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Research paper

Validation of housekeeping genes as an internal control for gene expression studies in *Giardia lamblia* using quantitative real-time PCR

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ABSTRACT

The analysis of transcript levels of specific genes is important for understanding transcriptional regulation and for the characterization of gene function. Real-time quantitative reverse transcriptase PCR (RT-qPCR) has become a powerful tool to quantify gene expression. The objective of this study was to identify reliable housekeeping genes in *Giardia lamblia*. Twelve genes were selected for this purpose, and their expression was analyzed in the wild type WB strain and in two strains with resistance to nitazoxanide (NTZ) and metronidazole (MTZ), respectively. RefFinder software analysis showed that the expression of the genes is different in the three strains. The integrated data from the four analyses showed that the NADH oxidase (NADH) and aldolase (ALD) genes were the most steadily expressed genes, whereas the glyceraldehyde-3-phosphate dehydrogenase gene was the most unstable. Additionally, the relative expression of seven genes were quantified in the NTZ- and MTZ-resistant strains by RT-qPCR, using the aldolase gene as the internal control, and the results showed a consistent differential pattern of expression in both strains. The housekeeping genes found in this work will facilitate the analysis of mRNA expression levels of other genes of interest in *G. lamblia*.

1. Introduction

Giardia lamblia, previously identified as *Giardia intestinalis* or *Giardia duodenalis*, is a unicellular eukaryotic parasite (Plutzer et al., 2010) that causes the disease giardiasis, which affects humans and a large number of vertebrates (Müller and von Allmen, 2005). *G. lamblia* has two stages in its life cycle, as a trophozoites responsible for clinical disease, and as a cyst, which is the mode of disease transmission (Lebwohl et al., 2003).

Giardia is an ancient protozoan sharing many characteristics with anaerobic prokaryotes (Plutzer et al., 2010), including some metabolic pathways. However, the synthesis of transcripts is very similar to that of eukaryotes, which is probably a result of horizontal gene transfer (Best et al., 2004; Morrison et al., 2007; Andersson, 2009), as concluded from different genomic and molecular studies, including the analysis of the promoter regions of genes including *Glutamate dehydrogenase* (Yee et al., 2000), *α2-Tubulin*, *Actin*, *Giardin*, *RAN*, (Elmendorf et al., 2001), histone genes (Yee et al., 2007), and the cyst wall protein genes *CWP1* and *CWP2*, involved in the encystation process (Huang et al., 2008). The study of genes encoding Variant-Specific Surface Proteins (VSPs) (Luján, 2008) provided important information on the regulation of gene expression in *G. lamblia*. Recently, the differential expression of genes encoding proteins involved in different processes (metabolism phosphatases, kinases, transcription/translation, cell cycle regulation, structural proteins) under conditions of oxidative stress was investigated using microarrays (Raj et al., 2014). A proteomic analysis of *G. duodenalis* strains resistant to albendazole (ABZ) showed

Abbreviations: mRNA, messenger RNA; cDNA, complementary DNA; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; RT-qPCR, real-time quantitative reverse transcriptase PCR; NTZ, nitazoxanide; MTZ, metronidazole; ABZ, albendazole; *CWP1*, cyst wall protein; *CWP2*, cyst wall protein 2; *VSP*, Variant-specific surface protein; *RAN*, ras-related nuclear protein; *ACT*, actin; *δ-GIA*, δ-giardin; *α2-TUB*, α2-tubulin; *NADH*, NADH oxidase; *ALD*, aldolase; *TPI*, triosephosphate isomerase; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *PKI*, pyruvate kinase I; *PKII*, pyruvate kinase II; *PPDK*, pyrophosphate-dependent pyruvate phosphate dikinase; *GDH*, NADP-dependent glutamate dehydrogenase; Ct, cycle threshold; NTC, no template control; SD, standard deviation; V, stability value; M, expression stability; ΔCt, delta Ct; CV, coefficient of variance; WtWB, wild type WB strain; bp, base pairs.

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that most of the antioxidant enzymes analyzed were deregulated, in accordance with the results for mRNA abundance obtained by reverse transcriptase-PCR (RT-PCR) (Argüello-Garcia et al., 2015).

The aforementioned studies made use of different techniques for determining gene expression and its regulation, ranging from microscopy (fluorescence- or chemically-labeled cells) (Fayer et al., 2000), proteomics, Northern blotting, microarrays and RT-PCR (Le et al., 2012). These methods can reliably detect expression levels, but some of them have limitations and may be laborious and expensive. In the last decade, quantification by RT-qPCR has been successfully used for evaluating gene expression in different organisms, including viruses, bacteria and parasites (Melo et al., 2015). The reliability of this method for gene expression studies strongly depends on the use of suitable reference genes, which are expressed constitutively and in steady amounts under different experimental conditions (Bustin et al., 2009; Cappelli et al., 2008).

As a general rule, genes involved in basic cellular processes (i.e., housekeeping genes) are chosen as reference genes. Genes such as those encoding tubulin (*TUB*), actin (*ACT*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and the *18S* rRNA gene have been widely used as internal controls in gene expression studies in eukaryotes (de Jonge et al., 2007; Kozera and Rapacz, 2013). However, it has been shown that the transcript levels of these genes may change significantly under some specific conditions (Kozera and Rapacz, 2013), and thus may not be appropriate as a universal reference for the analysis of gene expression in any organism under any given condition.

To date, no specific studies of possible reference genes and their stability in *Giardia lamblia* have been published. In this study, we analyze 12 housekeeping genes for *Giardia* involved in essential cellular and metabolic processes of the parasitic life cycle (*VSP*, *ACT*, *δ-GIA*, *NADH*, *ALD*, *TPI*, *PKI*, *PKII*, *PPDK*, *α2-TUB*, *GAPDH*, *GDH*) by qRT-PCR, to identify and quantify the expression values of different genes and propose them as candidates for use in gene expression studies. Additionally, we identify new reference genes and assess the expression of each of the transcripts in a wild type strain of *G. lamblia* (WB) and two strains resistant to nitazoxanide (NTZ) or metronidazole (MTZ). The results provide new insights into the gene regulation of this parasite, especially those genes involved in key processes such as infectivity and pathogenicity, which may be useful for future research.

2. Material and methods

2.1. Strains and experimental conditions

Trophozoites of the WB and the MTZ- and NTZ-resistant strains of *G. lamblia* were grown in culture medium TYI-S-33 (pH 7.3) supplemented with antibiotics. The WB strain was obtained from the American Type Culture Collection (ATCC). The strain resistant to MTZ was obtained as previously reported (Reyes-Vivas et al., 2014). The NTZ-resistant strain was isolated and axenized from a Mexican child previously treated with NTZ and clinically diagnosed as resistant to this drug. This NTZ strain was maintained and conserved in the presence of nitazoxanide (0.5 μg/mL). Trophozoites of all strains were grown in tubes containing 7 mL of medium and incubated at 37 °C. Upon reaching a confluent monolayer, the cells were placed on ice for 20 min and then collected by centrifugation at 3400 ×*g*; the medium was discarded completely and the cells were washed twice with phosphate buffered saline for RNA extraction.

2.2. Primer design

Twelve candidate genes to be used as references in RT-qPCR were analyzed in this study (Table 1). The sequences for primer design were obtained from GenBank, and then compared to reported sequences in the *Giardia* genome database (http://giardiadb.org/giardiadb/). The primer pairs were designed from mRNA sequences of the different genes with the programs DNAman and Primer3 (http://primer3.ut.ee) using the following parameters: length of 18–22 bp, GC content 45 to 55%, Tm 60 ± 2 °C, product size 60 to 250 bp, and avoiding the formation of secondary structures and dimers. Primer pairs were tested by endpoint PCR to analyze their specificity, using cDNA as the template (see next section) and the enzyme Q5™ High-Fidelity DNA polymerase (New England, BioLabs$_{inc}$) with the following amplification conditions: 30 s at 98 °C; 30 cycles of 10 s at 98 °C, 30 s at 60 °C, and 30 s at 72 °C; and finally 2 min at 72 °C. The amplified fragments were separated by electrophoresis in a 2% (*w*/*v*) agarose gel and visualized with GelRed (Nucleic Acid Gel, Biotium) on a MultiDoc-It (UVP).

2.3. RNA extraction and synthesis of first strand cDNA

RNA was extracted using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. The concentration and the purity of the isolated RNA were quantified using a Nanodrop ND-1000 Micro-Volume (NanoDrop Technologies, Wilmington, DE, USA). The 260/280 nm absorbance ratio of the extracted RNAs ranged from 2.0 to 2.2, and the purity of the RNA and its integrity were verified on 0.8% (*w*/*v*) agarose gels under denaturing conditions.

For cDNA synthesis, 1 μg of total RNA was first treated with 1 U of DNase enzyme (Thermo Scientific) in 1× DNase buffer. First strand cDNA synthesis was performed by mixing 1 μg of DNase-treated RNA, dNTP Mix (10 mM), oligo(dT)$_{18}$ primer and *Revertaid* reverse transcriptase (Thermo Scientific) in a final reaction volume of 20 μL, incubating at 42 °C for 60 min, and stopping the reaction by heating at 70 °C for 10 min. The cDNAs were stored at −20 °C until use.

Table 1
Genes analyzed in this study.

Gene symbol	Gene full name	Length (bp)	Function	GenBank
VSP (TSA417)	Variant-specific surface protein	702	Structural protein (Flagella and ventral disk)	U89152
ACT	Actin	1128	Cytoskeletal structural protein (all cell)	L29032
δ-GIA	δ-Giardin	851	Cytoskeletal structural protein (ventral disk)	AF331827
α2-TUB	α2-Tubulin	1365	Cytoskeletal structural protein (Flagella, median body, ventral disc)	AF331826
NADH	NADH oxidase	1455	O_2-Detoxifying enzyme	XM_001707922
ALD	Aldolase	972	Oxidoreductase in glycolysis	XM_001709998
TPI	Triosephosphate isomerase	764	Oxidoreductase in glycolysis	XM_001706778
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	1224	Oxidoreductase in glycolysis	XM_001703983
PKI	Pyruvate Kinase I	1662	Oxidoreductase in glycolysis	XM_001709477
PKII	Pyruvate Kinase II	1965	Oxidoreductase in glycolysis	XM_001704466
PPDK	Pyrophosphate-dependent Pyruvate phosphate dikinase	2655	Pyrophosphate-dependent glycolysis (generation ATP molecules)	XP_001705572
GDH	NADP-dependent glutamate dehydrogenase	1691	Glutamate metabolism	M84604