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Validation of reference genes for real-time quantitative RT-PCR studies in *Talaromyces marneffei*

Wiyada Dankai, Monsicha Pongpom, Nongnuch Vanittanakom *

Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

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ABSTRACT

Talaromyces marneffei (or *Penicillium marneffei*) is an opportunistic pathogen that can cause disseminated disease in human immunodeficiency virus (HIV)-infected patients, especially in Southeast Asia. *T. marneffei* is a thermally dimorphic fungus. Typically, *T. marneffei* has an adaptive morphology. It grows in a filamentous form (mould) at 25 °C and can differentiate to produce asexual spores (conidia). In contrast, at 37 °C, it grows as yeast cells that divide by fission. This study aimed to validate a quantitative reverse-transcription polymerase chain reaction (qRT-PCR) for gene expression analysis in *T. marneffei*. Analysis of relative gene expression by qRT-PCR requires normalization of data using a proper reference gene. However, suitable reference genes have not been identified in gene expression studies across different cell types or under different experimental conditions in *T. marneffei*. In this study, four housekeeping genes were selected for analysis: β-actin (*act*); glyceraldehyde-3-phosphate dehydrogenase (*gapdh*); β-tubulin (*benA*) and 18S rRNA. Two analysis programs; *BestKeeper* and *geNorm* software tools were used to validate the expression of the candidate normalized genes. The results indicated that the *actin* gene was the one which was most stably expressed and was recommended for use as the endogenous control for gene expression analysis of all growth forms in *T. marneffei* by qRT-PCR under normal and stress conditions.

1. Introduction

Talaromyces marneffei (formerly *Penicillium marneffei*) is a thermally dimorphic fungus. It can cause a fatal disseminated disease, especially as an opportunistic mycosis in human immunodeficiency virus (HIV)-infected patients. A high incidence of *T. marneffei* infection occurs in Southeast Asia, particularly Thailand, Northeastern India, Hong Kong, Southern China, Vietnam, Taiwan (Cooper and Vanittanakom, 2008), Malaysia (Nor-Hayati et al., 2012) and mainland China (Hu et al., 2013). *T. marneffei* growth can be divided into two phases. It grows as a filamentous (mould) form which can secrete a soluble red pigment on agar plates at 25 °C, and produce asexual spores (conidia) on specialized structures. In contrast, at 37 °C, the morphology of *T. marneffei* changes to grow as pathogenic unicellular yeast cells that divide by fission. Under suitable conditions at either 25 °C or 37 °C, the conidia accommodate isotropic growth, followed by polarized axis to produce a germ tube (this is termed germination). At 25 °C, the germ tube can continue growing apically to produce a hypha. The hyphae undergo cytokinesis to generate cellular compartments by septation. In contrast, at 37 °C, the germinated conidia develop into highly branching hypha and exhibit morphogenesis with the coupling of nuclear and cell division cycles to produce hyphae containing a single nucleus, the hyphae being known as pre-arthroconidia. The pre-arthroconidia develop to arthroconidia by the degradation of the double septa, liberating single cells. During arthroconidial formation, cells and nuclei are divided by fission into yeast cells (Boyce and Andrianopoulos, 2013).

T. marneffei infection appears to occur via inhalation of conidia from any source in the environment. Once the conidia are inhaled into host lungs, macrophages normally phagocytose and destroy the conidia. However, in immunocompromised patients, the conidia can survive and germinate into fission yeast cells and disseminate throughout the host (Vanittanakom et al., 2006). Many investigations have been focused on the molecular mechanisms that control the morphogenesis and pathogenicity of this fungus. However, many virulence factors of *T. marneffei* have yet to be identified.

A quantitative reverse-transcription polymerase chain reaction (qRT-PCR) is a useful technique for gene expression analysis. The benefit of this procedure includes its high sensitivity, specificity, accurate quantification and fast result. Although qRT-PCR is a powerful technique which enables the measurement of gene expression, there remain a number of problems, specifically, the differences between samples, sample preparation, RNA quantification and qualification, cDNA synthesis and internal control (Huggett et al., 2005). The analysis of relative gene expression by qRT-PCR requires normalization of data determined

* Corresponding author.

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by using the relative expression of the target gene compared with reference or housekeeping genes (HKGs). It is important to select appropriate HKGs to be a suitable reference for validating the level of gene expression.

The qRT-PCR technique has been widely used in gene expression assays of *T. marneffei*, however, different normalized genes were used in different laboratories; 18S ribosomal RNA (Wang et al., 2009), β-tubulin (Feng et al., 2010; Liu et al., 2007; Suwunnakorn et al., 2014), and *gapdh* gene (Nimmanee et al., 2015). This could cause problems for researchers when comparing results between laboratories including assessment of the reliability of the data. Moreover, the nature of *T. marneffei* itself, which has several forms and differential expression levels of housekeeping genes, make it difficult to compare the expression of the gene being investigated. The most commonly used HKGs include *act*, *gapdh* and 18S rRNA. Currently, no studies have been performed enabling the identification of appropriate reference genes which are stable across different cell types or under different conditions. The aim of this study is to validate reference genes suitable for analyzing gene expression in *Talaromyces marneffei* using qRT-PCR. Analytical software tools, such as *BestKeeper* (Pfaffl et al., 2004), and *geNorm* (Vandesompele et al., 2002) were used in this study to validate the expressions of the candidate normalization genes. The obtained results will be useful for the selection the appropriate housekeeping gene as an internal control for gene expression studies in *T. marneffei*.

2. Materials and methods

2.1. Fungal strains and culture conditions

The *Talaromyces marneffei* F4 strain (CBS119456), which was isolated from a patient at Maharaj Nakorn Chiang Mai Hospital, Thailand in 1998, and a G809 strain (Δ*ligD*::*pyrG*+ *niaD pyrG*, genically modified strain from ATCC18224, Bugeja et al., 2012) provided by Andrianopoulos (Department of Genetics, The University of Melbourne, Parkville, Victoria, Australia) were used in this study. The fungi were grown on *Aspergillus*-defined (ANM) agar containing 1% glucose for 10 days, and the conidia were collected by centrifugation (4 °C for 10 min at 4500 rpm). After collection, the 1×10^8 conidia were inoculated and allowed to grow in 100 ml Sabouraud's Dextrose Broth (SDB) (Difco) under culture conditions to generate germinating conidia, mycelia or yeast cells. The conditions for germinating the conidia were maintained by the culture being shaken at 150 rpm at either 25 °C or 37 °C for 12 h. For the mycelia or yeast forms, the conidia were grown at 25 °C for 2 days or 37 °C for 7 days, respectively. Expression of reference genes under stress conditions was evaluated following exposure to hydrogen peroxide (H_2O_2). All cultures with different forms of growth were incubated with 1 mM H_2O_2 (final concentration in 100 ml SDB) and were continuously shaken at 150 rpm for 1 h.

2.2. RNA extraction and cDNA synthesis

T. marneffei cells were collected by centrifugation. Extraction of the total RNA was performed by using a total RNA isolation kit (NucleoSpin® RNAII, Macherey-Nagel, GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. Then the extracted RNA was treated with rDNase prior to qRT-PCR analysis. The purity of RNA and RNA concentration was measured using a UV spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, Waltham, MA, USA). The A260/A280 ratio verification, assessing the average purity was expected to be in range from 1.80 to 2.20. The A260/A230 ratio was within the range 1.80 to 2.30. A PCR was performed to detect DNA contamination in RNA sample with Act1F and Act1R primers.

One microgram of the total RNA isolated from each fungal form was converted to cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Burlington, Canada). The reaction mixture contained 4 μl of 5× reaction buffer, 1 μl of random hexamer primer, 1 μg total RNA, 1 μl of ribolock RNase inhibitor, 2 μl of 10 mM dNTP, 1 μl of RevertAid M-MuLV RT reverse transcriptase and sterile nuclease free H_2O to give a final volume of 20 μl. cDNA was synthesized by incubating the reaction mixture for 5 min at 25 °C, followed by 60 min at 42 °C and then the reaction was terminated by heating at 70 °C for 5 min.

2.3. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

qRT-PCR for reference genes was performed using the SYBR Green qPCR mix (Thunderbird SYBR Green Chemistry, Toyobo, Japan) and the intensity of the fluorescence signal was detected by using a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA). The primers used to amplify 18S rRNA, β-actin, β-tubulin and *gapdh* genes are summarized in Table 1. Primers were used at 300 nM each for specific pairs of primers and the cDNA template at 25 ng in 20 μl total reaction. The reference genes were amplified according to the following conditions: one cycle of 95 °C for 60 s; followed by 40 cycles of 95 °C for 60 s, and 60 °C for 60 s; with dissociation (a melting curve) from 60 °C to 95 °C for verification of the single product (not primer dimer) of each primer.

To optimize the reaction, a standard curve was performed. The primers for standard curve analysis were used at 300 nM each for specific forward and reverse primers and 1 μl of serial dilution of cDNA (100 ng, 50 ng, 25 ng, 12.5 ng, 6.25 ng and 3.125 ng) in 20 μl total reaction. The reference genes were amplified according to the following conditions: one cycle of 95 °C, 60 s; followed by 40 cycles of 95 °C, 60 s and 60 °C, 60 s. The melting curve analysis was determined for the presence or absence of primer dimers which would indicate non-optimal primer annealing temperatures. PCR efficiency was determined by measuring the quantification cycle (Cq) value to a specific threshold for a serial dilution of cDNA. The amplification efficiencies of real-time PCR (E) were then calculated from the slope of each standard curve, according to the equation:

$$E = \left(10^{-1/slope} - 1\right) \times 100.$$

The statistical significance of the Cq values of these reference genes were calculated using the GraphPad Prism 5 program with an analysis of variance (ANOVA). Statistical significance was accepted when the p-value was less than or equal to 0.05. The experiments were repeated in triplicate for the F4 strain and in duplicate for G809.

Two analysis programs, *BestKeeper* and *geNorm* software tools were used to validate the suitably normalized gene. The *BestKeeper* was used to determine the best HKGs by using pair-wise correlation analysis of all pairs of candidate genes. The *geNorm* was used to define the internal control gene-stability by measuring M-values as the average pairwise variation of a particular gene with all other control genes.

2.4. Analysis of comprehensive time course of act gene expression levels in T. marneffei

To study comprehensive time course of *act* gene expression, 1×10^8 conidia of *T. marneffei* F4 strain were inoculated in 100 ml SDB (Difco) broth and incubated for 12 h, 1 day, 2 days, 3 days, 5 days, and 7 days at 25 °C or 37 °C. Expression of *act* gene under stress conditions was evaluated following exposure to H_2O_2. All cultures with different forms of growth were incubated with 1 mM H_2O_2 (final concentration in 100 ml SDB) and were continuously shaken at 150 rpm for 1 h. Then, *T. marneffei* cells were collected by centrifugation and the total RNA were extracted. One microgram of the total RNA isolated from each fungal form was converted to cDNA. The *act* gene expression was performed by qRT-PCR using the cDNA template of 25 ng in 20 μl total volume reactions.