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GENE EXPRESSION OF DRUG RESISTANCE GENES FOR NITAZOXANIDE AND METRONIDAZOLE IN *GIARDIA LAMBLIA* ISOLATED FROM CLINICALAND SUBCLINICAL PATIENTS

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Abstract

The work, herein was focused on gene expression of drug resistance genes for nitazoxanide and metronidazole in *Giardia lamblia* isolated from clinical and subclinical patients. Fifty stool samples were obtained from patients attended Al-Kut Hospitals and private clinic, Wasit, Iraq. Those samples were examined for the overexpression of the heat-shock protein (hsp70) and (hsp90) genes relying on using a real-time quantitative PCR (RT-qPCR) method. The results revealed increases in the expressions of the chaperone genes as 10 and 7 fold changes for both hsp70 and 90, respectively. The current study links the resistance against nitazoxanide and metronidazole in *Giardia lamblia* isolated from clinical and subclinical patients to the changes of expression in the chaperone genes.

Key words : Chaperone, Drug resistance, Heat shock protein, Hsp70, Hsp90.

Introduction

Giardia lamblia is a highly spread flagellated protozoan known for inducing diarrhea in people around the world. The parasite life cycle contains two main phases: the replicating trophozoite and the infective non-replicating cyst. The Cyst induces infections when taken perorally. In the upper part of the small intestine, the cyst break-release an excyzoite, a quickly transformable phase in the life cycle that is divided making four trophozoites immediately. *G. lamblia* are strongly relying on food component uptake from the intestine of the host using a group of organelles such as peripheral vacuoles (PVs) that work on endocytosis of fluid-phase and to release damaging materials into the intestine environment (Adam, 2001; Bernander, Palm and Svard, 2001; Shanda R Birkeland *et al*., 2010; Shanda R. Birkeland *et al*., 2010; Cernikova *et al*., 2018).

Metronidazole (Flagyl®) destroys trophozoites of *Gardia* by entering the trophozoites and reducing the chemical resulting in a toxic nitro radical via pyruvate : ferredoxin oxidoreductase (POR). The problem of drug resistance against these drugs are increasingly elevated as it has been revealed via resistant strains the were isolated from patients. The issue of developing new resistant strains was found to be due to deactivation in the work of the POR system (Gardner and Hill, 2001; Busatti *et al*., 2013; Weir and Le, 2019). The industry has been opened for thiazolide nitazoxanide (Alinia®). The first drug with documented effectiveness against cryptosporidiosis was authorized in the USA for the use in children and adults for the management of cryptosporidiosis and giardiasis. Nitazoxanide is quickly deacetylated and eventually metabolized into tizoxanide-glucoronide when orally ingested. Tizoxanide was recorded to show nitazoxanide comparable antimicrobial behaviors, while a percentage of microorganisms were completely inactive (Bailey and Erramouspe, 2004; Lopez-Velez *et al.*, 2010; Müller *et al*., 2013).

The work, herein, was focused on gene expression of drug resistance genes for nitazoxanide and metronidazole in *Giardia lamblia* isolated from clinical and subclinical patients. Fifty stool samples were obtained from patients attended Al-Kut Hospital, Wassit, Iraq.

Materials and Methods

Samples

Fifty stool samples were obtained from patients attended Al-Kut Hospitals and private clinic, Wasit, Iraq. The samples were cool-dry-transported in clean containers to a Lab for completing the analysis.

Total RNA extraction, processing and cDNA production

Total RNA from *G. lamblia* was extracted using Accuzol® reagent kit (Bioneer, Korea) with relying of in the instructions of the kit supplied by the company. A digestion step using a DNase I (Promega company, USA) for removing any remaining DNA following the kit's guidelines. The produced RNA was RNase-free-water-eluted and preserved for later work at -80°C. The concentration and the purity of the RNA was tested by a Nanodrop spectrophotometer (THERMO, USA). The cDNA production was enhanced using Qiagen Omniscript™ kit as shown in the company protocol. A concentration of RNA at 100ng/ul was recruited in the process of the cDNA synthesis. The thermocycler conditions were 1hr at 50°C for the cDNA synthesis step and 5mins at 95°C for the inactivation of heat.

Quantitative Real-Time PCR (qPCR)

Relative gene expression of the hsp70 and hsp90 genes was assessed using a RT-qPCR method for measuring their mRNA levelsby following the 2-ΔΔCT Livak method (Livak and Schmittgen, 2001). A Real-Time PCR system (BioRad, USA) was utilized for this part of the work employing SYBER Green dye qPCR master mix. Actin as a housekeeping gene was used. The primers, designed with the incorporation of the primer3 plus software are displayed in the table 1.

Table 1 : The RT-qPCR primers.

Primer		Sequence (5'-3')
HSP70	**F**	GAGGCGATCGTCCATCCC
	R	CCTTACACCACAGTTAGTCC
HSP90	**F**	ACGACGACGCAGAGAACCT
	R	CAAAGTAACACAACAAGTTCAC
Actin gene	**F**	ACATATGAGCTGCCAGATGG
	R	TCGGGGAGGCCTGCAAAC

AccuPower™ 2XGreen Star qPCR master mix kit (Bioneer, Korea) and its protocol were used for producing the master mix for each gene separately. An amount at 10ng of the cDNA was found to be suitable for the reaction. The thermocycler was employed using the conditions; 1 cycle for 1hr at 50°C as an initial denaturing step, 40 cycles of [20s at 95°C as a denaturing step and 30s at 60°C as an annealing\extension step, detection (scan)] and 1 cycle for 0.5s at 60-95°C as a melting step.

Results

The results revealed increases in the expressions of the chaperone genes as 10 and 7 fold changes for both hsp70 and 90, respectively (fig. 1).

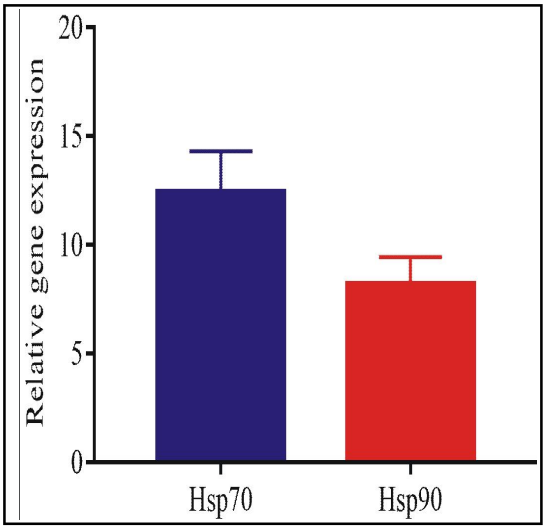

Fig. 1 : Relative gene expression of hsp70 and hsp90 genes in *G. lamblia*.

Discussion

In the present investigational work, the chaperone genes, hsp70 and hsp90 were checked for their relative expression in *G. lamblia* from giardiasis patients. The results demonstrated increases in the expressions of both genes with no significant differences between them.

The heat shock proteins, HSP72 and HSP27 are known for their roles in protecting the human cells against heat shock and the subsequent process of apoptosis. However, HSP functions in the current studied parasite are not understood. If *Giardia* has the same activities of those proteins, chaperones may play important roles in increasing thermotolerance and resistance against metronidazole/nitazoxanide as it has been recognized when a clone of this parasite was noticed to have such actions *in vitro* (Müller *et al.*, 2008).

Previous studies have demonstrated that nitazoxanide suppresses the activities of the giardial disulphide isomerases showing insufficient protein folding processes leading to destruction in the parasitic metabolic and regulatory machinery. Chaperone overexpression has been identified to perform strong activities in stabilizing of the cellular components. Although, some changes in

the level of gene expression related to proteins responsible for drug resistance are suggested, it has been mentioned that substitutions in the amino acids in those proteins may provide the resistant features in the tested parasites (Tejman-Yarden *et al*., 2011; Leitsch, 2015; Lalle and Hanevik, 2018).

It has been identified that *G. lamblia* can change it gene expression according to the environmental factors surrounding the protozoan. However, such resistance generated via those alterations may not induce vertical transmission of the resistance to other generations of the protozoan as explained by (Müller *et al*., 2008), who found that the excysted trophozoites were susceptible to metronidazole and nitazoxanide after prolonged exposure of the previous protozoan stage to both drugs. Interestingly, changes in the expression profiles of hsp70 and hsp90 were found to be relevant to the alterations of resistance to metronidazole and nitazoxanide (Müller *et al*., 2008).

The findings encourage the suggestion that *G. lamblia* handles itself well in different environments via its alterations of gene expression profiles especially in the presence of anti-protozoan drugs or antibodies. The current study links the resistance against nitazoxanide and metronidazole in *Giardia lamblia* isolated from clinical and subclinical patients to the changes of expression in the chaperone genes.

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