Dual Panel Multiplex PCR Assay for Rapid Detection of Medically Important Fungi and Resistant Species of Candida and Aspergillus

(Asai PCR Multiples Dual Panel untuk Pengesanan Segera Kulat yang Penting daripada Segi Perubatan dan Spesies Rintang Candida dan Aspergillus)

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ABSTRACT

Invasive fungal infections (IFIs) have risen dramatically in recent years among high risk immunocompromised patients. Rapid detection of fungal pathogens is crucial to timely and accurate antifungal therapy. Two multiplex polymerase chain reaction (PCR) assays were developed to detect major fungal species that cause invasive infections and identify resistant species. Genus specific primers for Candida, Aspergillus, Fusarium and species specific primers for Candida glabrata, Candida krusei and Aspergillus terreus which are known to be clinically resistant species, were designed from the internal transcribed spacer (ITS) regions of ribosomal ribonucleic acid (rRNA) gene complex. Both assays were performed simultaneously to promote rapid detection of fungal isolates based on distinct amplicon sizes. Inclusion of the universal fungal primers ITS 1 and ITS 4 in the genus specific assay produced a second amplicon for each isolate which served to confirm the detection of a fungal target. The limit of detection for the genus specific assay was 1 nanogram (ng) deoxyribonucleic acid (DNA) for Aspergillus fumigatus and Candida albicans, 0.1 ng DNA for Fusarium solani, while the species-specific assay detected 0.1 ng DNA of A. terreus and 10 picogram (pg) DNA of C. krusei and C. glabrata. The multiplex PCR assays, apart from universal detection of any fungal target, are able to detect clinically important fungi and differentiate resistant species rapidly and accurately, which can contribute to timely implementation of effective antifungal regime.

Keywords: Aspergillus; Candida; detection; Fusarium; multiplex PCR

ABSTRAK

Jangkitan kulat invasif telah meningkat sejak kebelakangan ini dalam kalangan pesakit terimunokompromi. Pengesanan segera patogen kulat adalah amat perlu supaya terapi anti kulat yang bersesuaian dapat diberikan. Dua asai tindak balas rantai polimerase multipleks telah dibangunkan untuk mengesankan spesies utama patogen kulat yang menyebabkan infeksi invasif dan mengenal pasti spesies resistan. Primer khusus untuk genus Candida, Aspergillus, Fusarium dan khusus untuk spesies Candida glabrata, Candida krusei dan Aspergillus terreus yang merupakan spesies rintang telah direka cipta berdasarkan jujukan mentranskripsi jarak dalaman (ITS) kompleks gen rRNA. Kedua-dua asai dijalankan serentak untuk mempercepatkan pengesan pencikan kulat berdasarkan saiz amplicon yang terhasil. Dalam asai khusus untuk pengesan genus, primer universal kulat disertakan bersama supaya amplicon kedua terhasil bagi setiap pencilan yang mengesahkan kehadiran kulat. Tahap pengesan untuk asai khusus genus adalah 1 nanogram (ng) asid deoksiribonukleik (DNA) Aspergillus fumigatus dan Candida albicans serta 0.1 ng DNA Fusarium solani, manakala asai khusus spesies dapat mengesan 0.1 ng DNA A. terreus dan 10 pikogram (pg) DNA C. krusei serta C. glabrata. Selain daripada pengesan semua kulat secara am, asai tindak balas rantai polimerase multipleks yang dibangunkan dapat mengesan kulat berkepentingan klinikal dan membezakan spesies rintang secara pantas dan tepat, justru boleh berperanan dalam penentuan aival ubatan anti kulat yang efektif bagi pesakit.

Kata kunci: Aspergillus; Candida; Fusarium; PCR multipleks; pengesan

INTRODUCTION

The last two decades has seen a growing number of fungal infections coincident with a dramatic increase in the population of severely immunocompromised patients due to human immunodeficiency virus infections, organ transplants, haematological disorders such as leukaemia and other malignancies (Oren & Paul 2014). Intensive and aggressive medical practices and treatments such as surgery, the use of catheters, injections, radiation, chemotherapy, antibiotics and steroids are risk factors for fungal infections (Galimberti et al. 2012). However, these procedures are necessary and therefore, the incidence of fungal infections are expected to increase.

Although Candida and Aspergillus species are major contributors to life-threatening infections, occurrence of resistant species and the emergence of previously rare fungal species is increasing, resulting from antifungal prophylaxis in high-risk patients (Preuner & Lion...
Candida albicans is still the predominant species causing infections, however an upward trend was observed for non-albicans Candida infections caused by C. glabrata, C. parapsilosis and C. krusei (Pfaller & Diekema 2007; Rishi & Clark 2011). These Candida species show reduced susceptibility towards newer echinocandin drugs such as caspofungin, while C. glabrata and C. krusei are widely resistant to the long-standing, most frequently used azole drug, fluconazole (Pfaller & Castanheira 2016). Prolonged caspofungin prophylaxis in neutropenic patients has contributed to a significant increase of infections caused by C. krusei and C. glabrata (Lortholary et al. 2011; Wisplinghoff et al. 2014). Aspergillus fumigatus continues to be the most frequent causative agent of invasive aspergillosis however, non-fumigatus Aspergillus infections increasingly occurred (Azaz et al. 2015; Bašková & Buchta 2012). In particular, A. terreus has been recognised as resistant towards amphoteracin B, the highly effective but also highly toxic broad-spectrum antifungal drug, with a high mortality rate for invasive infections (Blum et al. 2013; Steinbach & Perfect 2003). Less frequently observed fungal genera associated with very high fatality include Fusarium, Scedosporium and members of Mucorales, namely Rhizopus and Mucor (Bašková & Buchta 2012). In patients with haematological malignancies, Fusarium is the second or third most common mould causing infections (Tortorano et al. 2014) and are reported to have low susceptibility towards azole drugs (Alastrauey-Izquierdo et al. 2008). Fusarium solani is the species responsible for most human infections (50%) followed by F. verticillioides (20%) and F. moniliforme (Dignani & Anaissie 2004).

The standard approaches used for the diagnosis of invasive fungal infections include serological detection of circulating fungal antigens, culture of body fluids for fungal recovery followed by identification and histopathological examination of tissue sections for the presence of fungi. Although serological assays are widely used, each one is designed to detect a single fungal genus and tests for Aspergillus and Candida have shown variable sensitivity and specificity (Bašková & Buchta 2012; Denning 1998), which is similar to culture diagnosis. While histological analyses of biopsy tissues are highly sensitive and specific (Lenka et al. 2007), the method is frequently associated with bleeding complication in patients with severe thrombocytopenia (Denning 1998). Poor outcome in patients with invasive fungal infections (IFIs) is related to delayed institution of an effective antifungal regime and prescription of unnecessary toxic antifungal agents (Yeo & Wong 2002).

In recent years, nucleic acid detection techniques have been developed to provide an early diagnosis of mycotic infections and the identification of pathogenic fungi. Polymerase chain reaction (PCR) based methods including nested or semi-nested PCR assays are particularly promising because of their simplicity, specificity and sensitivity (Cerikçioglu et al. 2010; Than et al. 2012). Multiplex PCR has been used to detect Candida spp. (Mallus et al. 2013; Vahidnia et al. 2015) and Aspergillus spp. (Amini et al. 2015; Logotheti et al. 2009) as has real-time PCR (Emam & Abd El-salam 2015; Horváth et al. 2013). Several PCR techniques have targeted ribosomal DNA of Candida (Cerikçioglu et al. 2010; Mallus et al. 2013; Than et al. 2012) and Aspergillus (Walsh et al. 2011). Although these PCR methods have been useful for the identification of fungal species, they either only identify species within a particular genus or detect the fungus at genus level. Furthermore, real-time PCR requires the use of costly reagents and instrumentation.

Therefore, in this study multiplex PCR assays were developed for simultaneous detection of Candida, Aspergillus, Fusarium and the antifungal-resistant species A. terreus, C. glabrata and C. krusei through distinct amplicon sizes. An optimized multiplex PCR assay with gel electrophoresis detection is a very useful, low-cost method for the detection of major fungal targets, simultaneously.

**MATERIALS AND METHODS**

Primers were designed from internal transcribed spacer (ITS) regions of fungal ribosomal gene complex (Table 1). The rDNA sequences of the fungi were retrieved from GenBank database and were subjected to the software CLUSTALW (http://www.genome.jp/tools/clustalw) for multiple sequence alignment. The sequences were analyzed with BioEdit Sequence Alignment Editor Version 7.2.3 for nonhomologous regions among 101 different fungal strains (Table 2). The conserved regions within each targeted genus and species were compared for sequence consistency against multiple strains of each species. The primer regions were tested in silico using the BLAST program (http://blast.ncbi.nlm.nih.gov) to check for specificity towards the target fungi. Genus/species-specific primer sequences were analyzed in OligoAnalyzer 3.1 program (https://sg.idtdna.com/analyzer/Applications/OligoAnalyzer) to analyse its reverse complementary sequence, melting temperature, GC content and molecular weight. The selected primers were synthesized by IDT Singapore.

A total of 84 clinical fungal isolates were evaluated in this study. These include the filamentous fungi Aspergillus fumigatus (n=4), A. niger (n=9), A. flavus (n=3), A. terreus (n=1), Fusarium solani (n=11) and the yeast Candida albicans (n=27), C. tropicalis (n=8), C. parapsilosis (n=6), C. krusei (n=3), C. glabrata (n=9), C. kefyr (n=1), C. dublinensis (n=1) and Cryptococcus neoformans (n=1). Additionally, 6 ATCC (American Type Culture Collection) strains of C. albicans ATCC 10231, C. glabrata ATCC 66032, C. krusei ATCC 6258, C. parapsilosis ATCC 20246, A. fumigatus ATCC 204305 and F. solani ATCC 36031 were evaluated. Clinical fungal isolates were provided by the Mycology Unit, UKMMC (Universiti Kebangsaan Malaysia Medical Centre) and were identified using routinely used methods which include biochemical tests for yeasts and microscopic morphology for moulds. In addition, DNA from bacterial isolates namely Escherichia coli and Staphylococcus aureus was also evaluated for
control purpose. The bacterial isolates were obtained from the culture collection of the Novel Antibiotic Research Laboratory, UKM.

The fungal isolates were sub-cultured onto Potato-Dextrose Agar (PDA) or Sabouraud-Dextrose Agar (SDA) (Difco Laboratories, West Molesey, UK) media at 25-30°C; 2 days for the yeast and 2 weeks for the moulds. To extract DNA, two hundred microliters of fungal cell suspension was placed on QIAcard FTA Card (Qiagen, Hilden, Germany), allowed to dry and microwaved on high power for 30 s. Two discs of 3.0 mm diameter of QIAcard FTA was punched using Harris Uni-Core™ micro-puncher (Ted Pella, California, USA) followed by extraction with Extract-N-Amp™ Plant PCR kit (Sigma, Missouri, USA). The extracted genomic DNA was diluted ten-fold with nuclease free water before proceeding to PCR amplification (BIO-RAD T 100™).

### Table 1. Genus and species specific primers for fungal targets (patent pending)

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer designation</th>
<th>Sequence (5’→3’)</th>
<th>Approximate amplicon size (base pair)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Universal detection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All fungi</td>
<td>ITS1</td>
<td>TCC GTA GGT GAA CCT GCG G</td>
<td>500 for <em>Candida albicans</em>, <em>C. dubliniensis</em>, <em>C. parapsilosis</em>, <em>C. tropicalis</em>, <em>C. kefyr</em>, 480 for <em>C. krusei</em> &amp; 860 for <em>C. glabrata</em></td>
</tr>
<tr>
<td></td>
<td>ITS4</td>
<td>TCC TCC GCT TAT TGA TAT GC</td>
<td>550 for <em>Aspergillus fumigatus</em>, <em>A. niger</em>, <em>A. flavus</em>, <em>A. terreus</em> &amp; <em>Fusarium solani</em></td>
</tr>
<tr>
<td><strong>Genus-specific detection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus</em></td>
<td>GSAsp1</td>
<td>AAT CAC ACT CAG ACT GCA</td>
<td>200</td>
</tr>
<tr>
<td><em>Candida</em></td>
<td>GSCand1</td>
<td>GTA TYR CTC AAY ACC AAA C</td>
<td>340; 600 for <em>C. glabrata</em></td>
</tr>
<tr>
<td><em>Fusarium</em></td>
<td>GSFus1</td>
<td>TAC TAC GCW ATG GAA GCT</td>
<td>450</td>
</tr>
<tr>
<td><strong>Species-specific detection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus terreus</em></td>
<td>SSAter1</td>
<td>CAA GTT GCA GAT AAA TGC</td>
<td>510</td>
</tr>
<tr>
<td><em>Candida glabrata</em></td>
<td>SSCglab1</td>
<td>GCA GAT TAA TAG AGA AGC TTG</td>
<td>700</td>
</tr>
<tr>
<td><em>Candida krusei</em></td>
<td>SSCkrusei2</td>
<td>CTC TGC GCA CGC GCA AGA TG</td>
<td>280</td>
</tr>
</tbody>
</table>

### Table 2. Strains analysed and experimentally tested for genus specific primer design for Candida, Aspergillus, Fusarium and species specific primer for Aspergillus terreus, Candida glabrata, Candida krusei

<table>
<thead>
<tr>
<th>Genus (No. of strains analysed <em>in silico</em>)</th>
<th>(No. of strains experimentally tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida</em> spp. (Total = 32)</td>
<td><em>Candida</em> spp. (Total = 59)</td>
</tr>
<tr>
<td><em>C. albicans</em> (4)</td>
<td><em>C. albicans</em> (28)</td>
</tr>
<tr>
<td><em>C. dubliniensis</em> (1)</td>
<td><em>C. dubliniensis</em> (1)</td>
</tr>
<tr>
<td><em>C. tropicalis</em> (2)</td>
<td><em>C. tropicalis</em> (8)</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> (3)</td>
<td><em>C. parapsilosis</em> (7)</td>
</tr>
<tr>
<td><em>C. krusei</em> (11)</td>
<td><em>C. krusei</em> (4)</td>
</tr>
<tr>
<td><em>C. glabrata</em> (10)</td>
<td><em>C. glabrata</em> (10)</td>
</tr>
<tr>
<td><em>C. guilliermondii</em> (1)</td>
<td><em>C. kefyr</em> (1)</td>
</tr>
<tr>
<td><em>Aspergillus</em> spp. (Total = 31)</td>
<td><em>Aspergillus</em> spp. (Total = 18)</td>
</tr>
<tr>
<td><em>A. fumigatus</em> (8)</td>
<td><em>A. fumigatus</em> (5)</td>
</tr>
<tr>
<td><em>A. terreus</em> (9)</td>
<td><em>A. terreus</em> (1)</td>
</tr>
<tr>
<td><em>A. clavatus</em> (3)</td>
<td><em>A. niger</em> (9)</td>
</tr>
<tr>
<td><em>A. niger</em> (6)</td>
<td><em>A. flavus</em> (3)</td>
</tr>
<tr>
<td><em>A. flavus</em> (4)</td>
<td></td>
</tr>
<tr>
<td><em>A. nidulans</em> (1)</td>
<td></td>
</tr>
<tr>
<td><em>Fusarium</em> spp. (Total = 12)</td>
<td><em>Fusarium</em> spp. (Total = 12)</td>
</tr>
<tr>
<td><em>F. solani</em> (9)</td>
<td><em>F. solani</em> (12)</td>
</tr>
<tr>
<td><em>F. oxysporum</em> (3)</td>
<td></td>
</tr>
<tr>
<td>**Species (No. of strains analysed <em>in silico</em>)</td>
<td>(No. of strains experimentally tested)</td>
</tr>
<tr>
<td><em>C. glabrata</em> (9)</td>
<td><em>C. glabrata</em> (3)</td>
</tr>
<tr>
<td><em>C. krusei</em> (9)</td>
<td><em>C. krusei</em> (3)</td>
</tr>
<tr>
<td><em>A. terreus</em> (8)</td>
<td><em>A. terreus</em> (1)</td>
</tr>
</tbody>
</table>
Thermal Cycler, Germany).

The multiplex PCR reaction for fungal genus detection contained 2 μL of genomic DNA, 2× GoTaq® Green Master Mix that consisted of GoTaq® DNA Polymerase, 400 μM dNTP and 3 mM MgCl₂ (Promega, Madison, WI, USA), 0.1 μM of primer ITS4 and 0.4 μM of primer ITS1, genus specific Aspergillus (GSAp1), Candida (GSCand1), F. solani (GSFus1) and species specific C. krusei (SSCkrusei2), C. glabrata (SSCglabr1) and A. terreus (SSAter1) primers in a total volume of 10 μL. Multiplex PCR reaction for resistant species identification contained 2 μL of genomic DNA, 2× GoTaq® Green Master Mix and 0.4 μM of ITS1 primer and species specific C. krusei (SSCkrusei2), C. glabrata (SSCglabr1) and A. terreus (SSAter1) primers in a total volume of 10 μL. Optimized PCR amplification conditions were: 5 min initial 94°C step, followed by 34 cycles at 94°C for 1.5 min, 51.5°C for 2 min and 65°C for 3 min and a final extension step at 65°C for 10 min. A patent of the designed genus and species specific primers (Table 1) and multiplex PCR assay procedure submitted to the Intellectual Property Corporation of Malaysia (MyIPO) is pending (Malaysia Patent Application No. PI 2015001974). Amplification products were electrophoresed in agarose gels (3.0% w/v) (Vivantis, California, USA) in 0.5 × TBE buffer and stained with ethidium bromide (Nacalai Tesque, Kyoto, Japan).

The limit of detection of the multiplex PCR assays was determined using serial dilutions of fungal DNA extracted with Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA).

RESULTS

Each genus and species-specific primer was specific for its target as tested in multiplex PCR assays (Figures 1 and 2) and all the 83 fungal targets were correctly identified according to amplicon size. All Aspergillus species strains (n=17) were successfully amplified by ITS1-1-GSAp1 primer pair producing amplicons of approximately 200 base pair (bp). The Candida genus-specific primer pair ITS1-GSCand1 amplified an approximately 340 bp product for all Candida spp. Strains (n=43) except for C. glabrata strains (n=9) which had an approximate amplicon size of 600 bp (Figure 3). The Candida genus-specific primer did not amplify C. krusei strains. Therefore, C. krusei-specific primer pair ITS1-SSCkrusei2 was included in the PCR reaction and produced an approximately 310 bp amplicon for C. krusei strains (n=11). The F. solani strains (n=43) were amplified by Fusarium genus-specific primer pair ITS1-GSFus1 yielding approximately 450 bp amplicon. In the genus specific assay, the amplification product of the universal fungal primers, ITS1 and ITS4 was also detected, therefore the larger amplicon served to confirm the presence of a fungal target. A Cryptococcus neoformans isolate produced an amplicon of approximately 500 bp with the universal fungal primers, while the bacterial species tested were not detected. In the species differentiation assay, species specific primers together with the universal fungal primer ITS1 were used to identify A. terreus, C. glabrata and C. krusei. A strain of A. terreus was amplified.

**Figure 1.** Genus-specific multiplex PCR with mixed genomic DNA of F. solani, C. albicans, C. krusei and A. fumigatus (lane 2) compared with single genomic DNA multiplex PCR as a reference (lanes: 3-6). Lane 2: Universal fungal detection (ITS1 & ITS4 primers) for (arrows indicate, respectively, from top to bottom) filamentous fungi (~550 bp) and yeast (~500 bp), genus specific detection of F. solani (~450 bp), C. albicans (~340 bp), C. krusei (~310 bp) and A. fumigatus (~200 bp). Lane 3: Universal fungal detection of F. solani (~550 bp) and genus specific amplicon (~450 bp), lane 4: C. krusei (~500 bp & ~310 bp), lane 5: C. albicans (~500 bp & ~340 bp) and lane 6: A. fumigatus (~550 bp & ~200 bp).
using the primer pair ITS1-SSAter1 yielding approximately 510 bp amplicon and 9 strains of C. glabrata were amplified by the C. glabrata-specific primer pair ITS1-SSCglab1 producing amplicons of approximately 700 bp. C. krusei-specific primer pair ITS1-SSCkrusei produced the same amplicon size as before, of 310 bp.

Optimum cycling conditions was achieved with annealing temperature set at 51.5°C and extension temperature at 65°C for 35 cycles. The components of the PCR reaction were also optimized with ITS 4 primer concentration reduced to 0.1 μM whereas other primers were retained at 0.4 μM. Under these optimum conditions the limit of detection (LOD) for the genus specific assay was 1 ng DNA for A. fumigatus and C. albicans, 0.1 ng DNA for F. solani, while the species specific assay detected 0.1 ng DNA of A. terreus, 10 pg DNA of C. krusei and C. glabrata.

**DISCUSSION**

In this study, a dual-panel multiplex PCR assay was developed and tested for its accuracy and sensitivity in detecting Candida, Aspergillus and Fusarium spp. isolates. Three primers for individual genus detection and another three primers for C. glabrata, C. krusei and A. terreus were designed and optimised for multiplex detection of the fungal targets.

The ITS 1-5.8S-ITS 4 region was chosen for the design of genus and species-specific primers, due to high nucleotide variability among genera and species. The ITS region is a good molecular target for species level identification (Landlinger et al. 2009) and is extensively used as a universal DNA barcode in fungal taxonomy studies (Sulaiman et al. 2014). Furthermore, it is present at approximately 100 copies per genome (Henry et al. 2000) and is not found in
The multiplex PCR assays described here are able to differentiate three major clinically important fungal genera, namely *Candida*, *Aspergillus* and *Fusarium* and the resistant species *A. terreus*, *C. glabrata* and *C. krusei*. The deployment of a rapid, low cost yet reliable method for early diagnosis of invasive fungal infections is paramount to improving clinical management of the disease especially in hospitals with limited resources.

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SUPPLEMENTARY 2. Gel electrophoresis of PCR product using set of universal primers ITS 1 & ITS 4 (Lane 2) and set of *Candida* genus-specific primer, GSCand1 & ITS 1 with DNA isolates of different species (Lane 3 to Lane 10). Lane 1: 100 bp DNA ladder. Lane 2: positive control, *C. albicans* MM 1076. Lane 3: positive control, *C. parapsilosis* MM 1113. Lane 4: *A. fumigatus* MM 956. Lane 5: *A. niger* MM 2735. Lane 6: *A. flavus* MM 1938. Lane 7: *F. solani* MM 0020. Lane 8: *S. aureus*. Lane 9: S. *cauliflorum* sp. Lane 10: *E. coli*.

SUPPLEMENTARY 3. Gel electrophoresis of PCR product for sensitivity detection test using DNA isolate of *C. albicans* in multiplex PCR assay set 1. Lane 1: 100 bp DNA ladder. Lane 2: DNA concentration of 0.5 ng/μL. Lane 3: DNA concentration of 0.05 ng/μL. Lane 4: DNA concentration of 5.0 pg/μL. Lane 5: DNA concentration of 0.5 pg/μL. Lane 6: negative control.

SUPPLEMENTARY 4. Gel electrophoresis of PCR product for sensitivity detection test using DNA isolate of *C. krusei* in multiplex PCR assay set 1. Lane 1: 100 bp DNA ladder. Lane 2: DNA concentration of 0.5 ng/μL. Lane 3: DNA concentration of 0.05 ng/μL. Lane 4: DNA concentration of 5.0 pg/μL. Lane 5: DNA concentration of 0.5 pg/μL. Lane 6: negative control.
SUPPLEMENTARY 5. Gel electrophoresis of PCR product for sensitivity detection test using DNA isolate of *A. fumigatus* in multiplex PCR assay set 1. Lane 1: 100 bp DNA ladder. Lane 2: DNA concentration of 0.5 ng/μL. Lane 3: DNA concentration of 0.05 ng/μL. Lane 4: DNA concentration of 5.0 pg/μL. Lane 5: DNA concentration of 0.5 pg/μL. Lane 6: negative control

SUPPLEMENTARY 6. Gel electrophoresis of PCR product for sensitivity detection test using DNA isolate of *A. terreus* in multiplex PCR assay set 2. Lane 1: 100 bp DNA ladder. Lane 2: DNA concentration of 0.5 ng/μL. Lane 3: DNA concentration of 0.05 ng/μL. Lane 4: DNA concentration of 5.0 pg/μL. Lane 5: DNA concentration of 0.5 pg/μL. Lane 6: negative control

SUPPLEMENTARY 7. Gel electrophoresis of PCR product for sensitivity detection test using DNA isolate of *F. solani* in multiplex PCR assay set 1. Lane 1: 100 bp DNA ladder. Lane 2: DNA concentration of 0.5 ng/μL. Lane 3: DNA concentration of 0.05 ng/μL. Lane 4: DNA concentration of 5.0 pg/μL. Lane 5: DNA concentration of 0.5 pg/μL. Lane 6: negative control