Development of cycling probe-based real-time PCR system to detect *Fusarium* ~~sp.~~ **species** and
Fusarium solani species complex (FSSC)

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Abstract

In the present study, we developed a new real-time PCR system based on the cycling probe technology (CPT), which is composed of two **single tube** real-time PCR assays: the *Fusarium* genus-specific assay and the *Fusarium solani* species complex (FSSC)-specific assay **with primers targeting the 28s ribosomal RNA gene**. The *Fusarium* genus-specific assay was shown to be highly specific, detecting all reference *Fusarium* strains with no cross-reaction with other reference fungal strains, such as *Aspergillus* spp. and human DNA. The FSSC-specific assay also reacted very specifically with FSSC, except for a cross-reaction with *Fusarium lunatum*. To validate the real-time PCR system, we tested 87 clinical isolates of *Fusarium* spp. Identification results from the real-time PCR system were found to be 100% concordant with those from DNA sequencing of EF-1 α gene. The sensitivity testing also demonstrated high sensitivity, enabling detection of one copy of standard DNA with good reproducibility. Furthermore, both assays were shown to be extremely sensitive even when fungal cells were mixed with human cells, detecting ~~one~~ **3** germinated conidia spiked in ~~+~~ **3** ml of human blood. To apply our new real-time PCR system to the molecular diagnosis of fusariosis, we evaluated its efficacy using a mouse model of invasive *F. solani* infection. Plasma and whole blood samples of infected mice were tested using the real-time PCR system. The sensitivity of the real-time PCR system was found to be 100% (~~4/4~~ **n = 4**) in plasma samples. In contrast, no amplification signal was detected in whole blood samples. This system could provide a rapid and precise diagnostic tool for early diagnosis, which is necessary for appropriate treatment and improvement of prognosis of disseminated fusariosis.

Key Words

Real-time PCR, cycling probe technology, fusariosis, *Fusarium solani* species complex (FSSC)

Introduction

Species belonging to the genus *Fusarium* are well known as major plant pathogens and soil inhabitants (Summerell et al., 2011). In humans, *Fusarium* species are common pathogenic fungi that cause superficial (keratitis and onychomycosis), locally invasive, and disseminated infection (Wong et al., 1997; Godoy et al., 2004). In immunocompromised patients with haematological malignancies, particularly those who have undergone haematological stem cell transplantation (HSCT), disseminated fusariosis is a life-threatening infection (Boutati and Anaissie, 1997; Nucci et al., 2004; Aquino et al., 2010; Campo et al., 2010). *Fusarium solani* species complex (FSSC) are known to be the most common causative agents in fusariosis, followed by *Fusarium oxysporum* species complex (FOSC) and *Gibberella fujikuroi* species complex (GFSC) (Lortholary et al., 2010). The most notable causative species belonging to GFSC are *Fusarium verticillioides* (formerly *Fusarium moniliforme*) and *Fusarium proliferatum* (Lortholary et al., 2010). Although the gold standard for the diagnosis of fusariosis is isolation, culture, and identification of the organisms, the culture method requires over a week to yield results. Published reports indicate that antifungal agents including amphotericin B, voriconazole, and posaconazole generally show low minimum inhibitory concentrations (MIC) for *F. solani* and *F. oxysporum* (Muhammed et al., 2011). Systemic fusariosis is known for its poor outcome, and for immunocompromised patients in particular, an appropriate antifungal treatment based on early and precise diagnosis is critically important for better prognosis.

Recently, real-time PCR is becoming widely used for rapid detection of medically important pathogenic fungi, such as *Aspergillus* spp., from various clinical samples (Kami et al., 2001; McMullan et al., 2008; Simon et al., 2010). However, the development of a real-time PCR system for fusariosis is still limited. ~~In the present study, we developed a new real-time PCR system based on the cycling probe technology (CPT) for fusariosis. The system is composed of two real-time PCR assays: one is to detect *Fusarium* species (*Fusarium* genus specific assay) and the other to distinguish FSSC (FSSC specific assay) (Fig. 1). The system proved to be highly sensitive and specific, detecting fungal DNAs directly from blood of a fusariosis mouse model.~~ The objectives of the present work were to develop a new real-time PCR system based on the cycling probe technology (CPT) for the molecular detection of *F.* species and distinguishing FSSC with primers and probes targeting the 28s ribosomal RNA gene.

Materials and methods

Fungal strains. Fifteen FSSC reference strains, 11 non-FSSC *Fusarium* reference strains, and 38 non-*Fusarium* fungal reference strains used in this study were provided by the Medical Mycology Research Center, Chiba University, Chiba, Japan (Table 2). A total of 87 *Fusarium* clinical isolates, which were recovered from human blood cultures and ocular specimens, routinely processed at the ~~Micology~~ **Mycology** Laboratory - Clinical Pathology Division - Hospital and Clinics were provided by the State University of Campinas, São Paulo, Brazil.

DNA extraction from fungal cultures. ~~Total~~ **Fungal** genomic DNA was extracted from 7-14 day-old mycelia cultured on potato dextrose agar (PDA) using a modified version of the alkaline

heat-extracted method described in the product manual of Fungal rDNA (ITS1) PCR Kit Fast (Takara bio, Shiga, Japan,). Briefly, mycelia were suspended in 50 μ L of sterile distilled water and added with 50 μ L of 100 mM NaOH. The suspension was homogenized using a micropestle, incubated at 95°C for 15 min, and neutralized by adding 11 μ L of 1 M Tris-HCl buffer (pH 7.0). The neutralized solution was subsequently centrifuged at 5000 g for 2 min, and the resulting supernatant was used as DNA solution. DNA concentration was determined using a spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Yokohama, Japan). Two microliters of DNA solution diluted to 5 ng/ μ L with sterile distilled water were used as template DNA for PCR and real-time PCR assays.

DNA extraction from whole blood samples was essentially conducted as described by White et al (2010). Briefly, for the red cell lysis, 3 mL of human whole blood samples or 200 μ L of mouse whole blood samples were lysed with five times the volume of red cell lysis buffer [RCLB; 10 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 10 mM NaCl], incubated for 15 min, centrifuged for 10 min at 3,500 g, and the supernatant discarded. The red cell lysis step was repeated twice. For the white cell lysis, the pellet was resuspended in 1 mL white cell lysis buffer [WCLB: 10 mM Tris-HCl (pH 7.8), 10 mM EDTA, 50 mM NaCl, 0.2% SDS, 200 μ g/mL recombinant proteinase K (Roche Diagnostics, Tokyo, Japan)] and incubated at 65°C for 45 min, centrifuged at 5000 g for 10 min, and the supernatant discarded. The pellet was suspended in 300 μ L phosphate-buffered saline (PBS), added with 300 mg acid washed glass beads (710-1,180 μ m diameter, Sigma-Aldrich, Tokyo, Japan), and then fungal cells were disrupted using a Multi-beads shocker[®] (Yasui Kikai, Osaka, Japan) under the following conditions: 2500 rpm at 4°C, 5 cycles of 1 min on and 1 min off. DNA was extracted using a High Pure PCR template preparation kit (Roche Diagnostics) according to the manufacturer

instructions. Finally, DNA was eluted in 200 μ L of elution buffer (supplied in kit) and diluted tenfold with sterile distilled water. Two microliters of diluted DNA solution was used as template DNA for real-time PCR assays.

DNA extraction from mouse plasma samples of 240 μ L was performed using NucleoSpin[®] Plasma XS (Macherey Nagel, Diiren, Germany) according to the manufacturer's instructions following the high-sensitivity protocol. Finally, DNA was eluted in 20 μ L of elution buffer (supplied in kit) and diluted tenfold with sterile distilled water. Two microliters diluted DNA solution was used as template DNA for real-time PCR assays.

PCR. For fungal identification based on DNA sequencing analysis, a partial elongation factor-1 α (EF-1 α) gene was amplified using the PCR with HS392 [5'-TCAAAATGGGTAAGGA(A/G)GACAAGAC-3'] and HS393 [5'-GCCTGGGA(A/G)GTACCAGT(C/G)ATCATGTT-3'] which were designed from EF1 and EF2 primers (O'Donnell et al., 1998) by adding 5 more consensus nucleotides of *F. verticillioides* (FVEG02381) and *F. graminearum* (FGSG08811) in the *Fusarium* comparative genome project (Suga H. personal communication). The PCR was performed in a total volume of 20ul of the following mixture: 10 ng genomic DNA, 10 μ L 2 $\times$ PCR buffer for KOD FX neo (Toyobo, Tokyo, Japan), 4 μ L 2 mM dNTPs (Toyobo), 0.3 μ M each primer, and 0.4 U KOD FX neo (Toyobo). PCR was performed using the iCycler (Bio-Rad, Tokyo, Japan) under the following conditions: 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 53°C for 30 s, 72°C for 1 min, with a final extension at 72°C for 5 min. For the negative control, sterile distilled water was used in place of template DNA.

DNA sequencing. PCR products were purified using the ExoSAP-IT (Affymetrix USB, Ohio, USA) and sequenced by the direct sequencing method using the BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Tokyo, Japan) and the ABI 3130xl Genetic Analyzer (Life Technologies) according to the manufacturer's instructions. Primers HS392, HS393, EF11 (O'Donnell et al., 1998) and EF21 (O'Donnell et al., 1998) were used for the cycle sequencing. Sequences were assembled using the ATGC version 6.0 (Genetyx, Tokyo, Japan) and Genetyx[®]-win version 11 (Genetyx). BLAST searches of the sequence data were performed using the *Fusarium* MLST database (O'Donnell et al., 2012) (<http://www.cbs.knaw.nl/fusarium/>).

~~**Identification of clinical isolates by DNA sequencing of EF 1 α gene.** A total of 87 *Fusarium* clinical isolates were identified at the species complex or species level by DNA sequencing of a partial EF 1 α gene. A partial EF 1 α gene was amplified using the PCR with HS392 [5'-TCAAAATGGGTAAGGA(A/G)GACAAGAC 3'] and HS393 [5'-GCCTGGGA(A/G)GTACCAGT(C/G)ATCATGTT 3'] which were designed from EF1 and EF2 primers (O'Donnell et al., 1998) by adding 5 more consensus nucleotides of *F. verticillioides* (FVEG02381) and *F. graminearum* (FGSG08811) in the *Fusarium* comparative genome project (Suga H. personal communication). Sequencing primers, EF11 and EF21, were used for cycle sequencing (O'Donnell et al., 1998). DNA sequencing analyses were performed using an Applied Biosystems 3130xl Genetic Analyzer (Life Technologies, Tokyo, Japan). BLAST searches of the EF 1 α gene sequence data were performed using the *Fusarium* MLST database (<http://www.cbs.knaw.nl/fusarium/>). The sequence data obtained in this study were deposited in~~

~~GenBank with accession numbers AB817148–AB817234.~~

Primers and probes design. 28S rRNA gene sequences of 13 FSSC strains (*F. solani* DQ236678, DQ236813, DQ236594, EU719659, EU579657, AY097316, AY097318, AF130382, AB258994, AY819046, AY097317, *Fusarium falciforme* AY097326, *Fusarium lichenicola* AY097325), 8 non-FSSC *Fusarium* strains (*F. oxysporum* AB084299, FJ614650, HM210091, AF060383, *Fusarium graminearum* GZU34549, *Fusarium incarnatum* AF130380, *G. fujikuroi* GFU68130, AB084300), and 13 non-*Fusarium* fungal strains (*Aspergillus fumigatus* AFU28462, *Aspergillus nidulans* AF454168, *Aspergillus terreus* ATU28841, *Aspergillus niger* FJ184995, *Aspergillus versicolor* AF433059, *Penicillium aurantiogriseum* PAU15484, *Penicillium roqueforti* GQ458036, *Alternaria alternata* EF100960, *Paecilomyces variotii* AF222501, *Candida albicans* EF694598, *Paracoccidioides brasiliensis* DQ361104, *Ajellomyces capsulatus* AB176512, *Rhizopus oryzae* DQ466617) were obtained from GenBank and aligned using ClustalX version 2.1 (Thompson et al., 1997). Primer pair Fusa28sF1/Fusa28sR1 was designed from conserved regions of the genus *Fusarium*. *Fusarium* genus-specific cycling probe (Fprobe1) and FSSC-specific cycling probe (FSprobe1) were respectively designed for single-nucleotide polymorphisms (SNP) present on the target gene of *Fusarium* spp. and FSSC using CycleavePCR® Assay Designer online program (Takara bio, ~~Shiga, Japan~~, <http://www.takara-bio.co.jp/prt/snps/intro.htm>). Sequences and characteristics of primers and probes are shown in Table 1.

Standard DNA and internal positive control. A 222-bp product from the partial 28s rRNA gene was amplified from *F. solani* ATCC MYA-3939 with primer pair Fusa28sF1/Fusa28sR1. The PCR

product was inserted into pCR2.1 TA Cloning vector and transformed into TOP10 chemically competent *Escherichia coli* cells using TOPO[®] TA Cloning kit (Life technology). Plasmid DNA was extracted using GenElute[™] Plasmid Miniprep Kit (Sigma-Aldrich, ~~Tokyo, Japan~~). Plasmid DNA digested with restriction enzyme HindIII (Takara bio) was used as the standard DNA.

For the internal positive-control (IPC) DNA, primer pair Lambda-primerF1/Lambda-primerR2 and IPC cycling probe (Lambda-probe1) were designed from the λ DNA (Takara bio) using CycleavePCR[®] Assay Designer online program (Takara bio). An 392-bp product from the partial λ DNA was amplified with the primer pair Lambda-primerF1/Lambda-primerR2. The IPC DNA was constructed using the same methods as described above. Sequences and characteristics of primers and probe are shown in Table 1.

Real-time PCR assays. Each 20 μ L of PCR reaction mixture contained 10 μ L 2 $\times$ CycleavePCR[®] Reaction Mix (Takara bio), 0.2 μ M each primers, 0.08 μ M cycling probe, and template DNA. When testing blood samples, 0.2 μ M each IPC primers, 0.2 μ M IPC cycling probe, and 200 copies of IPC DNA were added to the PCR reaction mixture. Real-time PCR amplifications were carried out on an Applied Biosystems 7300 Real-Time PCR System (Life Technologies) under the following conditions: 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, 55°C for 10 s, 72°C for 30 s. For the negative control, sterile distilled water was used in place of template DNA. For the positive control, 1000 copies of standard DNA were used in place of the sample DNA.

Analytical sensitivity and specificity. The specificity of two real-time PCR assays was determined by testing 15 FSSC reference strains, 11 non-FSSC *Fusarium* reference strains, 38 non-*Fusarium*

198 fungal reference strains, and human DNA. All tests were run in duplicate. The sensitivity of
199 real-time PCR assays was determined by testing with a tenfold serial dilution of the standard DNA
200 using Easy dilution (Takara bio) ranging from 1 copy to 100,000 copies per reaction. The
201 repeatability of real-time PCR assays was determined by calculating the inter-assay coefficients of
202 variation (CV) of cycle threshold (Ct) value in five independent experiment runs.

203
204 **Detection from human whole blood sample spiked with germinated conidia.** *F. solani* ATCC
205 MYA-3939 was cultured on PDA for 10-14 days at 25°C. The microconidia were harvested using
206 sterile saline, filtrated using BD Falcon™ cellstrainer 70 µm (Becton, Dickinson and company,
207 Tokyo, Japan), and then incubated in RPMI 1640 medium at 37°C for 9 h. The germinated conidia
208 were washed in sterile saline by centrifugation, suspended in sterile saline, and then counted using
209 a haemocytometer. EDTA anti-coagulated whole blood samples from a healthy volunteer were
210 artificially spiked with a tenfold serial dilution of germinated conidia at a final concentration of ± 3
211 to ~~10,000~~ 30,000 per ± 3 ml whole blood. Whole blood sample without spiking was used as a
212 negative control. DNA extraction from whole blood samples and real-time PCR was performed as
213 described above. ~~DNA was extracted from whole blood samples essentially as described by White~~
214 ~~et al (2010). Briefly, for the red cell lysis, 3 mL of whole blood samples were lysed with five times~~
215 ~~the volume of red cell lysis buffer [RCLB: 10 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 10 mM NaCl],~~
216 ~~incubated for 15 min, centrifuged for 10 min at 3,500 g, and the supernatant discarded. The red cell~~
217 ~~lysis step was repeated twice. For the white cell lysis, the pellet was resuspended in 1 mL white cell~~
218 ~~lysis buffer [WCLB: 10 mM Tris-HCl (pH 7.8), 10 mM EDTA, 50 mM NaCl, 0.2% SDS, 200~~
219 ~~µg/mL recombinant proteinase K (Roche Diagnostics, Tokyo, Japan)] and incubated at 65°C for 45~~

~~min, centrifuged at 5000 g for 10 min, and the supernatant discarded. The pellet was suspended in 300 µL phosphate buffered saline (PBS), added with 300 mg acid washed glass beads (710-1,180 µm diameter, Sigma-Aldrich), and then fungal cells were disrupted using a Multi-beads shaker[®] (Yasui Kikai, Osaka, Japan) under the following conditions: 2500 rpm at 4°C, 5 cycles of 1 min on and 1 min off. DNA was extracted using a High Pure PCR template preparation kit (Roche Diagnostics) according to the manufacturer instructions. Finally, DNA was eluted in 200 µL of elution buffer (supplied in kit) and diluted tenfold with sterile distilled water.~~

Experimental mouse model of invasive *F. solani* infection. A clinical isolate, *F. solani* IFM 50956, was cultured on PDA for 14 days at 25°C. The microconidia were harvested using the same procedure as described above. Conidia were counted using a haemocytometer, and concentrations were confirmed by counting the colony-forming units (CFU) on PDA plate. Seven specific pathogen-free ICR mice (female, 5-week-old) weighing 15 to 20 g were purchased from Charles River Laboratories Japan (Kanagawa, Japan). Mice were housed in sterilized cages and provided with sterile food and water *ad libitum*. One day before infection, all mice were immunosuppressed using 5-fluorouracil intravenously at 150 mg/kg of body weight and cyclophosphamide intraperitoneally at 200 mg/kg. Five mice were infected by intravenous tail injection with 5.4×10^5 CFU of conidia in a 200 µL volume of sterile saline. Two control mice were immunosuppressed using the same procedure as described above and injected with 200 µL volume of sterile saline. When mice appeared moribund, blood samples (whole blood and plasma) and organs (lung, kidney, liver and spleen) were collected under anesthesia. **DNA extraction from whole blood and plasma samples and real-time PCR was performed as described above.** ~~DNA extracted from 200 µL whole~~

~~blood samples as described above was used for real-time PCR assays. DNA extraction from 240 µL~~
~~plasma samples was performed using NucleoSpin[®] Plasma XS (Macherey Nagel, Düren, Germany)~~
~~following the manufacturer's instructions, and 2 µL of eluted DNA was used for real-time PCR~~
~~assays.~~ A portion of organs homogenized in 200 µL sterile saline and 200 µL whole blood samples
were plated onto PDA plates supplemented with chloramphenicol (100 mg/L). Following 48 h of
incubation at 25°C, CFU per gram of tissue was calculated. The remaining organs were fixed with
buffered 10% formalin and routinely embedded in paraffin and sectioned at 4 to 5 µm thickness.
These sections were stained with hematoxylin and eosin (H&E) and Grocott's methenamine silver
stain. All procedures were approved by the Committee of Animal Welfare of Chiba University.

RESULTS

Analytical specificity. The specificity of the real-time PCR assays is shown in Table 2. The
Fusarium genus-specific assay specifically detected all *Fusarium* reference strains (FSSC: $n = 15$,
non-FSSC *Fusarium*: $n = 11$). The FSSC-specific assay could specifically detect all 15 FSSC
reference strains except *Fusarium lunatum* IFM 48434 (CBS 632.76). Amplification signal was not
detected in any of the 38 non-*Fusarium* fungal reference strains.

Analytical sensitivity. A standard curve was created by plotting copy number of standard DNA
logarithm values on X-axis against Ct values on Y-axis (Fig. 2 1). The plot showed good linearity
with R^2 value (*Fusarium* genus-specific assay: 0.9819, FSSC-specific assay: 0.9829). The
sensitivity limit of real-time PCR assays was determined using serial dilutions of standard DNA
with known copy numbers. The detection end point was one copy per reaction for both assays (Fig.

1). To confirm precision and reproducibility of real-time PCR assays, we determined inter-assay CV values of Ct values in five independent experiment runs. The CV values for the *Fusarium* genus-specific assay and the FSSC-specific assay were respectively less than 4.5% and 4.9%.

Identification of clinical isolates by the real-time PCR system. A total of 87 *Fusarium* clinical isolates were identified by DNA sequencing of partial EF-1 α gene and the real-time PCR system we developed. The identification results from the real-time PCR system were consistent with those of DNA sequencing. A Comparison of two identification results was shown in Table 3. The DNA sequence data were deposited in GenBank with accession numbers AB817148–AB817234.

~~Seventy five isolates were identified as FSSC (*Fusarium* cf. *solani*: 41, *F. falciforme*: 34), two isolates were identified as FOSC (*F. oxysporum*: 1, *F. sp.*: 1), four isolates were identified as FDSC (*Fusarium delphinoides*: 4), and six isolates were identified as GFSC (*Fusarium sacchari*: 1, *Fusarium proliferatum*: 2, *Fusarium napiforme*: 2, *Fusarium bactridioides*: 1) by DNA sequencing. Seventy five isolates were identified as FSSC and 12 isolates were identified as non-FSSC *Fusarium* spp. by the real-time PCR system, 100% in agreement with the identification results of DNA sequencing (Table 4).~~

Detection from human whole blood sample spiked with germinated conidia. The detection limit of both real-time PCR assays was shown to be one-3 germinated conidia per $\pm$ 3 ml of whole blood (Fig. 2). All real-time PCR assays were conducted as a duplex real-time PCR with the IPC assay in a single tube to monitor for false negative results that may occur in the presence of PCR inhibitors. The IPC amplification signals were detected for all samples (Fig. 2). No amplification signals were

detected in the negative controls.

Experimental mouse model of invasive *F. solani* infection. Four mice in the infection group appeared moribund and were sacrificed on the post-infection day 5. Whole blood and plasma samples were not collected for one mouse (No. 4) in the infection group that had already died early on the post-infection day 5. All mice in the no-infection control group had no symptoms of infection and were sacrificed on the post-infection day 5. Invasive *F. solani* infections in mice were confirmed histopathologically and by tissue culture. All mice in the infection group were shown to be tissue culture-positive (Table 4), and invasively growing fungal hyphae were observed by histopathological examination. In contrast, no fungal growth was observed in the whole blood culture except for one mouse (No. 5) in the infection group (Table 4). The real-time PCR system was performed using whole blood and plasma samples, and amplification signals were detected for all plasma samples from infected mice (Table 4). In contrast, no amplification signals were detected in all whole blood samples from infected mice. All samples from no-infection control mice were negative in the real-time PCR system.

Discussion

Fusarium species have recently come to the fore as one of the most important pathogenic filamentous fungi after *Aspergillus* spp. in patients with hematological malignancies (Marr et al., 2002; Pagano et al., 2007; Aquino et al., 2010). In immunocompromised patients with disseminated fusariosis, early diagnosis is extremely important in starting an appropriate antifungal treatment. The diagnosis of fusariosis has been based on fungal isolation and identification by observation of

morphological characteristics of fungal cultures. However, identification of *Fusarium* species is often difficult due to the variability among isolates (e.g. in shape and size of conidia, and colony color and texture) and the absence of characteristic macroconidia production. Therefore, morphological identification at the species level is difficult, requiring extensive microbiological experience and expertise. In recent years, molecular diagnostic and identification procedures, such as DNA sequencing, polymerase chain reaction (PCR), nested PCR and real-time PCR, have become important tools to facilitate morphology-based identification (Ahmad et al., 2010; Itahashi et al., 2010; Bernal-Martínez et al., 2012). For *Fusarium* species, however, the development of these molecular methods has not been fully investigated.

Fluorescent probe-based real-time PCR, such as TaqMan[®] probe-based real-time PCR, is widely used for the molecular detection of important disseminated fungal infections, such as aspergillosis and candidemia (Kami et al., 2001; McMullan et al., 2008; White et al., 2010; White et al., 2006), due to its high specificity and rapidity. Moreover, real-time PCR does not require agarose gel electrophoresis, thereby significantly decreasing contamination risks compared with conventional PCR and nested PCR. In the present study, we decided to develop a cycling probe technology (CPT)-based real-time PCR system for the rapid detection of medically important *Fusarium* species because of its extremely high sensitivity and specificity. We used a combination of a chimera probe, composed of RNA and DNA, and an RNase (Bekkaoui et al., 1996; Modrusan et al., 1998), which can recognize even an SNP (Bekkaoui et al., 1996; Suzuki et al., 2011). A CPT-based real-time PCR for detecting *Fusarium* species has been previously reported (Itahashi et al., 2010). However, the evaluation of analytical specificity is insufficient in this report. Therefore, we developed a new real-time PCR system based on CPT, which is composed of two real-time

PCR assays: the *Fusarium* genus-specific assay, which is designed for the general and sensitive detection of *Fusarium* species, and the FSSC-specific assay, which is designed for the specific detection of FSSC (Fig. 1).

The results show a high sensitivity, enabling detection of one copy of standard DNA with a good reproducibility. In terms of specificity, the *Fusarium* genus-specific assay proved to be highly specific, detecting all of the reference *Fusarium* strains, with no cross-reaction with other reference fungal strains and human DNA. The FSSC-specific assay also showed a very high specificity. The only exception is a cross-reaction with *F. lunatum* IFM 48434 (CBS 632.76), which belongs to *Fusarium dimerum* species complex. However, we believe this cross-reaction is of little clinical significance because the fungus is believed to be non-pathogenic, with no published reports of human infection. In fact, when we tested 87 *Fusarium* clinical isolates recovered from human blood and eye, no isolate of *F. lunatum* was found, and the identification results by the real-time PCR system were 100% concordant with those from DNA sequencing, validating the high utility and reliability of the real-time PCR system for identification of clinical isolates. These results indicate that our new real-time PCR system is a simple and time-saving method to achieve reliable results in identification when compared with conventional DNA sequencing methods for fungal cultures. This fact suggests that our new real-time PCR system can be used for the detection of *Fusarium* spp. as well as FSSC from filamentous fungi isolated from clinical samples.

In addition to the identification of cultured fungi, it would be very helpful if we could detect the fungal DNA directly from clinical samples, such as patients' blood. As the first step to know if the system works directly in clinical samples, we tested whole blood samples from healthy volunteers spiked with serial dilutions of germinated conidia from *F. solani*. Both assays could detect ~~one~~ 3

germinated conidia per 3 ml of whole blood, indicating a high potential for our system in detecting *Fusarium* species in clinical whole blood samples. Further, to apply our new real-time PCR system directly to clinical samples of fusariosis, we evaluated their efficacy using a mouse model of invasive *F. solani* infection. In the model, hyphae of *F. solani* were clearly visible histopathologically, and the fungus was also recovered from the lung, kidney, liver, and spleen tissue of all mice intravenously infected with *F. solani* conidia. When plasma and whole blood samples were collected from infected mice and tested with the real-time PCR system, the sensitivity was 100% (~~44~~ n = 4) in plasma samples, while no amplification signal was detected in whole blood samples.

In summary, our new real-time system enabled *Fusarium* species and FSSC to be identified more rapidly and precisely in comparison with conventional DNA sequencing methods. In addition, this system successfully detected *Fusarium* DNA from human whole blood samples spiked with the fungus and from plasma samples taken from infected animals. This system would provide a rapid and precise diagnostic tool for human clinical samples, which will be helpful for early diagnosis followed by appropriate treatment to achieve better prognosis in disseminated fusariosis patients.

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Legends to illustrations

~~Fig. 1~~

~~New real-time PCR system for detecting *Fusarium* spp. and *Fusarium solani* species complex (FSSC).~~

~~Fig. 2~~ Fig. 1

Analytical sensitivity of the real-time PCR assays determined by testing with a tenfold serial dilution of the standard DNA ranging from 1 copy to 10^5 copies per reaction. A. Amplification plot of the *Fusarium* genus-specific assay. B. Amplification plot of the FSSC-specific assay. C. A standard curve of the *Fusarium* genus-specific assay. D. A standard curve of the FSSC-specific assay.

~~Fig. 3~~ Fig. 2

~~Detection from whole blood samples artificially spiked with a tenfold serial dilution of *F. solani* germinated conidia at a final concentration of 1 to 10,000 per 1 ml whole blood. All tests were conducted as a duplex real-time PCR with the IPC assay in a single tube to monitor for false negative results that may occur in the presence of PCR inhibitors. 200 copies of IPC DNA were added to the PCR reaction. A. Amplification plot of the *Fusarium* genus-specific assay. B. Amplification plot of the FSSC-specific assay. C. Amplification plot of IPC assay performed with the *Fusarium* genus-specific assay as a duplicate real-time PCR. D. Amplification plot of IPC assay performed with the FSSC-specific assay as a duplicate real-time PCR.~~

Detection of *F. solani* from whole blood samples artificially spiked with *F. solani* germinated conidia by the real-time PCR system. All tests were conducted as a duplex real-time PCR with the IPC

484 assay in a single tube. A. Amplification plot of the *Fusarium* genus-specific assay. B.
485 Amplification plot of the FSSC-specific assay. C. Amplification plot of IPC assay performed
486 with the *Fusarium* genus-specific assay. D. Amplification plot of IPC assay performed with
487 the FSSC-specific assay.

1 Table 1 Oligonucleotide primers and cycling probes used in the real-time PCR system

Real-time PCR assay	Name	Sequences	Target gene	PCR products size (bp)
<i>Fusarium</i> -genus specific assay				
Forward primer	Fusa28sF1	5'-TGAAATCTGGCTCTCGGGCC-3'	28s rRNA	222
Reverse primer	Fusa28sR1	5'-GCTATCGGTCTCTGGCCGG-3'		
Cycling probe	Fprobe1	5' Eclipse-TCTGGT7(G)GA-FAM 3'		
FSSC-specific assay				
Forward primer	Fusa28sF1	5'-TGAAATCTGGCTCTCGGGCC-3'	28s rRNA	222
Reverse primer	Fusa28sR1	5'-GCTATCGGTCTCTGGCCGG-3'		
Cycling probe	FSprobe1	5' Eclipse-TTTGGTG(A)GG-FAM 3'		
IPC assay				
Forward primer	Lambda-primerF1	5'-AGCAGAGCATGTTGCGCCAG-3'	λDNA	392
Reverse primer	Lambda-primerR2	5'-CAACAAAGCTGCCTGATAG-3'		
Cycling probe	Lambda-probe1	5' Eclipse-CGCTC(G)TAA-ROX 3'		

2 RNA is parenthesized and SNP site is italicized.

3 Table 2 Reference strains tested by real-time PCR assays

Species (No. of strains tested)	Number of positive real-time PCR assays	
	<i>Fusarium</i> -genus specific assay	FSSC-specific assay
FSSC^a (n=15)		
<i>Fusarium solani</i> ¹ (n=15)	15	15
FOSC^b (n=2)		
<i>Fusarium oxysporum</i> ² (n=2)	2	0
FDSC^c (n=3)		
<i>Fusarium dimerum</i> ³ (n=2)	2	0
<i>Fusarium lunatum</i> ⁴ (n=1)	1	1
GFSC^d (n=4)		
<i>Fusarium proliferatum</i> ⁵ (n=1)	1	0
<i>Fusarium verticillioides</i> ⁶ (n=3)	3	0
FIESC^e (n=2)		
<i>Fusarium equiseti</i> ⁷ (n=1)	1	0
<i>Fusarium incarnatum</i> ⁸ (n=1)	1	0
Non- <i>Fusarium</i> (n=38)		
<i>Histoplasma capsulatum</i> ⁹ (n=1)	0	0
<i>Paracoccidioides brasiliensis</i> ¹⁰ (n=1)	0	0
<i>Aspergillus</i> spp. ¹¹ (n=7)	0	0
<i>Candida</i> spp. ¹² (n=8)	0	0
<i>Cryptococcus</i> spp. ¹³ (n=4)	0	0
<i>Rhizopus oryzae</i> ¹⁴ (n=1)	0	0
<i>Absidia corymbifera</i> ¹⁵ (n=1)	0	0
<i>Rizomucor pusillus</i> ¹⁶ (n=1)	0	0
<i>Trichosporon asahii</i> ¹⁷ (n=1)	0	0
<i>Scedosporium apiospermum</i> ¹⁸ (n=1)	0	0
<i>Paecilomyces variotii</i> ¹⁹ (n=1)	0	0
<i>Malassezia furfur</i> ²⁰ (n=1)	0	0
<i>Rhodotorula mucilaginosa</i> ²¹ (n=1)	0	0
<i>Alternaria alternate</i> ²² (n=1)	0	0
<i>Penicillium islandicum</i> ²³ (n=1)	0	0
<i>Sporothrix schenckii</i> ²⁴ (n=1)	0	0
<i>Epidermophyton floccosum</i> ²⁵ (n=1)	0	0
<i>Aureobasidium pullulans</i> ²⁶ (n=1)	0	0
<i>Exophiala dermatitidis</i> ²⁷ (n=1)	0	0
<i>Ochroconis gallopava</i> ²⁸ (n=1)	0	0
<i>Cladosporium cladosporioides</i> ²⁹ (n=1)	0	0
<i>Schizophyllum commune</i> ³⁰ (n=1)	0	0
Human DNA (n=1)	0	0

4 ^aFSSC: *Fusarium solani* species complex. ^bFOSC: *Fusarium oxysporum* species complex. ^cFDSC: *Fusarium dimerum* species complex. ^dGFSC:
5 *Gibberella fujikuroi* species complex. ^eFIESC: *Fusarium incarnatum-equiseti* species complex. ¹IFM 40718, IFM 48449, IFM 49196, IFM 49271,
6 IFM 49274, IFM 49275, IFM 49277, IFM 50956, IFM 51945, IFM 52139, IFM 54126, IFM 55596, IFM 57371, IFM 59638, IFM 59778. ²IFM
7 50912, IFM 48455. ³IFM 59549, IFM 51070. ⁴IFM 48434. ⁵IFM 52350. ⁶IFM 48456, IFM 59638, IFM 59558. ⁷IFM 48440. ⁸IFM 55589.
8 ⁹IFM 46159. ¹⁰IFM41621. ¹¹*Aspergillus fumigates* IFM 56857, *Aspergillus niger* IFM 47800, *Aspergillus flavus* IFM 56856, *Aspergillus terreus*
9 IFM 58692, *Aspergillus nidulans* IFM 47793, *Aspergillus restrictus* IFM 40845, *Aspergillus versicolor* IFM 5375 ¹²*Candida albicans* IFM 5801,
10 *Candida tropicalis* IFM 5797, *Candida glabrata* IFM 40217, *Candida parapsilosis* IFM 40205, *Candida krusei* IFM 5798, *Candida guilliermondii*
11 IFM 50462, *Candida kefyr* IFM 51432, *Candida lusitanae* IFM 49207. ¹³*Cryptococcus neoformans* IFM 45837, *Cryptococcus albidus* IFM 5794,
12 *Cryptococcus laurentii* IFM 5765, *Cryptococcus gattii* IFM 48635. ¹⁴IFM 40515. ¹⁵IFM 47664. ¹⁶IFM 41590. ¹⁷IFM 48575. ¹⁸IFM 5376.
13 ⁹IFM 58693. ²⁰IFM 48584. ²¹IFM 48980. ²²IFM 56185. ²³IFM 47462. ²⁴IFM 49820. ²⁵IFM 41142. ²⁶IFM 4802. ²⁷IFM 41479. ²⁸IFM
14 52977. ²⁹IFM 41447. ³⁰IFM 55055.

Table 3 Identification of *Fusarium* clinical isolates by EF-1 α sequencing and by the real-time PCR system

Species complex and species identified by EF-1 α sequencing (No. of isolates tested)	No. of isolates identified by the real-time PCR system			Agreement (%) [*]
	Non- <i>Fusarium</i>	FSSC	Non-FSSC <i>Fusarium</i>	
FSSC^a (n=75)				
<i>Fusarium</i> cf. <i>solani</i> (n=41)	0	41	0	100
<i>Fusarium falciforme</i> (n=34)	0	34	0	100
FOSC^b (n=2)				
<i>Fusarium oxysporum</i> (n=1)	0	0	1	100
<i>Fusarium</i> sp. (n=1)	0	0	1	100
FDSC^c (n=4)				
<i>Fusarium delphinoides</i> (n=4)	0	0	4	100
GFSC^d (n=6)				
<i>Fusarium sacchari</i> (n=1)	0	0	1	100
<i>Fusarium proliferatum</i> (n=2)	0	0	2	100
<i>Fusarium napiforme</i> (n=2)	0	0	2	100
<i>Fusarium bactridioides</i> (n=1)	0	0	1	100

^aFSSC: *Fusarium solani* species complex. ^bFOSC: *Fusarium oxysporum* species complex. ^cFDSC: *Fusarium dimerum* species complex.

^dGFSC: *Gibberella fujikuroi* species complex. *Agreement between identification results from EF-1 α sequencing and the real-time PCR system

21 Table 4 Experimental mouse model of invasive *F. solani* infection

Mouse no.	<i>F. solani</i> inoculum (CFU/mouse)	CFU of whole blood (log CFU/mL)	Real-time PCR system			
			Whole blood		Plasma	
			<i>Fusarium</i> genus- specific assay	FSSC-specific assay	<i>Fusarium</i> genus- specific assay	FSSC-specific assay
1	5.7 x 10 ⁵	NG ^a	- ^c	-	+ ^d	+
2		NG	-	-	+	+
3		NG	-	-	+	+
4		ND ^b	ND	ND	ND	ND
5	0	1	-	-	+	+
6		NG	-	-	-	-
7		NG	-	-	-	-

22 ^aNG: no growth. ^bND: not done. ^c -: negative. ^d+: positive.

Figure 1

Figure 2