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Reliable reference gene selection for quantitative real time PCR in *Haemonchus contortus*

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ABSTRACT

The aim of this work was to identify reliable reference genes for expression studies in adult *Haemonchus contortus*. Eleven candidate genes were identified and the stability of their expression was assessed in adult males and females of two genetically divergent *H. contortus* isolates: drug-susceptible (ISE) and multi-drug-resistant (WR). Five genes with the most stable expression patterns were further assessed for suitability as reference genes in anthelmintic-treated *H. contortus* adults versus non-treated controls. We identified important differences in the expression of a number of candidate genes in anthelmintic-treated samples, confirming the need for careful validation of control genes for such experiments. We propose the use of multiple reference genes for expression studies in this species and found *gpd*, *ama* and *far* most suitable for adult *H. contortus*.

1. Introduction

Haemonchus contortus is a blood-feeding gastrointestinal parasite of small ruminants, which severely compromises animal health and production. Recently, the genome and transcriptome have been published, independently by two groups [1,2], providing the resources for genome-wide expression studies. Despite intensive molecular biology research in *H. contortus*, the validation of reference genes (RGs) for expression studies in this species has not been described. The choice of RG is, however, an important consideration, especially when comparing gene expression between different isolates and drug-treatments, which are a major focus of current research.

Quantitative real-time PCR (qPCR) has become a ubiquitous tool in molecular biology. qPCR is the most reliable method for quantifying gene transcript levels [3]. Today, in the era of genome-wide analysis, qPCR is the method of choice for microarray and RNA-seq validation. Very often RGs are selected from known housekeeping genes, however those genes may vary under different experimental conditions [4]. Therefore, it is essential to have validated RGs for any experimental set up to ensure accurate normalization of gene expression. Several computer programs are available for the evaluation of candidate RGs such as geNorm [5], NormFinder [6], BestKeeper [7], and the comparative ΔΔCt method can also be used [8].

Anthelmintic resistance of parasitic nematodes is a major problem in the livestock industry, however the mechanisms underlying resistance are still not fully understood [9,10]. Comparison of gene expression in resistant and susceptible isolates of *H. contortus* may identify constitutive or anthelmintic-induced differences, which may be enlightening. Therefore, it is necessary to evaluate and identify reliable RGs in different isolates and anthelmintic treatments. In the present study, candidate gene stability was compared in two *H. contortus* laboratory isolates with different genetic backgrounds [11]: the susceptible ISE (Inbred-Susceptible-Edinburgh)

Abbreviations: 18S, ribosomal RNA 18S; ABZ, albendazole; act, β-actin; ama, RNA polymerase II large subunit; Cq, quantification cycle values; eef, elongation factor-2; far, fatty acid and retinol binding protein; gpd, glyceraldehyde 3-phosphate dehydrogenase; IVM, ivermectin; ISE, Inbred-Susceptible-Edinburgh, drug-susceptible *H. contortus* isolate; ncbp, nuclear-cap binding protein subunit 2-like; pfk, phosphofructokinase; qPCR, quantitative real-time PCR; RG, reference gene; rpl, ribosomal protein 2 large subunit; sod, superoxide dismutase; tnt, troponin T; WR, White River, multi-drug-resistant *H. contortus* isolate.

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isolate and the multi-drug-resistant WR (White River) isolate. Two anthelmintics, albendazole (ABZ) and ivermectin (IVM), were also used to assess the stability of candidate genes in a pharmacological context. These anthelmintics have different modes of action so are likely to differ in their effects on gene expression profiles. Benzimidazole anthelmintics, such as ABZ, prevent the polymerization of nematode β-tubulin. In a previous study, ABZ induced the transcriptional response of drug-metabolizing genes (e.g. cytochrome P450s and UDP-glucosyl transferases) in *Caenorhabditis elegans* [12]. IVM is a macrocyclic lactone, which irreversibly binds to and activates ligand-gated chloride channels, resulting in paralysis of the body wall and pharyngeal muscles [13]. IVM has been shown to induce the expression of a notably different subset of genes in *C. elegans,* particularly those associated with fat metabolism, thought to reflect a fasting response [14]. The effect of these anthelmintics on the stability of commonly used RGs in *H. contortus* has not been described.

In this study, 11 *H. contortus* genes were assessed for reliability as RGs in gene expression studies. Some of the candidate genes are commonly used as endogenous controls, others were identified bioinformatically based on stable expression in RNA-seq data.

2. Materials and methods

2.1. Animals and treatment

The maintenance of *H. contortus* ISE and WR isolates were as described in our previous reports [15–17]. Parasite-free Texel lambs were orally infected with 5000–6000 *H. contortus* L3. Seven weeks after infection, adult nematodes were removed from the abomasum at post-mortem, using the agar method [18] and sexed based on morphology. For the in vitro anthelmintic-exposure experiments, batches of ten males and batches of ten females (three biological replicates) were cultured in RPMI 1640 media at 37 °C. The media was supplemented with either ABZ or IVM dissolved in 0.1% DMSO (10 μM ABZ and 1 μM IVM) or 0.1% DMSO only for control samples [19]. These drug concentrations have been used in previous pharmacological studies [15,16,19]. After 24 h, worms were transferred directly to TriReagent and frozen in −80 °C for later use.

2.2. RNA isolation, cDNA Synthesis and Quantitative Real-time PCR

Total RNA was extracted from batches of ten males and ten females using a standard Trizol procedure (TriReagent; Molecular Research Center, USA). On average, 10 μg and 40 μg total RNA was isolated from males and females, respectively. One microgram of total RNA was used for the first strand cDNA synthesis using random hexamers and Protoscript II Reverse Transcriptase (NEB, USA). An identical reaction lacking the reverse transcriptase enzyme (noRT control) was carried out simultaneously. After initial heat denaturation (65 °C for 5 min) the reactions (20 μl) were incubated for 10 min at 25 °C, for 50 min at 42 °C, and for 5 min at 80 °C. The resulting cDNA was diluted 1 in 50 for use in the qPCR assay.

The candidate genes evaluated in this study were β-actin (*act*), RNA polymerase II large subunit (*ama*), elongation factor-2 (*eef*) fatty acid and retinol binding protein (*far*), glyceraldehyde 3-phosphate dehydrogenase (*gpd*), nuclear-cap binding protein subunit 2-like (*ncbp*), phosphofructokinase (*pfk*), ribosomal protein 2 large subunit (*rpl*), superoxide dismutase (*sod*), troponin T (*tnt*) and ribosomal RNA 18*S* (18*S*). Primers were designed and checked similarly as in our previous report [20], to produce a product between 50 and 250 bp in length using Primer3 software [21]. All primers were synthesized by Generi Biotech (Czech Republic). Reference genes, Genebank accession numbers, primer sequences, amplicon sizes and efficiencies (maintained between 91 and 104%) are listed in Table 1.

qPCR analyses were performed in an iQ5 Real-Time PCR Detection System (Bio-Rad, USA) using SYBR Green I detection. The 20 μl reaction mixture consisted of the reaction buffer, MgCl2, dNTPs, SYBR Green I, and HotGoldStar DNA polymerase from the qPCR Core kit for SYBR Green I (Eurogentec, Belgium), as specified by the manufacturer, forward and reverse primers (final concentration 100 nM), and 5 μl diluted cDNA (corresponding to 5 ng reverse-transcribed RNA). A single batch of diluted cDNA was subjected to qPCR to amplify all candidate genes. NoRT controls were analyzed simultaneously using *gpd* primers to exclude possible DNA contamination. The polymerase chain reactions were initiated by a denaturation step of 10 min at 95 °C, then 40 cycles of amplification as follows: denaturation for 15 s at 95 °C, annealing for 20 s at 60 °C, and extension for 20 s at 72 °C. Fluorescence data were acquired during the last step. A dissociation protocol with a gradient from 65 °C to 95 °C (0.5 °C every 30 s) was used to investigate the specificity of the reaction and presence of primer dimers. Gene-specific amplification was confirmed by a single peak in the melting curve analysis. All methodological details are in the Supplementary material S1 (MIQE guidelines). The average quantification cycle values (Cq) of the candidate RGs assessed in one cDNA batch ranged from 18.33 to 29.56; *far* was the most abundant transcript and *ncbp* was the least abundant (data not shown).

2.3. Data analysis

Data analysis were performed using the web-based comprehensive tool RefFinder (http://www.leonxie.com/referencegene.php), developed for evaluating and screening RGs from experimental datasets (in accordance with [22]). This software integrates a number of computer programs (BestKeeper, geNorm, NormFinder) and the ΔΔCt method. Based on the rankings from each program, RefFinder assigns an appropriate weight to an individual gene and calculates the geometric mean of their weights for the overall final ranking.

2.4. Ethic statement

Sheep were bred and housed in appropriate conditions in a collaborative farm in Hořiněves, Czech Republic in accordance with the Guide for the Care and Use of Laboratory Animals (Protection of Animals from Cruelty Act No. 246/92, Czech Republic). This breeding facility is accredited for this purpose by the Ministry of Agriculture of Czech Republic and all experiments were authorized by the Ethical Committee of Faculty of Pharmacy, Charles University (Permit Number: MSMT—25908 / 2014–9).

3. Results and discussion

Eleven candidate RGs were analyzed using the comprehensive tool RefFinder. The ranking of the candidate genes was slightly different with each individual program, since the calculations are based on differential assumptions and algorithms. However, all methods found *ama*, *far*, *gpd*, *ncbp* and *sod* to be the five most stable genes, except geNorm, which found *ama* and *eef* to be the most stable (Table 2). Therefore, the comprehensive ranking approach was used as the evaluation index. The genes with the most stable expression patterns were *ncbp* and *ama* followed by *sod*, *gpd* and *far* (Table 2). The observed variation of *tnt* (bold in Table 2) was anticipated from RNA-seq data, where the total raw count difference between ISE and WR females or ISE and WR males was not high, but the difference in total raw count between females and