RT-qPCR of Aspergillus

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Aspergillus is a genus of filamentous fungi with vast geographic and ecological distributions. Species within this genus are clinically, agriculturally and biotechnologically relevant, leading to increasing interest in elucidating gene expression dynamics of key metabolic and physiological processes. Reverse-transcription quantitative Polymerase Chain Reaction (RT-qPCR) is a sensitive and specific method of quantifying gene expression.

Keywords: reference gene ; RT-qPCR ; validation ; aflatoxin biosynthesis ; antifungal resistance ; actin ; beta-tubulin ; 18S rRNA ; glyceraldehyde 3-phosphate dehydrogenase

1. Introduction

The ascomycete genus *Aspergillus* is among the first described filamentous fungi, dating back to 1729, as recorded by Pier Antonio Micheli, an Italian biologist and priest. Under a microscope, the asexual spore-forming structure of these fungi looks like an aspergillum, a “holy water sprinkler”, and Micheli named these fungi in the genus *Aspergillus* [1][2]. Since then, over 300 species have been described and recognized in this genus [3]. These species differ in a diversity of morphological, physiological and phylogenetic characters. *Aspergillus* fungi are broadly distributed across the globe, and are found in diverse ecological niches such as soil, composts, water, buildings, air, and in or on plants [4]. Species in this genus have significant impacts on many fields, including biotechnology (e.g., antibiotics production) [5], fermented food production [6], food safety (e.g., mycotoxin production and food contamination) [7] and human health [2]. In addition, several *Aspergillus* species have been model organisms for understanding the fundamental biology, including physiology and genetics, of fungi and eukaryotes in general [8][9].

An emerging theme on the studies of these organisms is the regulations of gene expressions and metabolic pathways, and how the regulated expressions are related to their beneficial and detrimental effects to human welfares. For example, several *Aspergillus* species are employed frequently in the food and beverage industry as fermenters of soy, to make sake, miso and soy sauce [6]. Understanding how the genes are involved in producing these products could help develop strategies to control their expressions for optimal commercial productions. On the other hand, several other *Aspergillus* species are opportunistic human fungal pathogens (HFPs), including *Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Aspergillus terreus* and *Aspergillus nidulans* [10][11]. Globally, each year there are approximately 4.8 million cases of allergic bronchopulmonary aspergillosis (including asthma), 3 million cases of chronic pulmonary aspergillosis and 250,000 cases of invasive aspergillosis [12]. The dominant cause of aspergillosis in humans is *A. fumigatus* [10]. Furthermore, *A. flavus* can not only cause human infections, but along with *Aspergillus parasiticus*, can also produce aflatoxins that contaminate foods and severely impact human health, with long-term exposure leading to infertility and endocrine disorders [7].

Among human hosts infected with pathogenic *Aspergillus*, effective treatment often requires antifungal drugs such as itraconazole, voriconazole and amphotericin B [13]. Currently, itraconazole and voriconazole are the recommended first-line of treatment and prophylactic agent against invasive aspergillosis [14]. However, drug-resistant strains are increasing in both environmental and clinical populations of *A. fumigatus* and other opportunistic species [2]. Infections by drug-resistant fungal pathogens are associated with elevated length of hospitalisation and higher mortality [15]. Understanding the mechanism(s) of drug resistance in *Aspergillus* pathogens, including *A. fumigatus*, could help monitor and improve treatment options. In addition, there is increasing evidence that secreted enzymes in *A. fumigatus* play an important role in pathogen colonisation and host tissue damage. Intriguingly, strain Z5 of *A. fumigatus* has multiple xylanases [16], which can break down xylan into its constituents of xylose, arabinose and glucuronic acid, all of which can then be used in the production of biofuels [17]. Better control of the specific pathways involved in producing these beneficial enzymes could generate significant economic benefits.

Over the years, several approaches and techniques have been developed to monitor and quantify gene expression. These techniques include Northern blotting, microarray hybridisation, high throughput transcriptome sequencing and

reverse-transcription quantitative polymerase chain reaction (RT-qPCR). RT-qPCR quantifies the amount of mRNA in a biological sample, and takes this as a measurement of gene expression [18][19]. RT-qPCR possesses several advantages over other methods: it is quick, capable of high-throughput processing, and is highly sensitive and specific [18][19]. Additionally, RT-qPCR is useful for detecting low-abundance transcripts, as the high annealing temperature used during RT-qPCR allows for highly specific primer binding to the target gene [20]. Indeed, RT-qPCR is often used to confirm the results obtained using other approaches [21][22][23][24][25][26][27][28][29][30][31][32]. However, in order to accurately quantify gene expression using RT-qPCR, the normalisation of mRNA levels to validated reference genes is required [18]. Through normalisation using appropriate reference genes, the impact of differences in RNA yield (due to variation in extraction), cDNA yield (due to variation in reverse-transcription) and amplification efficiency on gene expression levels can be minimised [19]. Thus, by controlling for these differences, normalisation allows for the comparison of mRNA levels across different experimental treatments [19].

A good reference gene is one that is stably expressed under the experimental conditions being tested [19]. The Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines recommend using two or more validated reference genes to normalise gene expression data [19]. Using the geometric mean of multiple reference genes achieves high accuracy of normalisation, and is recommended over the arithmetic mean, as it better controls for differences in the amount of mRNA and outliers between genes [33]. When using multiple reference genes, it is also important to select genes that are not co-regulated, as co-regulated genes may lead to false positives as they lead to stable expression ratios [33]. Overall, inappropriate and/or insufficient reference genes can lead to the wrong interpretation of results, and reduce the reliability of experimental data [18][19]. Therefore, careful consideration should go into the selection of reference genes for RT-qPCR analysis.

2. Current Practices for Reference Gene Selection in RT-qPCR of *Aspergillus*

In a recent 2018 review of reference gene validation practices for RT-qPCR of insects, Shakeel and colleagues discuss the validation of reference genes thus far for select insect species, while emphasising the need for a comprehensive group of studies to be conducted under diverse experimental conditions for all species of insects [34]. They note that several studies of classical housekeeping genes show varying expression under different experimental conditions, and indicate ribosomal genes as a promising new set of genes for further stability analysis in insect-specific studies [34].

In a recent study, Archer and Xu discussed several methods employed by those who validated the reference genes for normalisation in their studies, including GeNorm [33], BestKeeper [35] and NormFinder [36] [37]. Shakeel and colleagues discussed the benefits of these and other methods, RefFinder and ΔCt, for assessing reference gene stability, and noted how each method may lead to slight differences in reference gene stability rankings [34]. As stated by the group, both GeNorm and Normfinder are excellent programs for the initial assessment of candidate reference gene stability, each with their own advantage, with GeNorm capable of determining the number of reference genes to use [33], and NormFinder computing the stability of each reference gene separately to avoid the consequences associated with co-regulation [36]. Given that different programs for determining reference gene stability may yield different stability rankings, we recommend that researchers use more than one program to validate the stability of the reference genes used under the experimental conditions being tested. In agreement with Shakeel and colleagues, because the results of some programs, such as GeNorm, may be biased due to co-regulation, care should be taken to select candidate reference genes that are not co-regulated.

Interestingly, Shakeel and colleagues noted nearly the same four reference genes as those most used in RT-qPCR studies in general across organisms: beta-actin, GAPDH, beta-tubulin and 18S rRNA, citing papers as early as 2004 [34]. The examination of 90 RT-qPCR studies, spanning 2001 to 2020, further demonstrates this to be the case for *Aspergillus*. The authors note that as of 2000, beta-actin and GAPDH were used 90% of the time without proper validation [34]. Out of the 30 usages of actin and 10 usages of GAPDH across the 90 studies we examined, actin was used without validation approximately 83% of the time, and GAPDH 60%. Similarly, of the 31 usages of beta-tubulin and 12 usages of 18S rRNA across the 90 studies we examined, beta-tubulin was used without validation approximately 81% of the time, and 18S rRNA, approximately 83%.

We hope that our critical review [37] will similarly stimulate future research on experimentally validating reference genes for gene expression studies in *Aspergillus* (and in fungi in general) using RT-qPCR. Without experimental validation of reference genes, it can be difficult to interpret the potential contributors to expression differences among strains, genes and treatments. **Figure 3** below summarises our recommended practice for reference gene selection.

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