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A highly sensitive and specific method to evaluate viable fungal burden of *Aspergillus fumigatus* in mice by RT-qPCR for 18S ribosomal RNA

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ABSTRACT

Potent fungicidal activity is one of the key factors of antifungals to overcome invasive pulmonary aspergillosis (IPA). To date, quantification of *Aspergillus* DNA in the lungs and galactomannan (GM) in serum or bronchoalveolar lavage fluid have been developed as general methods for measuring fungal burden in IPA animal models. However, GM quantification is not supposed to be a suitable method for precise evaluation of the fungicidal effects of antifungals, because killed *Aspergillus* hyphae can release GM for a certain period until they are eliminated by the host. Therefore, in terms of detecting viable fungal burden of *Aspergillus*, quantification of *Aspergillus* DNA has been thought to be a suitable method. Here, to obtain a method with much higher sensitivity, we applied reverse transcription quantitative PCR (RT-qPCR) for *A. fumigatus* 18S ribosomal RNA to measure the viable fungal burden in murine IPA models. Prior to *in vivo* tests, we confirmed that the sensitivity of 18S rRNA was nearly 50-fold higher than that of 18S ribosomal DNA *in vitro*. This highly sensitive method made it possible to evaluate the fungicidal effects of antifungals in a low-inoculation murine IPA model. In this model, single administrations of higher doses of voriconazole and posaconazole, which have fungicidal activity, were able to display fungicidal effects with $\geq$1 $\log_{10}$ reductions by 18S rRNA quantification, whereas significant reductions in serum GM were not observed. These results suggest that 18S rRNA quantification is a powerful tool for screening novel antifungals with potent fungicidal activity only after a single administration.

1. Introduction

Generally, colony forming units (CFUs) are used for evaluating fungicidal activity against the fungal burden of *Candida* and *Cryptococcus* in animal models (Abruzzo et al., 2000; Clemons and Stevens, 1998). However, counting *Aspergillus* CFUs is not optimal for measuring the fungal burden of its hyphae because elongation and killing of hyphae cannot be precisely reflected in CFUs (Bowman et al., 2001). Therefore, quantification of *Aspergillus fumigatus* DNA has been developed as a general technique to measure the fungal burden in invasive pulmonary aspergillosis (IPA) animal models. Quantification of 18S rDNA or *FKS1* gene as equivalent of the amount of *A. fumigatus* hyphae has been reported (Bowman et al., 2001; Lepak et al., 2013a, 2013b, 2013c), and the 18S rDNA quantification was 40-fold more sensitive than *FKS1* gene quantification (Herrera et al., 2009). However, its limited sensitivity requires high fungal inoculum *in vivo*, such as 10^6 conidia, leading to long-term antifungal treatments to achieve fungicidal activity due to high fungal burdens at the start of treatment (Lepak et al., 2013a, 2013b, 2013c).

In addition to *A. fumigatus* DNA, galactomannan (GM) has been used as a surrogate marker for the efficacy of antifungals in animal IPA models, because GM is a diagnostic marker for IPA in clinical settings (Mercier et al., 2018). Novel antifungal candidates under clinical trials, such as F901318 (olorofim), APX001 (fosmanogepix), and PC945, have been evaluated with GM semi-quantification, and have shown non-inferior efficacy to posaconazole (PSCZ) in murine IPA models (Gebremariam et al., 2020; Hope et al., 2017; Kimura et al., 2017). As for a limitation in GM semi-quantification, however, GM reductions from the beginning of the treatment may not be observed due to different secretion rates by the infected fungus and elimination from the lung or blood stream.

Although there is a need for new antifungals with fungicidal activity superior to existing azole drugs, existing non-clinical evaluation methods with quantification of *Aspergillus* DNA or GM cannot be suitable for screening novel antifungals with potent fungicidal activity due to the abovementioned limitations. Therefore, to detect strong fungicidal

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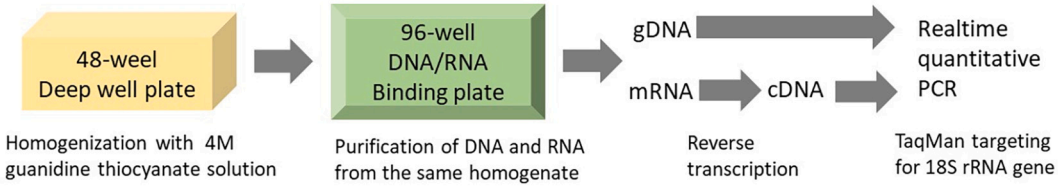

Fig. 1. Stepwise procedure for 18S rDNA or rRNA quantifications.

effects precisely, even in a short-term study, we have applied reverse transcription quantitative PCR (RT-qPCR) methods for *A. fumigatus* 18S rRNA, whose copy number is much higher than that of 18S rDNA, to measure the viable fungal burden in murine IPA models.

2. Materials and methods

2.1. Strains and media

A. fumigatus IFM47064 and IFM60237 (Hagiwara et al., 2014) were obtained from the Medical Mycology Research Center, Chiba University (Chiba, Japan). Conidia were grown on potato dextrose agar.

2.2. In vitro susceptibility testing

The MIC was determined by the broth microdilution method according to the CLSI M38-A2 standard. Conidia concentration was adjusted to approximately 1×10^4 CFU/mL, and were incubated for 48 h at 35 °C in RPMI1640 with 0.2% glucose buffered to pH 7.0, with 0.165 M morpholinepropanesulfonic acid. The MIC was defined as the minimal concentration of antifungal agents that exhibited no visible growth. The MEC was defined as the minimal concentration of antifungal agents that exhibited aberrant hyphal growth. The activities of voriconazole (VRCZ) and posaconazole (PSCZ) were shown as MICs, and the activities of caspofungin (CSFG) were shown as MECs. VRCZ and PSCZ were obtained from Tokyo Chemical Industry, and CSFG vials were purchased from Merck (Tokyo, Japan).

2.3. In vitro fungicidal assay

A. fumigatus IFM47064 was cultured for 16 h after inoculation with 1×10^4 CFU/mL in 200 μL of yeast nitrogen base medium without amino acids (Thermo Fisher Scientific) supplemented with 2% glucose, amino acids complete mix, and 50% heat-inactivated bovine serum (Thermo Fisher Scientific) in 96-well round plates. After 16 h of pre-incubation, VRCZ was treated for 48 h with two-fold serial dilutions, and then *A. fumigatus* cells and culture medium were collected with 2 mL of lysis solution from each well. The collected *A. fumigatus* cells in lysis solution were homogenized in a 48-well deep well plate with ShakeMaster Auto (BMS, Japan) for 5 min at 1100 rpm, and the resultant homogenates were applied to *A. fumigatus* 18S rDNA or rRNA quantification.

2.4. Invasive pulmonary aspergillosis (IPA) models in mice

Seven-week-old male DBA/2 N mice (Charles River, Japan) were immunosuppressed by intraperitoneal administration of cyclophosphamide at 150 mg/kg on day −4 and at 100 mg/kg on day −1. Approximately 1.0×10^4 conidia of *A. fumigatus* in 80 μL saline were inoculated intranasally on day 0 under anesthesia. Antifungals were single-administered on day 1, and lungs and serum were collected on day 2. Lungs were homogenized in lysis solution in a 48-well deep well plate with ShakeMaster Auto (BMS, Japan) for 5 min at 1100 rpm, and the resultant homogenates were applied to *A. fumigatus* 18S rRNA quantification. Serum was used for GM quantification. In the azole-susceptible strain (IFM47064) model, VRCZ and PSCZ were administered orally and CSFG was administered intravenously on day 1, and their efficacies were measured on day 2. All the antifungals were administered at low, medium, and high doses, where the middle doses were clinically equivalent doses as AUC. Plasma concentrations of VRCZ and PSCZ when administered orally in *A. fumigatus* infected DBA/2 N mice were shown in Supplemental Figure. In the azole-resistant strain (IFM60237) model, only PSCZ was administered orally in four doses on day 1, whose efficacy was measured on day 2. All animal experiments in this study were approved by Shionogi Institutional Animal Care and Use Committee. VRCZ tablets were purchased from Pfizer (Tokyo, Japan), PSCZ powder was purchased from Tokyo Chemical Industry, and CSFG vials were purchased from Merck (Tokyo, Japan).

2.4.1. A. fumigatus 18S rDNA or rRNA quantification

The procedure for 18S rDNA or rRNA quantification is shown in Fig. 1. Total DNA and RNA were simultaneously extracted from the same homogenates of intact *A. fumigatus* hyphae or intact infected lungs with lysis solution containing 4 M guanidine thiocyanate. The homogenates were filtered through a 96-well silica-membrane plate (Wizard® SV 96 Binding Plates, Promega), and total nucleic acids were eluted with distilled water after washing twice with 70% ethanol in 10 mM tris buffer. These nucleic acid eluates were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit® (Thermo Fisher Scientific) to quantify the 18S rRNA. Duplex real-time quantitative PCR was performed using TaqMan primer and probe sets targeting the *A. fumigatus* 18S rRNA gene. Forward primer: 5'-GGGCCG CTGGCTTCTTAG-3′, reverse primer: 5'-TGTTATTGCCGCGCACTTC-3′, and probe: 5′-(FAM)- ACTATCGGCTCAAGCC MGB-3′ were used. Murine GAPDH was used as the normalizer for relative quantification with the standard curve method using Mouse GAPD (GAPDH) Endogenous Control® (VIC®/MGB Probe) (Thermo Fisher Scientific).

2.5. Serum GM quantification

Serum GM was quantified using PLATELIA ASPERGILLUS Ag kit® (BIO-RAD) according to the manufacture's protocol. GM quantities were shown as absorbance at 450 nm. Undiluted serum samples were used for ELISA. When the absorbance of undiluted serum samples reached over 1.0, the samples were diluted by 10-fold and absorbance was re-measured.

2.6. Statistical analyses

Dunnett's test was used for comparison between control groups (beginning of treatment or untreated) and antifungal-treated groups using SAS version.9.4.

Table 1
MIC values of VRCZ, PSCZ and CSFG for *A. fumigatus* IFM47064 and IFM60237.

MIC or MEC (μg/mL)	VRCZ[a]	PSCZ[a]	CSFG[b]
A. fumigatus IFM47064	0.25	0.125	0.125
A. fumigatus IFM60237	1	1	0.125

[a] The values of VRCZ and PSCZ were MICs.
[b] The values of CSFG were MECs.

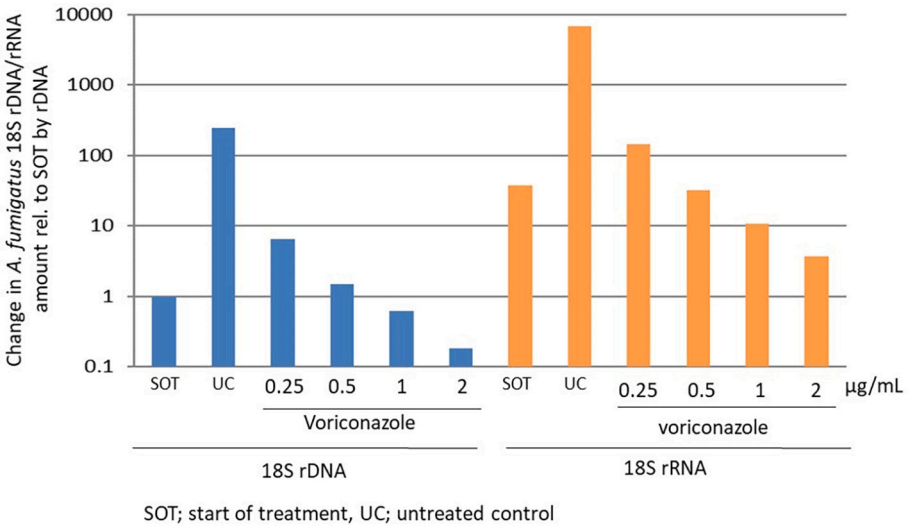

Fig. 2. *In vitro* fungicidal assay with 18S rDNA and rRNA quantifications.
Genomic DNA and total RNA were extracted from *A. fumigatus* hyphae and the culture medium from each well. For 18S rRNA quantification, the total RNA was reverse transcribed. Genomic DNA and cDNA from total RNA were subjected to TaqMan qPCR using the mentioned primer set for detecting *A. fumigatus* 18S rRNA (FAM probe). The relative amounts of 18S rDNA or rRNA were calculated by the comparative cycle threshold (ΔΔCT) method compared to the SOT (start of treatment) of 18S rDNA.

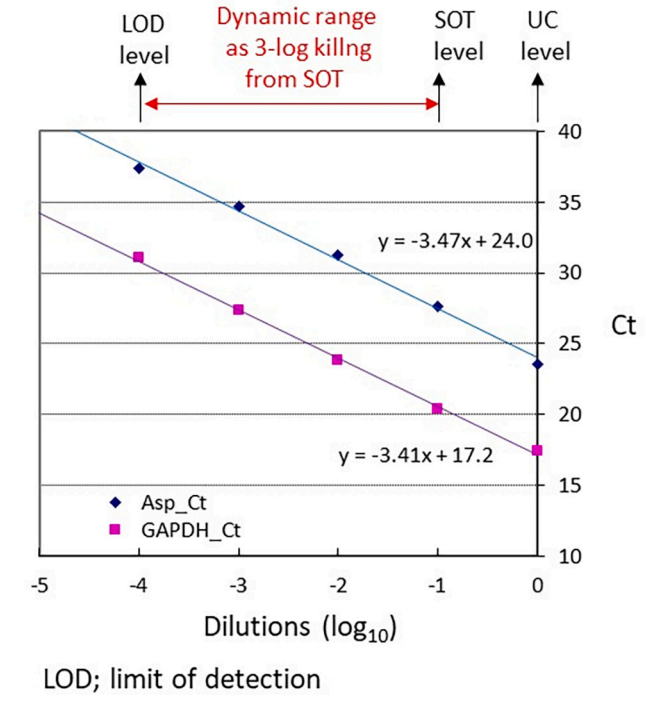

Fig. 3. Standard curve of untreated control sample for C_ts of *A. fumigatus* 18S rRNA and murine GAPDH.
Total RNA was extracted from the lung samples of the untreated control and reverse transcribed. The resultant cDNA was serially diluted from 10^0 to 10^{-4}. The serially diluted cDNA was applied to TaqMan duplex qPCR using mentioned primer sets for detecting *A. fumigatus* 18S rRNA (FAM probe) and murine GAPDH (VIC probe). The approximate curves of *A. fumigatus* 18S rRNA and murine GAPDH were obtained using Excel analysis.

3. Results and discussions

3.1. In vitro susceptibility and fungicidal assay

According to a previous report (Hagiwara et al., 2014), *A. fumigatus* IFM60237 is an itraconazole-resistant strain. In this study, we confirmed that IFM60237 showed a reduced susceptibility to PSCZ with a MIC value of 1 μg/mL when compared with the susceptibility of ATCC 204305 with a MIC value of 0.125 μg/mL (Table 1). In the *in vitro* fungicidal assay, 18S rRNA levels were nearly 50-fold higher than that of 18S rDNA at every condition in the VRCZ-treated and untreated groups (Fig. 2). Both 18S rDNA and 18S rRNA quantifications showed almost same dose-dependent antifungal activities from 0.25 to 2 μg/mL for VRCZ (Fig. 2). These results suggest that 18S rRNA quantification is a much more sensitive method for measuring viable fungal amounts of *A. fumigatus* when compared to 18S rDNA quantification.

3.2. In vivo efficacies measured with 18S rRNA

To measure viable fungal burdens in the lungs using a relative quantification method, a standard curve was created using an untreated control sample. The 10-fold serial dilutions of cDNA from untreated control samples were used for qPCR. From 10^0 to 10^{-4} dilution points, the C_t values of *A. fumigatus* were detected under a C_t of 40, where less than 10 copies of cDNA were detected, and the standard curve was linear (Fig. 3). As the viable fungal burden level at the beginning of treatment was approximately at 10^{-1} dilution point, the efficacious dynamic range was approximately 3 $\log_{10}$ killing by the limit of detection (Fig. 3).

In the azole-susceptible strain (IFM47064) model, the clinical equivalent doses of VRCZ, PSCZ, and CSFG showed only static effects on day 2 compared to the viable fungal burdens at the beginning of treatment on day 1 (Fig. 4A). Higher doses of VRCZ and PSCZ displayed fungicidal effects with ≥1 $\log_{10}$ reductions and statistical significance, whereas only a static effect was observed by CSFG on day 2 (Fig. 4A). These results indicated that clinically equivalent doses of VRCZ and PSCZ were unable to exhibit maximum fungicidal effects even against the azole-susceptible strains *in vivo*. A strong order of antifungal efficacy with 18S rRNA quantification was also observed with serum GM quantification, whereas fungicidal effects of higher doses of VRCZ and PSCZ were not observed with serum GM quantification (Fig. 4B). Interestingly, an exacerbation effect of higher dose of CSFG was observed only with serum GM quantification (Fig. 4B). As synthesis and secretion of GM could be increased when azole or echinocandin drugs were used (Geißel et al., 2018; Petraitiene et al., 2002), viable fungal burdens after treatment with these drugs might not be measured precisely by serum GM.

In the azole-resistant strain (IFM60237) model, the clinically equivalent dose of PSCZ showed only a moderate suppression in the growth of infected fungus, and higher doses of PSCZ, which showed a 2 $\log_{10}$ killing effect in the azole-susceptible strain model, showed only a fungistatic effect (Fig. 5A). Serum GM quantification showed almost same efficacy as that of 18S rRNA quantification (Fig. 5B). These results demonstrated the ability of 18S rRNA quantification to monitor the

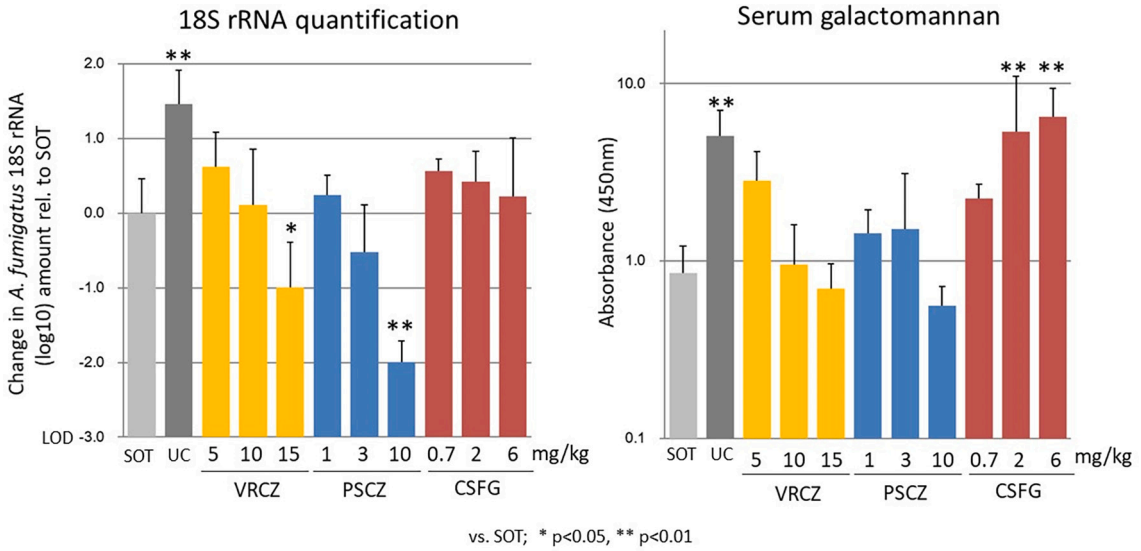

Fig. 4. Efficacies of VRCZ, PSCZ, and CSFG in azole-susceptible (IFM47064) strain model.
SOT (start of treatment) was sampled at 24 h, and UC (untreated control) and antifungal-treated groups were sampled at 48 h. Each bar represents the average fungal burden with 18S rRNA or GM amounts, with absorbance at 450 nm ± the standard deviation from five mice. Data were analyzed by using Dunnett's test with UC and each treatment group compared to SOT. * $p < 0.05$ and ** $p < 0.01$.

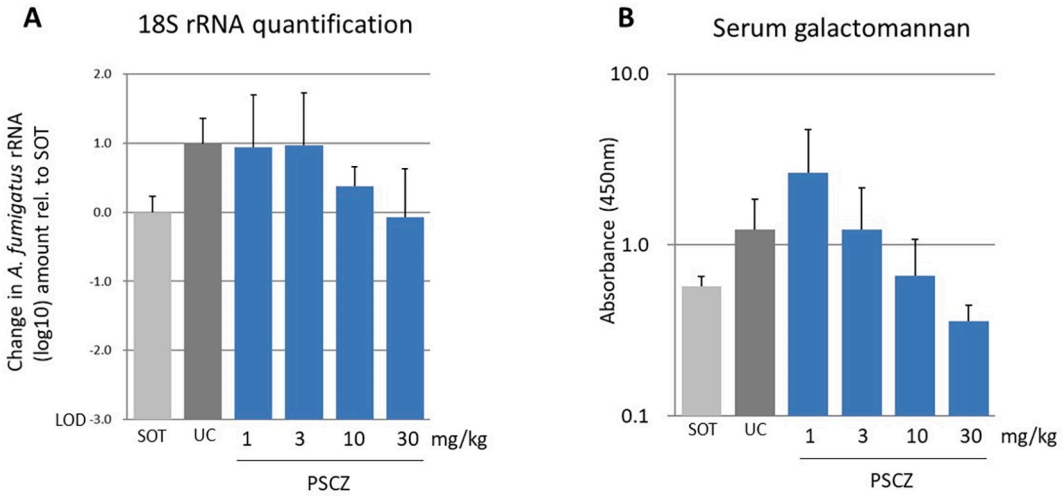

Fig. 5. Efficacies of PSCZ in azole-resistant (IFM60237) strain model.
SOT (start of treatment) was sampled at 24 h, while UC (untreated control) and antifungal-treated groups were sampled at 48 h. Each bar represents the average fungal burden with 18S rRNA or GM amounts, with absorbance at 450 nm ± the standard deviation from five mice.

decrease in *in vivo* susceptibility due to the decrease in *in vitro* susceptibility to antifungals.

4. Conclusions

In conclusion, 18S rRNA quantification was shown to be a powerful tool for evaluating the fungicidal effects of existing antifungals in a murine low-inoculation IPA model. This novel quantification method can provide highly efficient *in vivo* screening strategies for the discovery of novel antifungal candidates with potent fungicidal activities.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106214.

Declaration of Competing Interest

All authors are employees of Shionogi & Co., Ltd., and we report no conflicts of interest related to this study.

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