Accurate and Rapid Identification of Candida spp. Frequently Associated with Fungemia by Using PCR and the Microarray-Based Prove-it Sepsis Assay

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The rapid identification of microbes responsible for bloodstream infections (BSIs) allows more focused and effective therapies and outcomes. DNA sequence-based methods offer an opportunity for faster, accurate diagnosis and for effective therapy. As our objective of the study, the ability of the Prove-it Sepsis platform, already proven as a rapid PCR- and microarray-based assay for the majority of sepsis-causing bacteria, was extended to also rapidly identify clinically relevant yeasts in blood culture. The performance characteristics of this extended platform are described. We found that the extended diagnostic Prove-it Sepsis platform was found to be highly accurate when analyzing primary isolates, spiked blood cultures, nucleic acid extracts from a retrospective blood culture data set, and primary blood cultures. Comparison of the blood culture results from the Prove-it Sepsis platform with those from conventional culture-based methods or by gene sequencing demonstrated a sensitivity of 99% and a specificity of 98% for fungal targets (based on analysis of a total of 388 specimens). Total assay time was 3 h from DNA extraction to BSI diagnosis. These results extend the performance characteristics of the Prove-it platform for bacteria to the easy, rapid, and accurate detection and species identification of yeasts in positive blood cultures. Incorporation of this extended and rapid diagnostic platform into the tools for clinical patient management would allow possibly faster identification and more focused therapies for BSIs.

The incidence of fungemia, particularly candidemia, continues to increase steadily, especially in hospitalized patients (4, 5, 8, 12, 30, 34). Candida spp. are the 4th most common causative agents of nosocomial bloodstream infections (BSIs) in the United States and the 5th to 10th most common cause in Europe and Australia (6, 8, 36). Candida albicans remains the predominant species in these infections, although recent data suggest an increase in the proportion of Candida species other than C. albicans, such as C. tropicalis, C. parapsilosis, C. glabrata, and C. krusei (3, 4, 8, 16, 17, 34).

Candidemia is associated with a high mortality rate, a prolonged length of hospital stay, and high hospital costs (13, 15, 17, 26, 29, 35). Appropriate antifungal therapy administered in a timely fashion impacts morbidity and mortality and costs of treatment (14, 15, 20, 25). Currently, suspected candidemia is diagnosed by blood culture, subsequent Gram staining, and various diagnostic practices, including a germ tube assay and various morphological and biochemical identification methods. These methods are all time-consuming, operator dependent, and costly, delaying effective and focused therapy and leading to avoidable additional mortality (20). In order to respond to the need for improved diagnostic tools, several diagnostic strategies and platforms have emerged for sepsis diagnostics, and some of them take advantage of the combination of PCR with microarray hybridization (19, 21, 27).

In recent work by Tissari and coworkers (33), the Prove-it Sepsis assay was validated in a clinical setting, which consisted of a broad-range PCR and microarray-based platform and positive blood cultures. That study indicated the assay was 99% specific and 95% sensitive with a pathogen panel covering over 50 Gram-negative and Gram-positive bacterial species as well as the methicillin resistance marker meca. To respond to the considerable contribution of Candida spp. in the microbial etiology of BSIs, the pathogen coverage of this assay has now been extended to include seven of the most clinically relevant Candida spp. The extended panel includes the species C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. guilliermondii, C. lusitaniae, and C. krusei, together with a higher pan-yeast taxon, covering at least the species C. dubliniensis, C. pelliculosa, C. kefyr, C. norvengensis, C. haemulonii, and Saccharomyces cerevisiae. This study evaluated the performance of the extended Prove-it Sepsis assay for the identification of fungal targets.

MATERIALS AND METHODS

Clinical isolates for performance analysis. In order to analyze the platform’s performance, 160 blinded fungal clinical isolates were sent to Mobidiag, Finland, for fungal Prove-it microarray analysis. First, the isolates were cultured for 48 h aerobically at 35°C on Sabouraud dextrose agar (Labema, Kerava, Finland) and then stored at -80°C in LyphoMatrix (Labema). All cultured strains were stored on Sabouraud dextrose agar at 4°C until DNA was extracted.

Spiked blood culture samples for performance analysis. A total of 69 BacT/Alert SA standard aerobic (bioMérieux, Marcy l’Etoile, France) blood culture bottles were spiked with different yeast strains representing eight Candida species. The strains were clinical isolates previously identifi-
fied to the species level by conventional methods (germ tube, API 20C AUX and/or API32, as well as morphological characteristics on corn meal-Tween 80 agar) in the Helsinki University Central Hospital Laboratory (HUSLAB), Finland. Strains were first subcultured on Sabouraud dextrose agar for 48 h aerobically at 35°C, followed by preparation of suspensions in 0.9% NaCl. After adjusting the suspensions to be equivalent to that of a McFarland 3.5 standard (≈10.5 × 10⁶ CFU/ml), 0.5 ml of the each suspension in 5 ml of sheep blood was inoculated into blood culture bottles, which were then incubated in a blood culture instrument (Bact/T  Alert 3D) until flagged as positive.

**Blood culture material.** Altogether, 3,318 blood culture samples from patients with suspected sepsis were collected in the routine microbiology laboratories of HUSLAB and University College London Hospitals (UCLH) in 2008. Blood culture bottles (Bact/Alert 3D [bioMérieux] and BacT 924 [Becton, Dickinson, NJ]) were incubated for a total of 6 days or until flagged as positive, whereafter organisms were identified by conventional methods. DNA extracts of samples positive for yeasts (44 samples), randomly selected samples positive for bacteria (16 samples), and samples negative for bacteria or yeasts (82 samples) were selected and anonymized for the fungal microarray analysis. Additionally, 19 blood culture samples from patients with suspected fungal sepsis (HUSLAB in 2010) were included in this study.

**DNA extraction.** For clinical fungal/bacterial isolates, one microbiological colony was picked from the agar plate and suspended in 2 ml of NucliSENS lysis buffer (bioMérieux). Alternatively, 100 µl of the spiked or clinical blood culture sample was taken and suspended in 2 ml of NucliSENS lysis buffer. Subsequently, DNA was extracted using an eluate volume of 55 µl in an automated nucleic acid extraction instrument (Nuclisens easyMAG; bioMérieux) according to the manufacturer's protocol (Genetic 2.0.1). A negative control was included in each test series.

**Analysis with PCR and microarray.** DNA samples were analyzed with the Prove-it Sepsis assay (Mobidiag, Helsinki, Finland) according to the manufacturer's instructions by using 1.5 µl of DNA extract for the PCR with the Mastercycler ep gradient S thermal cycler (Eppendorf, Hamburg, Germany). A proprietary multiplex PCR was designed with conserved regions within internal transcribed spacers (ITS) of the rRNA gene complex of Candida species. In silico primer design was compiled according to guidelines set out in a novel method for producing single-stranded DNA during the PCR step (18). The PCR methodology of the bacterial Prove-it Sepsis assay was similarly applied for the fungal PCR, yielding fungal amplicons of ~400 bp. Fungal and bacterial PCR mixtures were kept separate (for use in a total of two reactions) to ensure efficient fungal amplification and to avoid compromising the performance of bacterial targets of the assay. Subsequently, the amplicons from both of the PCRs were subjected to one microarray area of the Prove-it StripArray microarray. After hybridization, positive hybridization-based reaction products were imaged and analyzed by using the StripArray reader and Prove-it Advisor analysis software (version 1.0). Target identification was interpreted using specific rules and parameters built into the Prove-it Advisor software. These rules included the assessment of several internal control probes designed to validate assay parameters, without which a result is not authorized. The PCR control of the assay was a low-copy-number non-bacterial/fungal DNA fragment, which is added for every PCR to verify and validate amplification conditions. Hybridization and reagent controls confirmed the success of DNA hybridization and the functionality of the used reagents during the microarray step. For a sample series to be accepted, for each run the negative controls included in the extraction and/or PCR step are required to be negative. The internal controls for the PCR and hybridization are required to be positive.

**DNA sequencing.** Discrepancies in identifications between the reference and microarray methods were variously addressed by repeating the test, DNA sequencing, and querying the original identifications with HUSLAB and UCLH in order to resolve issues of discrepancies. PCR product samples for DNA sequencing were run on an agarose gel, from which they were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Sequencing was performed using cycle sequencing with the BigDye Terminator kit (version 3.1; Applied Biosystems [ABI], CA). Reactions were run on the ABI 3130xl capillary sequencer according to the manufacturer's instructions. Sequences were edited and analyzed with the Vector NTI Advance (Invitrogen, CA) and BioEdit (http://www .mbio.ncsu.edu/BioEdit/bioedit.html) programs and the ClustalW alignment algorithm, version 1.4 (32). The BLAST algorithm (1) was used to search for homologous sequences in the European Bioinformatics database as well as in both the National Center for Biotechnology Information database (http://www.ncbi .ac.uk/Tools/ssg/nciblast/nucleotide.html) and Mobidiag's proprietary sequence database.

**Statistical analysis.** The results were compared, and the sensitivity, specificity, and confidence interval (CI) values were calculated according to the Clinical and Laboratory Standards Institute's guidelines (10). Briefly, these analyses were performed using the following definitions: true positive (TP; identification to the species at taxon or species level as indicated), true negative (TN; negative array result either from yeasts not included in the repertoire of the assay, from bacterial samples, or from samples containing no microbes), false negative (FN; negative test result from yeasts included in the test repertoires or yeast identification only at the taxon level instead of the expected species-level identification), and false positive (FP; positive test result from samples expected to be negative or with incorrect species identification). The sensitivity and specificity were calculated as follows: \[ TP/(TP + FN) \times 100 \] \[ TN/(TN + FP) \times 100 \], respectively.

**RESULTS**

**Assay design and specificity of the fungal oligonucleotide probes.** The specificities of the fungal oligonucleotide probes were studied by applying DNA extracts of the Prove-it Sepsis bacterial panel to the newly configured microarray. Bacterial species were identified correctly, and no cross-hybridization with the fungal oligonucleotide probes was observed (data not shown).

**Blind analysis of clinical fungal isolates.** A total of 159 clinical fungal isolates were cultured on Sabouraud dextrose agar and analyzed in a blinded fashion. All Candida isolates were identified by either the pan-yeast or species-specific probes (Table 1). Specifically, 100% of C. glabrata and C. parapsilosis isolates were correctly reported at the species level. Similarly, 48/50 C. albicans isolates were identified at the species level, but 2 samples were identified only at the pan-yeast taxon level. Confirmatory sequencing revealed these samples to be C. glabrata and C. albicans. The latter sample finally was identified as C. dubliniensis by gene sequencing. The new assay also identified correctly 14/15, 7/8, and 4/5 C. tropicalis/C. krusei, and C. guilliermondii isolates, respectively. In the remaining cases, the microarray detected double findings of C. tropicalis/C. glabrata, C. krusei/C. parapsilosis, and C. guilliermondii/C. lusitaniae, respectively. In the subculturing of the original samples, the detected double findings were confirmed by conventional identification methods (Table 1). Two out of three samples originally identified as C. lusitaniae were named correctly at the species level by the new assay, whereas one sample was flagged only at the pan-fungal level. Confirmatory sequencing revealed this strain was C. oleophila. The microarray identified at the pan-fungal taxon level 14 samples altogether, representing strains of C. kefyr, C. haemulonii, C. norvegensis, C. dubliniensis, and S. cerevisiae (Table 1), as expected. One C. kefyr isolate was identified as C. guilliermondii by the microarray. This finding was also confirmed to be correct by gene sequencing. As expected, 8/10 samples related to the species Cryptococcus albidus, Cryptococcus neoformans, Trichosporon asahii,
Trichosporon mycotoxinivorans, Trichosporon mucoides, and Trichosporon inkin were reported as negative in the microarray analysis. However, two samples was flagged as positive. One of these false-positive findings was confirmed to be Trichosporon mucoides by conventional methods. Another one couldn’t be clarified by either sequencing or conventional identification methods.

Altogether, after confirmatory testing, the fungal microarray correctly reported 149 samples in the test repertoire and produced only two false-positive test results. No cross-hybridization between bacterial and fungal targets, or vice versa, was observed.

Evaluation of microarray performance by using spiked blood cultures. The fungal microarray workflow was challenged by spiking BacT/Alert SA standard aerobic blood culture bottles with eight different Candida spp. that originated from 69 different clinical isolates. These were incubated in a standard manner until flagged as positive. Incubation time was on average 9 h for all the samples, and no exceptions were noted that were related to Candida spp. Extraction of DNA from the spiked blood bottles followed by PCR and microarray analysis resulted in 100% concordance (Table 2).

Evaluation of microarray performance using DNA extracts from positive blood cultures. For the study, a total of 62 samples containing yeast elements were selected. These included 43 DNA extracts from blood cultures positive for yeasts. The samples originated from the previous bacterial microarray performance study, in which 3,318 blood cultures were examined (33). Additionally, 19 blood culture specimens positive for yeast (HUSLAB, Finland) extracts from blood cultures positive for yeasts. The samples originated from the previous bacterial microarray performance study, in which 3,318 blood cultures were examined (33). Additionally, 19 blood culture specimens positive for yeast (HUSLAB, Finland)
were included. The fungal samples were anonymized and incorporated as part of a randomly selected, larger number of DNA extracts containing either bacterial elements (including Enterobacter aerogenes, Enterococcus faecalis, Escherichia coli, Haemophilus influenzae, Pseudomonas aeruginosa, Staphylococcus epidermidis, and Streptococcus pneumoniae) or lacking any bacterial or fungal elements (98 samples).

The fungal microarray identified yeast species in 58/62 (93.5%) samples with yeast elements (Table 3). The four samples that remained negative in the microarray consisted of two Rhodotorula samples and also one sample that originally contained an unidentified yeast and another one whose detection was based entirely on Gram staining with negative culture results. The remaining samples were either reported as negative or flagged as bacteria, as expected. In summary, a total of 102 samples were reported as true negatives (2 Rhodotorula, 2 unidentified samples, and 98 blood culture-negative or bacteria-positive samples). The platform identified correctly all C. albicans (36), C. glabrata (9), and C. tropicalis (2) samples. C. parapsilosis was identified at the species level in 2 out of 3 samples, whereas 1 sample gave a result only at the pan-yeast level. Confirmatory sequencing did not bring any further information. One C. lusitaniae sample was identified as C. albicans. Sequence analysis of the sample identified it as C. lusitaniae. C. dubliniensis, C. pelliculosa, and S. cerevisiae (one sample each) were correctly identified only at the pan-yeast level. One sample, reported by conventional methods as a summary finding of C. albicans and C. glabrata, had been provided as separate samples from aerobic and anaerobic blood culture bottles. The Prove-it test identified C. albicans in one sample and C. glabrata in another one. Another C. albicans/C. glabrata multi-infection sample was identified only as C. albicans and was included in the false-negative group. All the negative and bacteria-positive blood culture samples included in the study yielded a negative yeast microarray result.

**DISCUSSION**

The performance of the newly designed Prove-it Sepsis microarray (33), extended to include the detection of clinically relevant yeasts, was assessed by analyzing 388 samples (clinical isolates, spiked blood cultures, and DNA from a retrospectively collected and well-characterized blood culture data set from patients with suspected sepsis) and demonstrated accurate identification for the vast majority of the seven most clinically relevant yeasts.

In the analysis of clinical fungal isolates, four conflicting identification results were observed compared to conventional identification. The capability of the new assay was emphasized by the subsequent DNA sequencing, which confirmed the four microarray results as correct. Additionally, the identification power of the new microarray over the conventional methods was demonstrated in three samples where a double finding was detected instead of a single one. Although sample contamination cannot be excluded, this finding reflects the difficulty of a primary conventional identification, where repeated subculturing may be needed in order to find a double infection. In these three cases, the double finding could be confirmed also by conventional identification.

Accuracy of the new test was further shown by analyzing spiked blood samples. All of the 69 spiked yeasts were identified correctly either at the species or taxon level. Among 59 clinical samples, there were, however, one C. parapsilosis isolate that was incorrectly identified at the taxon level and one C. lusitaniae isolate that was identified as C. albicans by the microarray. Taken together, the fungal microarray was 99% sensitive and 97% specific when the 388 samples were analyzed, and correct fungal target identification with no deterioration in bacterial target performance was demonstrated (Table 4). This additional target range expands the current generation of the Prove-it Sepsis microarray to beyond 90% of all microbes found in the original, observational study (31). Total assay time was 3 h, in addition to the DNA extraction step, which required 1 h.

Many groups have published similar applications as that used here to demonstrate the applicability of various microarray technologies in clinical fungal diagnostics, with equivalent performance results (7, 22, 23, 31, 37). Only Yoo and coworkers (37), however, demonstrated simultaneous detection of bacterial and fungal pathogens among clinical specimens, which is the main focus of this study. The new assay is a relative improvement with an added value for the rapid identification of the most frequent and clinically relevant yeasts that are associated with the majority of bloodstream infections. The potential for a microarray-based clinical diagnostic test that can detect bacterial and fungal pathogens simultaneously is promising, and it may help to reduce the need for ancillary hemocultures and at the same time may improve patient outcomes.

### Table 3 Yeast species identified from the clinical blood culture specimens

<table>
<thead>
<tr>
<th>Fungal pathogen identified by blood culture</th>
<th>No. of specimens</th>
<th>No. positive by microarray</th>
<th>Reported identification by microarray</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>36</td>
<td>36</td>
<td>C. albicans</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>9</td>
<td>9</td>
<td>C. glabrata</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>3</td>
<td>2</td>
<td>C. parapsilosis</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>2</td>
<td>2</td>
<td>C. tropicalis</td>
</tr>
<tr>
<td>C. lusitaniae</td>
<td>3</td>
<td>2</td>
<td>C. lusitaniae</td>
</tr>
<tr>
<td>C. albicans, C. glabrata</td>
<td>2</td>
<td>2</td>
<td>C. albicans</td>
</tr>
<tr>
<td>C. pelliculosa</td>
<td>1</td>
<td>1</td>
<td>Pan-yeast</td>
</tr>
<tr>
<td>C. dubliniensis</td>
<td>1</td>
<td>1</td>
<td>Pan-yeast</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>1</td>
<td>1</td>
<td>Pan-yeast</td>
</tr>
<tr>
<td>Rhodotorula sp.</td>
<td>2</td>
<td>0</td>
<td>Negative</td>
</tr>
<tr>
<td>Unidentified yeast species</td>
<td>2</td>
<td>0</td>
<td>Negative</td>
</tr>
<tr>
<td>Negative for yeast</td>
<td>98</td>
<td>0</td>
<td>Negative</td>
</tr>
<tr>
<td>Total</td>
<td>160</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Results included 55 true positives, 2 false negatives, 102 true negatives, and 1 false positive.

### Table 4 Sensitivity and specificity of the fungal and bacterial Prove-it Sepsis oligonucleotide microarrays relative to the reference methods

<table>
<thead>
<tr>
<th>Result with reference method</th>
<th>No. of samples (category) with indicated Prove-it Sepsis result</th>
<th>Sensitivity or specificity (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>No. of samples</td>
<td>Sensitivity (97.9–99.8)</td>
</tr>
<tr>
<td>Negative or positive</td>
<td>273 (true positive)</td>
<td>2 (false negative)</td>
</tr>
<tr>
<td></td>
<td>3 (false positive)*</td>
<td>110 (true negative)</td>
</tr>
</tbody>
</table>

*Blood culture-positive samples, including a pathogen not covered by the Prove-it Sepsis assay.

**Table 4** Sensitivity and specificity of the fungal and bacterial Prove-it Sepsis oligonucleotide microarrays relative to the reference methods.
fungal targets on the same microarray. We note that there is a lack of widespread and routine use of microarrays, and only a few diagnostic applications based on the technology are currently commercially available. These remarks may be due to complicated and labor-intensive protocols and/or to the necessity for relatively expensive consumables and microarray imaging equipment. It has to be emphasized that neither of these conditions applies to this extended Prove-it Sepsis platform, which makes the platform suitable also for small clinical laboratories, avoiding the high investment costs and devices with big footprints associated with the newly emerged, more rapid diagnostic solutions that are based on matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF/MS) and pyrosequencing.

Several studies have reported various proportions of yeasts versus bacterial isolates in positive blood cultures. The vast majority of yeast isolates in blood are within the Candida genus, with C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, and C. krusei representing up to 97% of cases with the remaining 3 to 5%, comprising of 12 to 14 rare species (5, 12, 16, 24, 28, 34, 35). In clinical work, reliable identification of the causative fungal species is often sufficient to guide the choice of antifungal therapy, as resistance profiles for many types of yeast are consistent and predictable. Consequently, fluconazole (FLC) still remains an economic alternative to the more expensive echinocandins in many cases of candidemia (14, 15, 17, 28, 34). There have been several reports on how adequate initial empirical treatment is crucial in terms of outcome for patients with a Candida bloodstream infection (9, 25). When all C. krusei and a proportion of C. glabrata isolates are resistant to FLC, their rapid primary identification as the infection-causing yeast is of paramount importance (12, 34). In this respect, commonly used fungal identification methods are either too slow or unspecific to allow rapid and optimal antifungal therapy.

Patients with extreme immunosuppression (associated with acute myeloid leukemia or bone marrow transplantation) might have highly unusual organisms, such as Scedosporium spp. and Fusarium spp., in their blood (24, 28). The new microarray in its current configuration is unable to identify these organisms, although their Gram-staining properties might indicate a mold infection, which in turn would trigger the need for a different antifungal therapy for these very difficult-to-identify organisms. It is also acknowledged that Cryptococcus spp. remain important pathogens, especially in countries with high rates of late-stage HIV disease. In the present configuration, the new microarray does not detect these organisms, but this way to overcome this shortfall in an otherwise-extensive fungal panel are now under development.

Arendrup and colleagues (2) reported a study in which various simulated fungal specimen types were sent blindly to clinical mycology laboratories in four Nordic countries via Nordic External Quality Assessment programs. The samples, consisting of monomicrobial or polymicrobial combinations of Candida, Cryptococcus neoformans, Aspergillus fumigatus, and some bacterial species were routinely processed in each laboratory. There were significant variations and inaccuracies of identification, especially in the case of C. glabrata. Similar misidentifications have also been reported by Hajieh and colleagues (16), suggesting a need for higher-quality and consistent mycological diagnostics that can be applied in all laboratories.

BSI remains a life-threatening condition, and early initiation of effective antimicrobial therapy at least for bacterial infection is a major predictor of outcome (14, 20, 25). Mortality from fungemia is as high if not higher than bacteremia mortality, and prompt initiation of the correct antifungal therapy is likely to be equally important for the outcome (11). This study on the modified Prove-it Sepsis platform demonstrates its extended capacity as a simple and robust identification method for bacteria and for the vast majority of yeasts. These qualities in turn prompt the possibility of faster and more evidence-based management of patients.

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Vanya Gant, Michael Petrov, Pentti Kuusela, and Minna Mäki designed and led the study. All authors supervised several aspects of the study, analyzed and interpreted the data, and helped develop and edit the manuscript.

Anne Aittakorpi and Minna Mäki are associated with Mobidiag, the manufacturer of the test products used in this study. All other authors declare no conflicts of interests.

REFERENCES


