MZ). Pyruvate: ferredoxin oxidoreductase (PFOR), the membrane-localized enzyme, plays a key role in the development of resistance to drugs. The aim of the present study was to evaluate the difference in the levels of PFOR gene expression between MZ-resistant and MZ-susceptible strains of *G. intestinalis*.

**Methodology:** From 159 samples with *G. intestinalis* cysts, 48 strains were successfully cultivated. Using specific pair primers, PFOR gene expressions were estimated in different groups of *Giardia*. The polymerase chain reaction (PCR) data were analyzed with Bayesian analysis of qRT-PCR data using MCMC.qpcr package, with relative expression software tool (REST) and quantitative PCR CopyCount web source.

**Results:** In the group of *Giardia* with minimum inhibitory concentration (MIC) of 6.3 µM, the level of PFOR gene expression was downregulated and compared with controls, differed by 1.5 to 2.8 times. At the same time, there was no significant difference in PFOR gene expression between the control (susceptible) group and the group with MIC of 3.2 µM.

**Conclusions:** Though there is association between PFOR gene expression and metronidazole resistance of *Giardia intestinalis*, the level of PFOR gene expression cannot be a strong genetic marker to predict level of resistance to metronidazole based on MICs.

**Key words:** *G. intestinalis*; metronidazole resistance; pyruvate ferredoxin oxidoreductase (PFOR) expression.

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### Introduction

*Giardia intestinalis* (syn. *Giardia lamblia*, *Giardia duodenalis*) is considered to be the most common intestinal protozoan in the world [1], parasitizing different hosts, including mammals and birds [2]. *G. intestinalis* has two different forms during life cycle: the cyst, which is capable of causing infection and being resistant to many environmental conditions, and the trophozoite, which is the vegetative form of *G. intestinalis* and colonizes the intestinal mucosa, causing symptoms of diarrhea and malabsorption [3]. The main path of distribution of this parasite is the fecal-oral route through ingestion of cysts in contaminated food or water. The clinical course of giardiasis may be asymptomatic or may lead to dehydration, causing diarrhea and abdominal

discomfort. Some recent studies [4,5] showed that chronic diarrhea that lasts for several months leads to increased mortality among malnourished children and among children with immune deficiency in the first three years of life.

Since the introduction of nitroheterocyclic drugs in the 1950s, metronidazole became the most widely used drug for the treatment of infections caused by *G. lamblia*, *T. vaginalis*, and *E. histolytica* [6,7]. Today there is data available that shows the appearance of resistant protozoa to this drug [7,8].

Attempts to find the genetic markers that are responsible for resistance to metronidazole led to the discovery of the correlation between PFOR gene expression and drug resistance [9]. As shown previously [10], the level of PFOR activity was much

lower in metronidazole-resistant *Giardia* compared to that in metronidazole-susceptible protozoa.

The aim of this study was to evaluate the difference in the levels of PFOR gene expression in metronidazole-resistant and metronidazole-susceptible strains of *G. intestinalis* using the relative gene expression approach and absolute polymerase chain reaction (PCR) quantification.

## Methodology

### Sample collection

Fecal samples (n = 187) were gathered from 2013 to 2014. The samples were positive for the presence of cysts of *Giardia* based on microscopic results. Cysts were extracted from samples using zinc sulfate (specific gravity of 1.18) as described by Georgi *et al.* [11]. The control of obtained cysts was performed with bright field microscopy at 200× magnification. The commercial PCR real-time kit RIDAGENE Parasitic Stool Panel (R-Biopharm, Darmstadt, Germany) was used for cyst identification. Ultimately, 159 of 187 samples were positive for *Giardia* cysts.

### Cultivation and phenotypic identification of metronidazole resistance of *G. intestinalis*

Fresh culture medium (TYIS-33) with penicillin (100 U/mL), streptomycin (100 mg/mL), amphotericin B (0-8 µg/mL), and 10% adult bovine serum was used for *G. lamblia* trophozoites cultivation (at 37°C and anaerobic condition), based on the methods of Diamond *et al.* [12]. Growth of trophozoites and signs of bacteria or yeast were controlled microscopically, and the medium was changed every 24–48 hours.

Phenotypic detection of resistance to metronidazole was performed by cultivating *G. intestinalis* trophozoites in the presence of metronidazole as described by Upcroft *et al.* [13]. Two concentrations of metronidazole (6.3 µM as a mean and 3.2 µM as a minimum value from the range specified by Upcroft *et al.* [13]) were used to define the resistance of *Giardia*. The strain was defined as a resistant (R) if there was growth in the media with 6.3 µM of metronidazole. About 20% coverage of the well surface was considered to be a successful growth. Intermediate resistance of *Giardia* (I) was defined as cases with growth of *Giardia* in the presence of 3.2 µM of metronidazole. All others were considered to be susceptible (S) strains.

### PFOR gene expression in MZ-resistant and MZ-susceptible strains of *G. intestinalis* by PCR

Total RNA was extracted using a Pure Link RNA Mini Kit (Ambion, Carlsbad, USA). The reverse transcription reaction was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, USA). The PFOR (target gene) and ACT (endogenous gene) genes of *G. intestinalis* were amplified by real-time PCR using SYBR Select Master Mix (Applied Biosystems) and primer pairs GL PFOR F/GL PFOR R for PFOR gene detection and GL ACT F/GL ACT R primer pair for actin gene detection (forward GL PFOR: 5'-CTACGACATTGACTTTGCTG-3', and reverse GL PFOR: 5'-CCCATCTTCTTGTCCTTGAC-3'; forward GL ACT: 5'-CAAGGAGCTTACAGACTTCTG-3' and reverse GL ACT: 5'-AACGGACTCATAATCAAGGG-3'); designed and synthesized by ACGT lab, Astana, Kazakhstan). The values of cycles were determined with alternative Cy0 approach as described by Guescini *et al.* [14] and integrated in the qpcR package of Ritz *et al.* [15]. The efficiency of each PCR reaction was calculated with the linear regression of efficiency method also integrated in the qpcR package [15]. The relative gene expression was analyzed with two approaches: Bayesian analysis of qRT-PCR data using the MCMC.qpcr package (author, Mikhail V. Matz), and relative expression software tool (REST) designed by Qiagen (Hilden, Germany). The absolute PCR quantification was realized using qPCR CopyCount (DNASoftware, Ann Arbor, USA).

Statistical analyses were performed using MedCalc for Windows, version 10.2 (MedCalc Software, Ostend, Belgium). The level of significance (p value) was < 0.05.

## Results

From 187 different human fecal samples containing cysts (based on microscopy data) collected from an isolation hospital in Karaganda during 2013–2014, 159 samples (85%) were positive for the presence of *Giardia intestinalis* cysts by PCR.

The rate of successful cultivation, when axenic cultures were obtained, was 30.2% (48 strains of 159). Thirteen strains of *G. intestinalis* showed growth at 6.3 µM of metronidazole and were considered to be resistant (R). Eleven samples had intermediate resistance, and 24 were susceptible to metronidazole and were considered to be controls.

**Table 1.** The values of cycles for each group of *G. intestinalis* strains detected with Cy0 method

	Gene								
	Act (3.2 μM)			Act (6.3 μM)			Act (control)		
	Mean	95% CI	SD	Mean	95% CI	SD	Mean	95% CI	SD
Cy0	20.062	19.935 – 20.189	0.2864	20.279	20.110 – 20.449	0.4196	20.230	20.155 – 20.306	0.2601
	PFOR (3.2 μM)			PFOR (6.3 μM)			PFOR (control)		
Cy0	20.957	20.804 – 21.111	0.3466	22.567	22.323 – 22.810	0.6034	20.992	20.890 – 21.094	0.3511

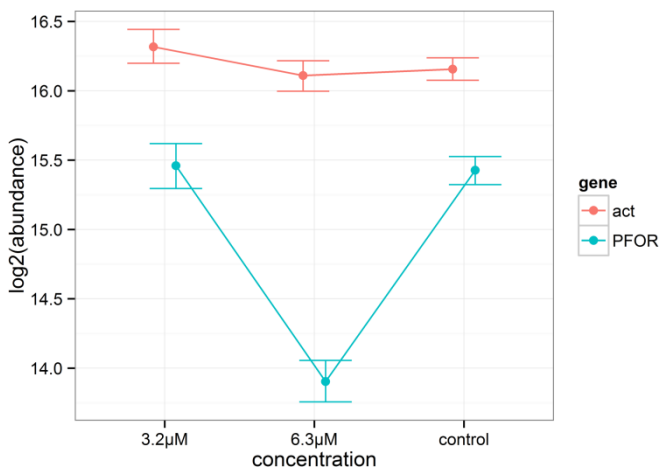
**Table 2.** Relative gene expression calculated with REST software.

Comparison groups	Gene	Type	Reaction Efficiency (%)	expression	Std. error	95% CI	p(value)
3.2 μM/control	PFOR	target	95	0.914	0.544-1.493	0.337-2.127	0.294
	Actin	reference	95		1		
6.3 μM/control	PFOR	target	95	0.361	0.210-0.664	0.101-1.255	<0.000
	Actin	reference	95		1		

**Table 3.** Absolute PCR quantification based on qPCR CopyCount (DNAsoftware).

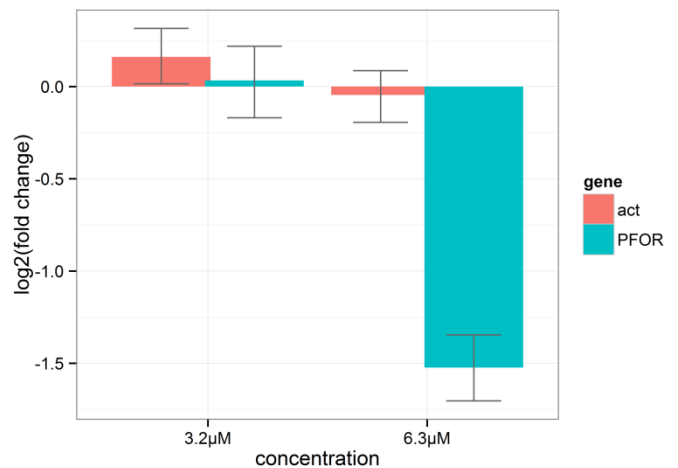
		PFOR (3.2 μM; n=21)			
Mean	95% CI	SD	Minimum	Maximum	
51,746.4	46,430 – 57,062.7	11,990.6	32,546	75,777	
		PFOR (6.3 μM; n=23)			
Mean	95% CI	SD	Minimum	Maximum	
17,625.8	14,800.9 – 20,450.7	6,993.9	6,596	34,139	
		PFOR (control; n=24)			
Mean	95% CI	SD	Minimum	Maximum	
50,604.7	46,737.3 – 54,472.2	13,319	33,580	76,430	

**Figure 1.** Gene expression of PFOR and actin in different groups of *Giardia* strains.



This figure describes the changes in PFOR gene expression and control gene actin in different minimum inhibitory concentrations of metronidazole. The abundance is presented on axis Y; the inhibitory concentrations of metronidazole is presented on axis X.

**Figure 2.** Relative gene expression in comparison with control.



This figure describes changes of relative PFOR gene expression compared with controls. The level of relative expression is presented on axis Y; the inhibitory concentrations of metronidazole is presented on axis X.

The results of  $Cy_0$  values of PCR reactions for three groups of *G. intestinalis* are presented in Table 1.

The efficiency of each PCR reaction was determined with the qpcR package of R statistics (author, Andrej-Nikolai Spiess) using the linear regression of efficiency method calculated on fitted nonlinear sigmoidal models with seven parameters [16]. The mean efficiency for two genes (PFOR and actin) of *Giardia* was 95%.

Two gene expressions, obtained using a generalized linear mixed model analysis of qRT-PCR data integrated in the MCMC.qpcr package, are shown in Figure 1.

As seen in Figure 1, the levels of actin gene expression (chosen as the housekeeping gene) were expressed at relatively constant levels, while levels of PFOR gene expression in the resistant group of *G. intestinalis* (MIC 6.3  $\mu\text{M}$ ) were different.

The results of changes in relative gene expression in the control group (strains with susceptibility to metronidazole) are shown in Figure 2. The level of PFOR gene expression was downregulated and ranged from -1.7 to -1.34 in the group of *Giardia* with MIC of 6.3  $\mu\text{M}$  ( $p < 0.05$ ), while the level of relative expression of PFOR gene did not differ ( $p > 0.05$ ) in the group with MIC of 3.2  $\mu\text{M}$ .

The analysis of relative gene expression was performed using Relative Expression Software Tool; data are presented in Table 2.

As shown in Table 2, PFOR gene was also downregulated in the sample group (MIC of 6.3  $\mu\text{M}$ ) compared with the control group by a mean factor of 0.361 (standard range was 0.210–0.664) ( $p < 0.000$ ).

Using raw data of PCR reactions and some additional information such as length of amplicons, total volume of reaction, and type of real-time PCR, the absolute quantification was calculated with qPCR CopyCount. The obtained data are presented in Table 3.

Student-Newman-Keuls test for all pairwise comparisons of DNA molecule numbers of the PFOR gene showed a significant difference ( $p < 0.05$ ) in the group of *G. intestinalis* with MIC 6.3  $\mu\text{M}$ . In the resistant group, the amount of DNA was in 2.87 times lower than in the control group.

## Discussion

*Giardia intestinalis* (also known as *G. duodenalis* or *G. lamblia*) is the most common flagellated protozoan causing persistent diarrhea throughout the world. The prevalence of *G. intestinalis* has been reported to range from 2%–8% in developed countries

[17] to 20%–60% in underdeveloped countries [1]. The symptoms of giardiasis are quite variable and range from none to chronic diarrhea and malabsorption, which can lead to increased mortality in some cases [4].

Since the mid-twentieth century, nitroimidazoles, and especially metronidazole, have been widely used for the treatment of giardiasis because of their effectiveness, low cost, and safety profile. Their use has led to the appearance of strains of *Giardia* resistant to metronidazole; according to some studies [16–19], this trend continues to grow.

Investigations into the mechanism of metronidazole resistance have revealed the key role of PFOR, the membrane-localized enzyme. Metronidazole, as an antiprotozoal drug, is activated after the 5-nitro group has been reduced by ferredoxin that has, in turn, been reduced by PFOR, which generates a toxic nitro radical for parasites. The downregulation of PFOR activity decreases the electron flow to ferredoxin and facilitates the survival of *G. intestinalis* in the presence of the drug [10]. The important function of the PFOR enzyme in developing resistance to metronidazole has been proven in *in vitro* studies [20,21], where the active effect of PFOR enzyme purified from *G. intestinalis* cultivated in the presence of metronidazole has been shown.

In this study, the expression of PFOR gene between giardiasis with different MICs (3.2 and 6.3  $\mu\text{M}$ ) of metronidazole using RT-PCR was investigated. The results were analyzed using two approaches: Bayesian analysis of qRT-PCR data using MCMC.qpcr package of R statistics, and REST software to estimate the relative gene expression between groups with different resistant and susceptible strains of *Giardia* along with CopyCount, a web source, to calculate the absolute amount of transcriptions. No significant difference was found in PFOR gene expression between the control (susceptible) group and the group with MIC of 3.2  $\mu\text{M}$ . At the same time, in the group of *Giardia* with MIC of 6.3  $\mu\text{M}$ , the level of PFOR gene expression was downregulated. The levels ranged from 1.5 (based on Bayesian analysis of qRT-PCR data) to 2.8 (based on REST software and CopyCount web source of DNAsoftware) times those from the control group.

Though previous studies [7,9] have also reported the association of downregulated level of PFOR gene and resistance *Giardia* to metronidazole, the gene expression in the strains with susceptibility was not significant.

## Conclusions

Taking these findings into account, we conclude that albeit there is a relationship between PFOR gene expression and metronidazole resistance of *Giardia intestinalis* based on some studies, the level of PFOR gene expression cannot be used as a strong genetic marker to predict the MICs of metronidazole.

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