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Evaluation of a Commercially Developed Semiautomated PCR–Surface-Enhanced Raman Scattering Assay for Diagnosis of Invasive Fungal Disease

P. Lewis White,[a] Samantha J. Hibbitts,[b] Michael D. Perry,[a] Julie Green,[c] Emma Stirling,[c] Luke Woodford,[c] Graeme McNay,[c] Ross Stevenson,[c] Rosemary A. Barnes[b]

Public Health Wales, Microbiology Cardiff, UHW, Cardiff, United Kingdom[a]; School of Medicine, Cardiff University, UHW, Cardiff, United Kingdom[b]; Renishaw Diagnostics Ltd., Glasgow, United Kingdom[c]

Nonculture-based tests are gaining popularity in the diagnosis of invasive fungal disease (IFD), but PCR is excluded from disease-defining criteria because of limited standardization and a lack of commercial assays. Commercial PCR assays may have a standardized methodology while providing quality assurance. The detection of PCR products by a surface-enhanced Raman scattering (SERS) assay potentially provides superior analytical sensitivity and multiplexing capacity compared to that of real-time PCR. Using this approach, the RenDx Fungiplex assay was developed to detect *Candida* and *Aspergillus*. Analytical and clinical evaluations of the assay were undertaken using extraction methods according to European *Aspergillus* PCR Initiative (EAPCRI) recommendations. A total of 195 previously extracted samples (133 plasma, 49 serum, and 13 whole blood) from 112 patients (29 with proven/probable IFD) were tested. The 95% limit of detection of *Candida* and *Aspergillus* was 200 copies per reaction, with an overall reproducibility of 92.1% for detecting 20 input copies per PCR, and 89.8% for the nucleic acid extraction–PCR-SERS process for detecting fungal burdens of <20 genome equivalents per sample. A clinical evaluation showed that assay positivity significantly correlated with IFD ($P < 0.0001$). The sensitivity of the assay was 82.8% and was similar for both *Candida* (80.0%) and *Aspergillus* (85.7%). The specificity was 87.5% and was increased (97.5%) by using a multiple (≥2 samples) PCR-positive threshold. In summary, the RenDx Fungiplex assay is a PCR-SERS assay for diagnosing IFD and demonstrates promising clinical performance on a variety of samples. This was a retrospective clinical evaluation, and performance is likely to be enhanced through a prospective analysis of clinical validity and by determining clinical utility.

The use of nonculture-based tests can enhance the diagnosis of invasive fungal disease (IFD) (1, 2). While antigen detection (galactomannan enzyme immunoassay [EIA] and [1,3]-β-D-glucan) is widely used, molecular-based detection (PCR) has been excluded from the revised European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) disease-defining criteria; this is a consequence of limited standardization and a paucity of commercially manufactured assays (3).

Since the publication of the disease-defining criteria, there have been several advances to standardize molecular diagnostics. The minimum information required for the publication of quantitative PCR experiments (MIQE) guidelines have been proposed in order to provide a standardized generic approach when developing and reporting real-time PCR assays (4). Specific to mycology, the European *Aspergillus* PCR initiative (EAPCRI) has offered recommendations to provide optimal methodology when testing whole-blood and serum samples by *Aspergillus* PCR (5, 6). An international standard has been developed, providing reference material for assay comparison and validation (7).

Commercial assays have become available for diagnosing invasive aspergillosis (IA) and invasive candidiasis (IC), providing standardized methodology and quality-controlled development in line with commercially available antigen-based tests (8–10). Most commercial PCR tests utilize real-time technology, providing rapid results for an individual target or a limited number of targets plus an internal control in a single reaction. Multiplexing is restricted by the limited number of fluorescent channels available on the PCR platform. Consequently, they are often inadequate for syndromic approaches, for which performing multiple PCRs per sample is neither cost- nor time-efficient. When real-time technology has been applied, for example, in the investigation of sepsis using the Roche SeptiFast assay, cases of IFD have been limited, providing only anecdotal information regarding performance (11, 12).

Recently, a novel approach to genotyping human papillomavirus infection utilized surface-enhanced Raman scattering (SERS) (13). SERS uses sensitive spectroscopic detection to generate analyte-specific fingerprint spectra and is particularly suited to the simultaneous detection of multiple targets (14).

To provide a standardized diagnostic approach, a new SERS spectroscopy platform, RenDx (Renishaw Diagnostics Limited, Glasgow, United Kingdom), has been developed for detecting nucleic acid targets, including semiautomated postamplification processing and multiplex detection using Raman spectroscopy, and it is capable of detecting up to 10 targets per reaction. Using

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Editor: D. J. Diekema
Address correspondence to P. Lewis White, lewis.white@wales.nhs.uk.

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purpose-designed assays, or by modifying existing PCR protocols, SERS detection can differentiate between multiple etiological targets by detecting the presence of oligonucleotides specific for each pathogen. The essential components for a screening assay targeting a low-incidence disease are high sensitivity and concordant negative predictive value to exclude disease. SERS multiplexing capacity and analytical sensitivity have the potential to exceed fluorescent-based technologies, making it ideal for such an application (14).

Using this approach, an assay was developed (RenDx Fungiplex) for diagnosing IA using a generic *Aspergillus* probe, and for diagnosing IC by combining a broad-range *Saccharomycetales* yeast probe with a probe specific for pathogenic species belonging to the diploid arm of the *Candida* clade (*C. albicans*, *C. dubliniensis*, *C. parapsilosis*, and *C. tropicalis*) (15). The assay has the added benefit of differentiating potentially antifungal-resistant species (*Candida glabrata*, *Candida krusei*, and *Aspergillus terreus*) through specific probes. This paper describes the process of analytical and clinical evaluations of the assay when testing multiple sample types, as well as nucleic acid (NA) extraction methods in keeping with EAPCRI recommendations.

MATERIALS AND METHODS

Analytical performance. A summary of the process used to determine analytical performance is shown in Fig. 1.

Determining the limit of detection of PCR-SERS. To evaluate the limit of detection (LOD) of PCR amplification combined with SERS detection, 40 replicates of two concentrations (200 and 20 copies per PCR) and nine replicates of two lower concentrations (2 and 1 copy per PCR) of quantified plasmids containing the 18S or 28S rRNA PCR target, for a range of *Candida* and *Aspergillus* species, respectively, were tested. To determine assay robustness, PCR amplification of the two higher concentrations (200 and 20 copies per reaction) was performed on five separate occasions using four different types of PCR instruments (Bio-Rad MyCycler X2, Bio-Rad T100, Applied Biosystems Veriti, and GRI G-Storm); replicate testing was split equally ($n = 8$) across the platforms (Table 1). At the lower concentrations (2 and 1 copy per reaction), amplification was performed on the Bio-Rad MyCycler only.

Determining the limit of detection of the entire molecular process (extraction–PCR-SERS detection). To investigate the influence of NA extraction on the LOD, various simulated sample types (EDTA whole blood [WB], serum, and plasma) containing clinically relevant concentrations (100 to 10 genome equivalents per ml of sample) of the pertinent fungal pathogens were prepared. A total of 93 simulated samples were tested (Table 2). The WB samples were spiked with quantified fungal organism (*Aspergillus* conidia or *Candida* blastospore), whereas genomic DNA was used for the serum/plasma samples. NA was extracted according to EAPCRI recommendations (5, 6). Three-milliliter samples of WB were extracted using red and white blood cell lysis, followed by mechanical disruption of the fungal cells by bead beating and automated NA purification/precipitation using the tissue kit on the Qiagen EZ1 Advanced XL instrument (Qiagen, United Kingdom). For serum/plasma, NA was extracted from 0.5-ml volumes using the Qiagen EZ1 DSP virus kit on the same platform. All NA was eluted in 50 to 60 μl. PCR amplification was performed using the Applied Biosystems Veriti instrument (Life Technologies, United Kingdom) (Fig. 2).

Determining the range of detection and cross-reactivity. To determine the analytical specificity (detection range/cross-reactivity), a panel of genomic DNA extracted from large quantities ($>10^6$ organisms) of the following fungal genera/species was tested: *Absidia* spp., *Cunninghamella* spp., *Rhizopus* spp., *Fusarium oxysporum*, *Fusarium dimerum*, *Scedosporium apiospermum*, *Scedosporium prolificans*, *Penicillium* spp., *Exophiala* spp., *Epidermophyton floccosum*, *Trichophyton mentagrophytes*, *Trichophyton tonsurans*, *Trichophyton rubrum*, *Microsporum* spp., *Aspergillus fumigatus* ($n = 5$), *A. terreus* ($n = 4$), *A. flavus* ($n = 4$), *Aspergillus niger* ($n = 2$), *Aspergillus glaucus*, *Aspergillus versicolor*, *Aspergillus ustus*, *Aspergillus candidus*, *C. albicans* ($n = 5$), *C. krusei* ($n = 5$), *C. glabrata* ($n = 5$), *C. tropicalis* ($n = 5$), *C. parapsilosis* ($n = 2$), *Candida lusitaniae*, *Candida viswanathii*, *Candida kefyr*, *Saccharomyces cerevisiae*, *Bipolaris* spp., *Scopulariopsis brevicaulis*, *Cladosporium* spp., *Acremonium* spp., and *Blastoschizomyces capitatus*.

PCR amplification and SERS detection. PCR-SERS testing was performed, according to the manufacturer's instructions, using 10 μl of DNA template in a final reaction volume of 50 μl, targeting the 18S rRNA and 28S rRNA genes, and generating an amplicon ranging from 200 to 500 bp, dependent on species. A no-template control (NTC) was included in every PCR run and, for experiments testing simulated samples, quantified plasmids containing the PCR target were included in order to assess PCR efficiency. To monitor for sample inhibition, a quantified amount of plasmid containing a potato gene was added, and a control PCR was performed. Given the low concentrations of fungal DNA in blood samples, the inhibition PCR was performed as a separate reaction to prevent competition for reagents that might occur if both the fungal and control PCR were performed in a single tube. In doing so, any potential reduction in the sensitivity of the Fungiplex assay was avoided. All testing was performed with the user blinded to the original identity of the sample. An overview of the process is shown in Fig. 2. The total process, including extraction, was completed within one working day (8 h), with the PCR amplification-SERS analysis taking approximately 6 h to complete, and a hands-on time of approximately 1 to 2 h, including extraction, was needed. The results (positive, negative, or inhibitory) were interpreted automatically by the instrument, using a direct classical least-squares model of analysis, which requires no manual spectroscopic expertise.

Determination of clinical performance. Fungal PCR is offered as a routine diagnostic test for patients at high risk of developing IFD (1, 17). On completion of the tests, NA extracts and surplus serum/plasma samples are retained for internal quality control and performance assessment purposes. A total of 195 NA extracts from 112 patients were tested by the Fungiplex assay. The patients were a combination of hematology patients ($n = 83$), abdominal surgery patients ($n = 27$), a renal transplant patient, and one patient suffering from chronic granulomatous disease. Forty-four hematology patients received allogeneic stem cell transplantation. The basic patient demographics are shown in Table 3. IFD was defined according to the revised EORTC/MSG criteria, with PCR not used in determining a diagnosis (3). Twenty-eight samples were from 14 patients diagnosed with proven/probable IA, and nine samples were from three cases of possible IA. Twenty-eight samples were from 15 patients with a proven IC diagnosis. One hundred thirty samples were from 80 patients having no evidence of fungal disease (NEF). The study was conducted as a retrospective anonymous case-control study to assess the performance of the Fungiplex assay, with no impact on patient management.

The NA extracts from patients previously tested by fungal PCR were tested by the Fungiplex assay with the user blinded to the previous PCR result and to the diagnosis of IFD (17). NA was extracted from 0.5 ml plasma/serum using the Qiagen EZ1 DSP virus kit, or from 3 ml of WB according to EAPCRI recommendations combined with the Qiagen EZ1 tissue kit, with NA eluted in 50- to 60-μl volumes (5, 6). For all extractions, positive and negative extraction controls were incorporated at the time of extraction to monitor assay performance. On completion of the routine testing, the extracts were stored at −80°C before being tested by the Fungiplex assay. PCR testing was performed on these extracts as described above using the Applied Biosystems Veriti instrument, and an inhibition control was used for every sample. For every run-quantified plasmid, NTCs and simulated samples were included to assess PCR performance. On completion of the testing, the results were independently compared against the original diagnosis to determine assay performance.

Statistical analysis. To determine the validity of the Fungiplex assay results, the positivity rate of samples originating from cases was compared

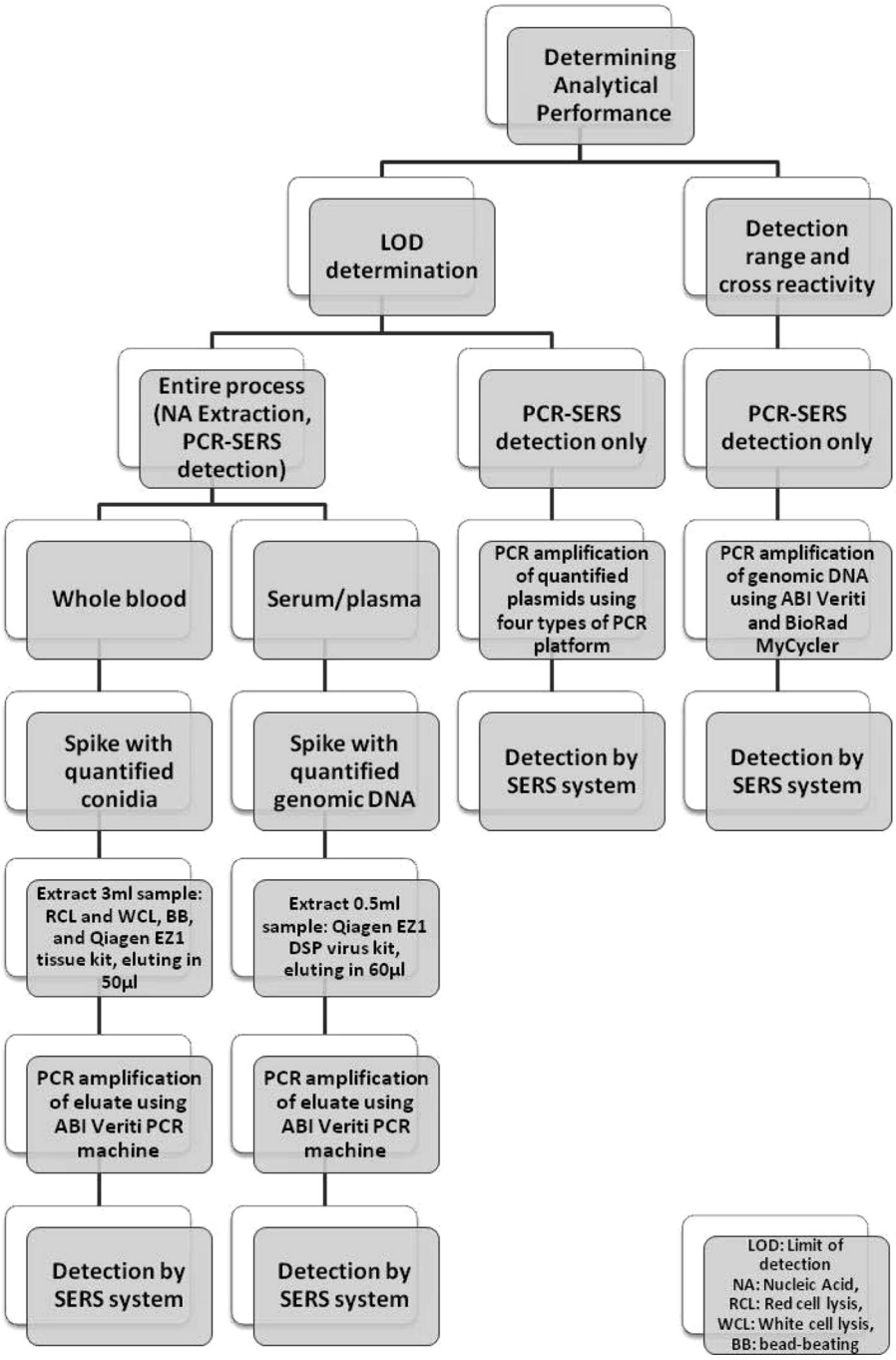

FIG 1 Overview of the analytical process.

to the false-positivity rate of the control samples. To determine the clinical performance (sensitivity, specificity, positive and negative likelihood ratios, and diagnostic odds ratio) of the Fungiplex assay, 2 by 2 tables were constructed, using both proven/probable IFD and proven/probable/possible IFD as true cases and NEF patients as the control population. For all patients, including those with multiple samples tested, only a single positive was required to consider the patient positive. Given the case-control study design and the artificially high prevalence of IFD (28.6%), predictive values were not used. Ninety-five percent confidence intervals were generated for each proportionate value and, where required, P values (Fisher's exact test; $P = 0.05$) were used to determine the significance of the difference between the rates (18). The agreement between the Fungiplex assay and the original real-time PCR results was determined by calculating the kappa statistic and observed agreement (17, 19).

RESULTS

Limit of detection of PCR amplification and SERS detection. The LOD of the PCR-SERS process was determined by identifying the lowest plasmid concentration that achieved a ≥95% reproducibility of detection, and this is summarized in Table 1. When testing a plasmid input of 200 copies per PCR, 11 of the 12 plas-

TABLE 1 Reproducibility of detection of the PCR-SERS process when testing quantified plasmid containing the PCR target

Target	Data for indicated total input copies per PCR							
	200 ($n = 40$)		20		2 ($n = 9$)		1	
	No.	% (95% CI)	No.	% (95% CI)	No.	% (95% CI)	No.	% (95% CI)
C. albicans	40	100 (91.2–100)	38[a]	95.0 (83.5–98.6)	3	33.3 (12.1–64.6)	1	11.1 (2.0–43.5)
C. glabrata	40	100 (91.2–100)	37[b]	92.5 (80.1–97.4)				
C. krusei	40	100 (91.2–100)	32[c]	80.0 (65.2–89.5)				
C. guilliermondii	40	100 (91.2–100)	36[d]	90.0 (77.0–96.0)	5	55.6 (26.7–81.1)	1	11.1 (2.0–43.5)
C. parapsilosis	39[e]	97.5 (87.1–99.6)	38[f]	95.0 (83.5–98.6)	4	44.4 (18.9–73.3)	1	11.1 (2.0–43.5)
C. lusitaniae	40	100 (91.2–100)	36[g]	90.0 (77.0–96.0)	1	11.1 (2.0–43.5)	1	11.1 (2.0–43.5)
C. tropicalis	40	100 (91.2–100)	37[h]	92.5 (80.1–97.4)	4	44.4 (18.9–73.3)	1	11.1 (2.0–43.5)
C. dubliniensis	40	100 (91.2–100)	39[i]	97.5 (87.1–99.6)	4	44.4 (18.9–73.3)	1	11.1 (2.0–43.5)
A. fumigatus[j]	40	100 (91.2–100)	38[k]	95.0 (83.5–98.6)	1	11.1 (2.0–43.5)	1	11.1 (2.0–43.5)
A. terreus	40	100 (91.2–100)	39[l]	97.5 (87.1–99.6)				
A. niger	40	100 (91.2–100)	33[m]	82.5 (68.1–91.3)	5	55.6 (26.7–81.1)	1	11.1 (2.0–43.5)
A. flavus	40	100 (91.2–100)	39[n]	97.5 (87.1–99.6)	3	33.3 (12.1–64.6)	1	11.1 (2.0–43.5)
Overall	479/480	99.8 (98.8–100)	442/480	92.1 (89.3–94.2)	30/81	37.0 (27.3–47.9)	9/81	11.1 (6.0–19.8)

[a] One replicate missed using the Bio-Rad MyCycler, and one replicate missed using the GRI G-Storm.
[b] One replicate missed using the Bio-Rad MyCycler, and two replicates missed using the GRI G-Storm.
[c] One replicate missed using the Bio-Rad MyCycler, two replicates missed using the Bio-Rad T100, and five replicates missed using the GRI G-Storm.
[d] One replicate missed using the Bio-Rad MyCycler, and three replicates missed using the GRI G-Storm.
[e] One replicate missed using the Applied Biosystems Veriti.
[f] One replicate missed using the Bio-Rad MyCycler, and one replicate missed using the Bio-Rad T100.
[g] One replicate missed using the Bio-Rad MyCycler, two replicates missed using the Bio-Rad T100, and one replicate missed using the Applied Biosystems Veriti.
[h] Two replicates missed using the Bio-Rad MyCycler, and one replicate missed using the Applied Biosystems Veriti.
[i] One replicate missed using the Bio-Rad T100.
[j] For comparative purposes, the mean number of copies of the rRNA genes per genome of *A. fumigatus* is 54 (16). Consequently, 200 copies per reaction equates to approximately 4 genomes, whereas 20, 2, and 1 copy equates to <1 genome per reaction. One *A. fumigatus* conidium contains one genome.
[k] Two replicates missed using the Applied Biosystems Veriti.
[l] One replicate missed using the GRI G-Storm.
[m] One replicate missed using the Bio-Rad MyCycler, one replicate missed using the GRI G-Storm, and five replicates replicate missed using the Applied Biosystems Veriti.
[n] One replicate missed using the Bio-Rad MyCycler.

TABLE 2 Reproducibility of detection of the entire molecular process (nucleic acid extraction, PCR amplification, and SERS detection) by testing simulated samples with various concentrations of fungal burden

Target	Load (GE) by sample type at the indicated copy no.					
	Serum/plasma		EDTA whole blood		Combined	
	50–100	10–20	50–100	10–20	50–100	10–20
C. albicans	4/4	4/4	1/1	2/2	5/5	6/6
C. glabrata	4/4	2/4	1/1	2/2	5/5	4/6
C. krusei	3/4	3/4	1/1	2/2	4/5	5/6
C. guilliermondii	2/2	3/3			2/2	3/3
C. parapsilosis	4/4	3/3			4/4	3/3
C. lusitaniae	4/4	3/3			4/4	3/3
C. tropicalis	2/2	2/2			2/2	2/2
C. dubliniensis	2/2	2/2			2/2	2/2
A. fumigatus[a]	1/1	8/9	4/4	4/4	5/5	12/13
A. terreus		2/3		2/2		4/5
A. niger		3/3		1/2		4/5
A. flavus		3/3		2/2		5/5
Overall	26/27	38/43	7/7	15/16	33/34	53/59

[a] For comparative purposes, the mean number of copies of the rRNA genes per genome of *A. fumigatus* is 54 (16). Consequently, 10 genomes per sample equates to 540 copies per sample. When testing a sample with a 10 GE or conidial burden and assuming 100% extraction efficiency, combined with an elution volume of 60 μl and a PCR template input volume of 10 μl, the total input equals 90 copies or 1.6 GE/conidia per reaction. One *A. fumigatus* conidium contains one genome.

mids (eight *Candida* and four *Aspergillus* targets) achieved 100% reproducibility, with one target (*C. parapsilosis*) achieving 97.5% reproducibility. At the lower concentration of 20 input copies per reaction, six targets achieved the prerequisite 95% reproducibility limit, with four targets (*C. glabrata*, *C. guilliermondii*, *C. lusitaniae*, and *C. tropicalis*) achieving >90% reproducibility, and two targets (*C. krusei* and *A. niger*) achieving 80% and 82.5% reproducibility, respectively. The overall reproducibility for all targets at this input was 92.1% (95% confidence interval [CI], 89.3 to 94.2%). The 38 false-negative results were distributed evenly between the different PCR platforms used (Bio-Rad MyCycler, 10; Bio-Rad T100, 6; GRI G-Storm, 13; ABI Veriti, 9). At an input of two plasmid copies per reaction, reproducibility ranged from 1/9 (11.1%; 95% CI, 2.0 to 43.5%) for *A. fumigatus* and *C. lusitaniae*, to 5/9 (55.6%; 95% CI, 26.7 to 81.1%) for *A. niger* and *C. guilliermondii*, with an overall reproducibility of 37.0% (95% CI, 27.3 to 47.9%). At one input copy per reaction, the reproducibility was 11.1% for all targets.

Reproducibility of detection of the entire molecular process. The reproducibility of detection of the entire molecular process (NA extraction–PCR amplification-SERS detection) was determined by testing 93 simulated samples and is summarized in Table 2. At moderate (50 to 100 genomic equivalents [GE] per sample) and low (10 to 20 GE per sample) fungal burdens, the overall reproducibility of detection rates were 97.1% (95% CI, 85.1 to 99.5%) and 89.8% (95% CI, 79.5 to 95.3%), respectively, with no significant difference in performance between the different sam-

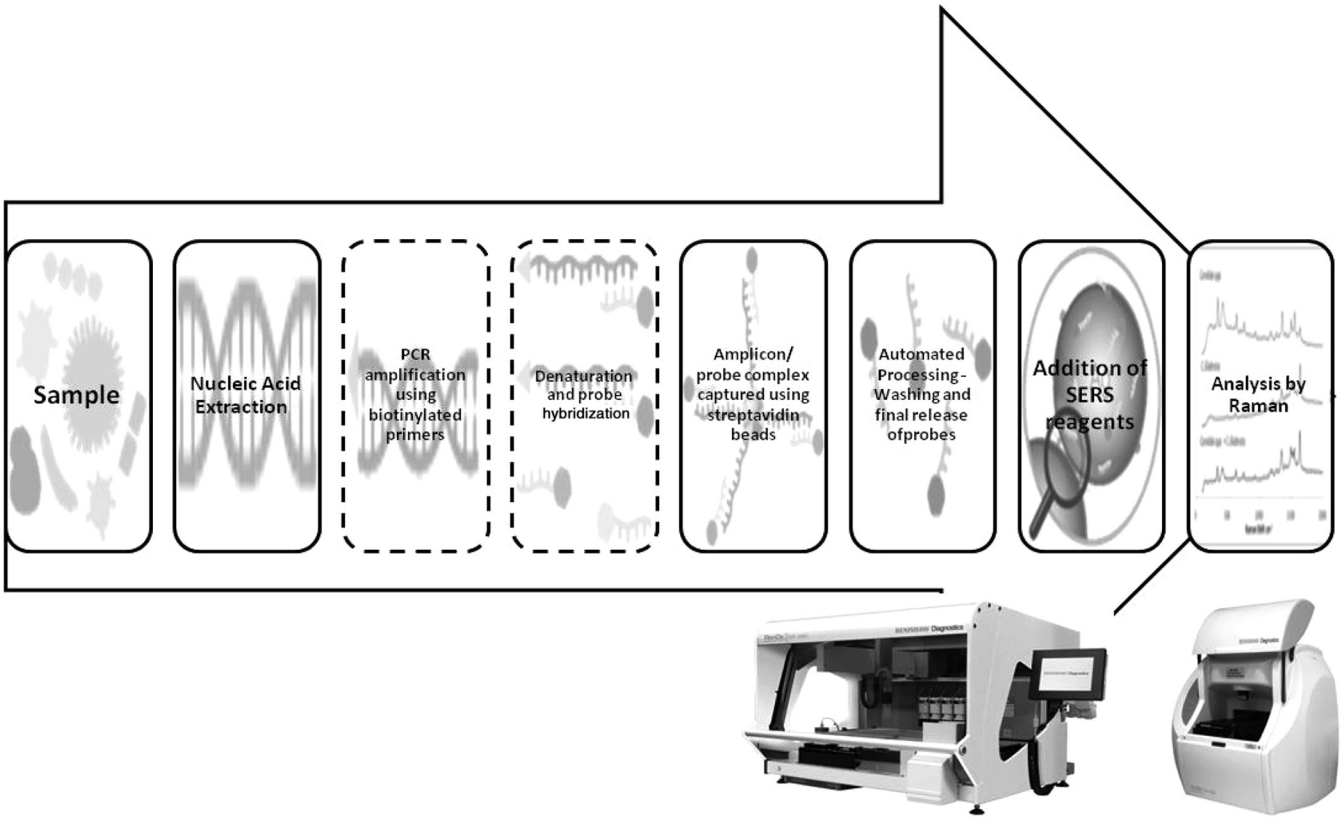

FIG 2 Overview of the Fungiplex assay process. DNA is extracted from the clinical sample according to EAPCRI recommendations. PCR is performed using biotinylated oligonucleotides, after which the PCR products are subsequently denatured. Specific and optimized multiplex SERS probes are then added and, if DNA from a target organism is present, the appropriate probe is hybridized to the biotinylated PCR product. The samples are then transferred to the RenDx SP-1000 for automated processing. Labeled DNA is captured onto streptavidin-coated magnetic beads (via the biotin label), which are then immobilized, allowing the excess and residual probes to be removed by washing. The amplicon-probe complexes are then eluted and transferred to a clean 96-well detection plate, while the magnetic beads and attached DNA are discarded. At the final stage, the SERS detection reagents (silver colloidal suspension) are added and the plate is transferred to the SA-1000 for Raman spectroscopic analysis. The manual steps are in the boxes with dashed lines. The nucleic acid extraction step can be automated; this is dependent on the method used and sample tested. For whole-blood samples, when performed according to EAPCRI recommendations, the process is semiautomated (5).

ple types at either burden level (moderate burden, $P = 1.0$; low burden, $P = 0.2199$).

Analytical specificity: detection range and cross-reactivity. In testing the robustness of the detection range, a total of 44 strains, comprising eight species of both *Candida* and *Aspergillus*, were tested. Five strains of both *C. krusei* and *C. glabrata* were correctly identified by their specific probes. All other species of *Candida* were detected by one or both of the generic probes. Four

TABLE 3 Patient demographics with EORTC/MSG diagnosis of aspergillosis

	Data by fungal infection type (*n*)[a]			
Demographic	Proven/probable IA (14)	Possible IA (3)	IC (15)	No IFD (80)
Male/female ratio	0.75/1	2/1	1.1/1	1.1/1
Mean age (range) (yr)	53.8 (25–72)	69.3 (66–73)	58.8 (27–82)	53.1 (20–84)
Underlying condition (*n*)	AML/MDS (5), ALL (2), AA (2), lymphoma (2), CML/CLL (2), renal transplant (1)	AML/MDS (3)	Abdominal surgery (15)	AML/MDS (43), abdominal surgery (12), ALL (10), lymphoma (6), AA (2), CML/CLL (2), CGD (1), Other (4)
HSCT[b]	Allo (5)	Allo (0)	NA	Allo (39), auto (1)
Fungal disease manifestation	IPA (9), IPA/sinusitis (1), sinusitis (2), IPA/aspergilloma (1), ABPA (1)	IPA (3)	Candidemia (7), peritonitis (6), abdominal abscess (2)	None
No. of extracts tested	28 plasma	9 plasma	15 serum, 13 whole blood	96 plasma, 34 serum

[a] IA, invasive aspergillosis; IC, invasive candidiasis; IFD, invasive fungal disease; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; lymphoma, Hodgkin, non-Hodgkin lymphoma, lymphoprofilerative disorder, and diffuse large B cell lymphoma; AA, aplastic anemia; CML, chronic myeloid leukemia; CLL, chronic lymphoblastic leukemia; Other, other nonspecified hematological malignancy; CGD, chronic granulomatous disease; allo, allogeneic; NA, not applicable; auto, autologous; IPA, invasive pulmonary aspergillosis; ABPA, allergic bronchopulmonary aspergillosis.
[b] HSCT, hematopoietic stem cell transplantation.

strains of *A. terreus* were correctly identified by the specific probe, and six of the seven remaining *Aspergillus* species were detected by the generic probe. *A. ustus*, despite being successfully amplified by PCR, did not generate a positive SERS signal, and the sequencing of the amplicon showed only 83.3% similarity with the generic *Aspergillus* probe.

Potential cross-reactivity was noted for one of the 23 additional fungal species tested, with *F. oxysporum* cross-reacting with both the *Saccharomycetales* probe and the probe specific for pathogenic *Candida* (*C. albicans*, *C. dubliniensis*, *C. parapsilosis*, and *C. tropicalis*). A sequence alignment of the oligonucleotides showed 90.9% and 86.4% similarities with the forward and reverse *Candida* primers, respectively, and 100% and 80.8% similarities with the *Saccharomycetales* and generic *Candida* probes, respectively. Repeat testing of the original DNA confirmed this finding. However, the sequencing of both amplicons provided an identification of *C. parapsilosis*, suggesting sampling error or contamination with *C. parapsilosis* DNA. This cross-reactivity was not seen with other *Fusarium* species tested. *S. cerevisiae* was also detected by the *Saccharomycetales* probe but was not considered cross-reactivity, as it is part of this order. An additional sequence alignment showed that potential cross-reactivity between *Penicillium* species and the *Aspergillus* assay and *Trichosporon* species and the generic *Candida* assay might occur. Upon testing *Penicillium* species, intermittent false positivity was noted, and further investigations are ongoing.

Clinical performance: sample positivity rates. Of the 28 samples originating from patients with IC, 18 generated a positive *Candida* SERS signal (positivity rate, 64.3%; 95% CI, 45.8 to 79.3%). For patients with proven/probable IA, the sample positivity rate was 20/28 (71.4%; 95% CI, 52.9 to 84.8%) compared to 2/9 (22.2%; 95% CI, 6.3 to 54.7%) for patients with possible IA. The overall positivity rate for the samples originating from cases of proven/probable IFD was 38/56 (69.7%; 95% CI, 54.8 to 78.6%), and for proven/probable/possible IFD, it was 40/65 (61.5%; 95% CI, 49.4 to 72.4%).

When testing serum, plasma, and WB samples, the positivity rates were 8/15 (53.3%; 95% CI, 30.1 to 75.2%), 20/28 (71.4%; 95% CI, 52.9 to 84.8%), and 10/13 (76.9%; 95% CI, 49.8 to 91.8%), respectively, for samples originating from cases of proven/probable IFD.

The false-positivity rate (i.e., a positive signal in the samples from NEF patients) was 12/130 (9.2%; 95% CI, 5.4 to 15.4%) and was significantly lower than the true positivity rates (*Candida* difference, 55.1%; 95% CI, 35.6 to 70.6%, $P < 0.0001$; *Aspergillus* difference, 62.2%; 95% CI, 42.7 to 76.1%; $P < 0.0001$; overall difference, 58.6%; 95% CI, 44.2 to 70.0%; $P < 0.0001$), showing a significant association between assay positivity and proven/probable IFD ($P < 0.0001$). False positivity was significantly greater in serum (9/34 [26.5%]) than in plasma (3/96 [3.1%]) (difference, 23.4%; 95% CI, 10.2 to 40.1%; $P = 0.0022$) samples. No WB samples from NEF patients were tested. False positivity was split equally between *Candida* ($n = 6$) and *Aspergillus* ($n = 5$) organisms, with one sample generating a positive signal for both targets.

Clinical performance: concordance between PCR assays. The overall observed agreement between the original real-time PCR result and the retest RenDx result was 80.5% (95% CI, 73.4 to 87.6%), generating a kappa statistic of 0.552, representing moderate agreement between the assays (Table 4). The kappa statistics specifically for *Candida* and *Aspergillus* detection were 0.416 (moderate agreement) and 0.596 (good agreement), respectively. The kappa values specifically for serum and plasma testing were 0.315 (fair agreement) and 0.605 (good agreement), respectively (results not shown). The observed agreement was significantly greater when confirming the results for the samples originating from patients without IFD (117/130 [90.0%]) compared to the results for the samples originating from patients with IFD (40/65 [61.5%]) (difference, 28.5%; 95% CI, 15.9 to 41.3%, $P < 0.0001$). Consequently, 115/124 (92.7%) originally negative real-time PCR results were also negative by the Fungiplex assay, and 42/71 (59.2%) of the originally positive real-time PCR results were confirmed by the Fungiplex assay (difference, 33.5%; 95% CI, 21.4 to 45.7%; $P < 0.0001$).

TABLE 4 Comparison of Fungiplex and original real-time PCR results

Sample subset (total $n = 195$)[a]	No. with real-time positive result	No. with real-time negative result
Fungiplex positive	42	9
Fungiplex negative	29	115
Samples from patients at risk of IA ($n = 133$)		
Fungiplex positive	23	2
Fungiplex negative	18	90
Samples from patients at risk of IC ($n = 62$)		
Fungiplex positive	19	7
Fungiplex negative	11	25

[a] IA: invasive aspergillosis; IC, invasive candidiasis.

Clinical performance parameters. The performance of the RenDx Fungiplex assay is summarized in Table 5. The sensitivity was similar for detecting both *Aspergillus* (85.7%) and *Candida* (80.0%). The specificity was 87.5% and was improved (97.5%; 95% CI, 87.7 to 99.7%) by using a more stringent positivity threshold (≥2 samples required to be RenDx Fungiplex positive), albeit at the expense of sensitivity (31.0%; 95% CI, 13.5 to 55.8%). The sensitivity varied with IC disease manifestation and was higher for cases of candidemia (100%; 95% CI, 64.6 to 100%) than for those from other IC infections, such as peritonitis or abdominal abscesses (62.5%; 95% CI, 30.6 to 86.3%), although the numbers were limited. Sensitivity also varied with sample type and was lowest when testing NA extracted from serum (53.3%; 95% CI, 30.1 to 75.2%), followed by WB (76.9%; 95% CI, 49.7 to 91.8%) and plasma (85.7%; 95% CI, 60.1 to 96.0%).

DISCUSSION

A preliminary clinical evaluation of the RenDx Fungiplex assay shows promising clinical performance, and it is coupled with the benefits of commercial manufacture to ISO13485, requiring a comprehensive quality management system for the design and manufacture of medical devices. The overall sensitivity of 82.8% (95% CI, 58.1 to 94.8%) for detecting cases of both IC and IA was not unduly influenced by the performance of either arm of the assay. The sensitivity for detecting cases of IA (85.7; 95% CI, 48.7 to 98.2%) was comparable to the sensitivities generated by meta-analyses evaluating the performance of *Aspergillus* PCR assays for testing both blood (88%) and bronchoalveolar lavage (BAL) fluid (79%) samples (20, 21). The sensitivity for detecting cases of IC was 80.0% (95% CI, 44.6 to 96.0%) but was 100% (95% CI, 64.6 to 100%) for detecting cases of candidemia, similar to the results

TABLE 5 Clinical performance of the RenDx Fungiplex assay

Parameter[a]	Data by Fungiplex comparison[b]			
	Proven/probable IFD vs NEF ($n = 109$)	Proven/probable/possible IFD vs NEF ($n = 112$)	Proven/probable IA vs NEF and IC ($n = 109$)	IC vs NEF and proven/probable IA ($n = 109$)
Sensitivity (no. detected/total no., % [95% CI])	24/29, 82.8 (58.1–94.8)	26/32, 81.3 (57.9–93.6)	12/14, 85.7 (48.7–98.2)	12/15, 80.0 (44.6–96.0)
Specificity (no. detected/total no., % [95% CI])	70/80, 87.5 (74.7–94.5)	70/80, 87.5 (74.7–94.5)	83/95, 87.4 (74.7–94.5)[c]	84/94, 89.4 (74.7–94.5)
LR positive	6.62	6.50	6.86	6.4
LR negative	0.20	0.21	0.16	0.23
DOR	33.60	30.33	42.00	28.00

[a] CI, confidence interval; LR, likelihood ratio; DOR, diagnostic odds ratio.
[b] IFD, invasive fungal disease; NEF, no evidence of fungal disease; IA, invasive aspergillosis; IC, invasive candidiasis.
[c] Two patients diagnosed with IC were positive for both *Candida* and *Aspergillus* by the Fungiplex assay. These were considered false positives with regard to the *Aspergillus* result.

generated in a meta-analysis of *Candida* PCR, in which the sensitivity fell from 95 to 100% for candidemia to 73% for proven/probable/possible IC (22). When localized disease is suspected and the patient is not fungemic, testing samples specific to the particular site may be beneficial, as biomarker availability in the circulation may be limited.

The specificity was 87.5% (95% CI, 74.7 to 94.5%), comparing favorably with the specificities determined in the various meta-analyses (range, 75% to 94%) (20–22). False positivity was associated equally with the two pathogens (*Candida*, $n = 6$; *Aspergillus*, $n = 5$) but more with serum testing ($P = 0.0022$). However, serum testing was associated with post-abdominal surgery patients, and it may be the patient population rather than the sample type causing this false positivity. Biomarker false positivity has been noted in this population, hypothetically associated with translocation of flora from the gastrointestinal (GI) tract. In the study by León et al. (23), combining two biomarker assays improved sensitivity but reduced specificity compared to that for (1,3)-β-D-glucan testing. In half of the false-positive cases generated by the Fungiplex assay, other biomarker assays were positive, perhaps representing undiagnosed disease. Specificity was increased by using a multiple-positive threshold (≥2 samples positive), with only two cases generating two false-positive results, although this significantly compromises sensitivity (Table 5).

The sensitivity as determined by retrospective analysis is likely too low for the assay to be used as a screening test. However, the Fungiplex assay performance may be further enhanced by a prospective evaluation. In the initial retrospective case-control study of the MycAssay *Aspergillus* assay, the sensitivity for testing serum samples was 60 to 70% but increased to 100% on prospective testing (9, 10). The potential improvement in sensitivity through prospective evaluation maybe sufficient for the Fungiplex assay to be used as a screening assay to exclude IFD. Long-term storage of samples and the effect of freeze-thawing may result in the degradation of DNA. If DNA was already at a concentration close to the limit of reproducible real-time PCR detection when originally tested, degradation might result in false negativity on retesting. This might also explain why the concordance between the initial positive PCR results from the cases was lower than that for the negative PCR results from the controls. Unfortunately, the limited original NA eluate and availability of stored samples prevented further retesting. Consequently, in this study, it is not possible to determine if SERS detection provides superior sensitivity over real-time technology, as proposed elsewhere (14). Being a case-control study, it is not designed to determine the clinical utility of the Fungiplex assay, and a prospective cohort study is required to provide accurate clinical validity and utility.

A major strength of the evaluation is that it is in accordance with the EAPCRI recommendations for the extraction of fungal DNA from whole blood and serum, allowing a standardized approach for both sample processing and, through commercial manufacture of the Fungiplex assay, PCR detection. The commercial kits are quality controlled, and the methodology is standardized in line with other commercial fungal biomarker tests (galactomannan [GM] enzyme-linked immunosorbent assay [ELISA] and [1,3]-β-D-glucan). Analytical performance should therefore be robust across study centers, providing identical performance without local optimization and expertise required to develop an in-house test. Several commercial assays are available, although most target either an individual fungal pathogen (MycAssay *Aspergillus* and T2MR *Candida*) or, despite targeting both *Candida* and *Aspergillus*, have received validation for one pathogen only (Roche SeptiFast) (8–12). The Fungiplex assay has received validation for both targets and has the added benefit of being an open platform with broad multiplexing capacity (currently up to 10 targets), allowing the user to develop bespoke assays targeting other fungal pathogens, as directed by local epidemiology.

In conclusion, this is the first evaluation of the novel, commercially manufactured, and quality controlled RenDx Fungiplex assay. Initial evidence suggests that the assay provides promising clinical performance, although further clinical validity and utility need to be determined through a prospective evaluation. By providing a commercial PCR option, in combination with EAPCRI recommendations, PCR testing for IFD can be standardized.

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REFERENCES

1. **Barnes RA, Stocking K, Bowden S, Poynton MH, White PL.** 2013. Prevention and diagnosis of invasive fungal disease in high-risk patients within an integrative care pathway. J. Infect. **67:**206–214. http://dx.doi.org/10.1016/j.jinf.2013.04.020.
2. **Morrissey CO, Chen SC, Sorrell TC, Milliken S, Bardy PG, Bradstock KF, Szer J, Halliday CL, Gilroy NM, Moore J, Schwarer AP, Guy S, Bajel A, Tramontana AR, Spelman T, Slavin MA, Australasian Leukaemia Lymphoma Group, Australia and New Zealand Mycology Interest Group.** 2013. Galactomannan and PCR versus culture and histology for directing use of antifungal treatment for invasive aspergillosis in high-risk haematology patients: a randomised controlled trial. Lancet Infect. Dis. **13:**519–528. http://dx.doi.org/10.1016/S1473-3099(13)70076-8.
3. **De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, Pappas PG, Maertens J, Lortholary O, Kauffman CA, Denning DW, Patterson TF, Maschmeyer G, Bille J, Dismukes WE, Herbrecht R, Hope WW, Kibbler CC, Kullberg BJ, Marr KA, Muñoz P, Odds FC, Perfect JR, Restrepo A, Ruhnke M, Segal BH, Sobel JD, Sorrell TC, Viscoli C, Wingard JR, Zaoutis T, Bennett JE, European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group, National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group.** 2008. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin. Infect. Dis. **46:**1813–1821. http://dx.doi.org/10.1086/588660.
4. **Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT.** 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. **55:**611–622. http://dx.doi.org/10.1373/clinchem.2008.112797.
5. **White PL, Bretagne S, Klingspor L, Melchers WJ, McCulloch E, Schulz B, Finnstrom N, Mengoli C, Barnes RA, Donnelly JP, Loeffler J, European *Aspergillus* PCR Initiative.** 2010. *Aspergillus* PCR: one step closer to standardization. J. Clin. Microbiol. **48:**1231–1240. http://dx.doi.org/10.1128/JCM.01767-09.
6. **White PL, Mengoli C, Bretagne S, Cuenca-Estrella M, Finnstrom N, Klingspor L, Melchers WJ, McCulloch E, Barnes RA, Donnelly JP, Loeffler J, European *Aspergillus* PCR Initiative (EAPCRI).** 2011. Evaluation of *Aspergillus* PCR protocols for testing serum specimens. J. Clin. Microbiol. **49:**3842–3848. http://dx.doi.org/10.1128/JCM.05316-11.
7. **Lyon GM, Abdul-Ali D, Loeffler J, White PL, Wickes B, Herrera ML, Alexander BD, Baden LR, Clancy C, Denning D, Nguyen MH, Suqrue M, Wheat LJ, Wingard JR, Donnelly JP, Barnes RA, Patterson TF, Caliendo AM, AsTeC, IAAM, EAPCRI Investigators.** 2013. Development and evaluation of a calibrator material for nucleic acid-based assays for diagnosing aspergillosis. J. Clin. Microbiol. **51:**2403–2405. http://dx.doi.org/10.1128/JCM.00744-13.
8. **Neely LA, Audeh M, Phung NA, Min M, Suchocki A, Plourde D, Blanco M, Demas V, Skewis LR, Anagnostou T, Coleman JJ, Wellman P, Mylonakis E, Lowery TJ.** 2013. T2 magnetic resonance enables nanoparticle-mediated rapid detection of candidemia in whole blood. Sci. Transl. Med. **5:**182ra54. http://dx.doi.org/10.1126/scitranslmed.3005377.
9. **Torelli R, Sanguinetti M, Moody A, Pagano L, Caira M, De Carolis E, Fuso L, De Pascale G, Bello G, Antonelli M, Fadda G, Posteraro B.** 2011. Diagnosis of invasive aspergillosis by a commercial real-time PCR assay for *Aspergillus* DNA in bronchoalveolar lavage fluid samples from high-risk patients compared to a galactomannan enzyme immunoassay. J. Clin. Microbiol. **49:**4273–4278. http://dx.doi.org/10.1128/JCM.05026-11.
10. **White PL, Perry MD, Moody A, Follett SA, Morgan G, Barnes RA.** 2011. Evaluation of analytical and preliminary clinical performance of Myconostica MycAssay *Aspergillus* when testing serum specimens for diagnosis of invasive aspergillosis. J. Clin. Microbiol. **49:**2169–2174. http://dx.doi.org/10.1128/JCM.00101-11.
11. **Lamoth F, Jaton K, Prod'hom G, Senn L, Bille J, Calandra T, Marchetti O.** 2010. Multiplex blood PCR in combination with blood cultures for improvement of microbiological documentation of infection in febrile neutropenia. J. Clin. Microbiol. **48:**3510–3516. http://dx.doi.org/10.1128/JCM.00147-10.
12. **Steinmann J, Buer J, Rath PM, Paul A, Saner F.** 2009. Invasive aspergillosis in two liver transplant recipients: diagnosis by SeptiFast. Transpl. Infect. Dis. **11:**175–178. http://dx.doi.org/10.1111/j.1399-3062.2009.00367.x.
13. **Hibbitts SJ, White PL, Green J, McNay G, Graham D, Stevenson R.** 2014. Human papilloma virus genotyping by surface-enhanced Raman scattering. Anal. Methods **6:**1288–1290. http://dx.doi.org/10.1039/c4ay00155a.
14. **Faulds K, Barbagallo RP, Keer JT, Smith WE, Graham D.** 2004. SERRS as a more sensitive technique for the detection of labelled oligonucleotides compared to fluorescence. Analyst **129:**567–568. http://dx.doi.org/10.1039/b406423b.
15. **Butler G, Rasmussen MD, Lin MF, Santos MAS, Sakthikumar S, Munro CA, Rheinbay E, Grabherr M, Forche A, Reedy JL, Agrafioti I, Arnaud MB, Bates S, Brown AJ, Brunke S, Costanzo MC, Fitzpatrick DA, de Groot PW, Harris D, Hoyer LL, Hube B, Klis FM, Kodira C, Lennard N, Logue ME, Martin R, Neiman AM, Nikolaou E, Quail MA, Quinn J, Santos MC, Schmitzberger FF, Sherlock G, Shah P, Silverstein KA, Skrzypek MS, Soll D, Staggs R, Stansfield I, Stumpf MP, Sudbery PE, Srikantha T, Zeng Q, Berman J, Berriman M, Heitman J, Gow NA, Lorenz MC, Birren BW, Kellis M, Cuomo CA.** 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. Nature **459:**657–662. http://dx.doi.org/10.1038/nature08064.
16. **Herrera ML, Vallor AC, Gelfond JA, Patterson TF, Wickes BL.** 2009. Strain dependent variation in 18S ribosomal DNA copy numbers in *Aspergillus fumigatus*. J. Clin. Microbiol. **47:**1325–1332. http://dx.doi.org/10.1128/JCM.02073-08.
17. **White PL, Linton CJ, Perry MD, Johnson EM, Barnes RA.** 2006. The evolution and evaluation of a whole blood polymerase chain reaction assay for the detection of invasive aspergillosis in hematology patients in a routine clinical setting. Clin. Infect. Dis. **42:**479–486. http://dx.doi.org/10.1086/499949.
18. **Newcombe RG.** 1998. Improved confidence intervals for the difference between binomial proportions based on paired data. Stat. Med. **17:**2635–2650.
19. **Viera AJ, Garrett JM.** 2005. Understanding interobserver agreement: the kappa statistic. Fam. Med. **37:**360–363.
20. **Mengoli C, Cruciani M, Barnes RA, Loeffler J, Donnelly JP.** 2009. Use of PCR for diagnosis of invasive aspergillosis: systematic review and meta-analysis. Lancet Infect. Dis. **9:**89–96. http://dx.doi.org/10.1016/S1473-3099(09)70019-2.
21. **Tuon FF.** 2007. A systematic literature review on the diagnosis of invasive aspergillosis using polymerase chain reaction (PCR) from bronchoalveolar lavage clinical samples. Rev. Iberoam. Micol. **24:**89–94. http://dx.doi.org/10.1016/S1130-1406(07)70020-7.
22. **Avni T, Leibovici L, Paul M.** 2011. PCR diagnosis of invasive candidiasis: systematic review and meta-analysis. J. Clin. Microbiol. **49:**665–670. http://dx.doi.org/10.1128/JCM.01602-10.
23. **León C, Ruiz-Santana S, Saavedra P, Castro C, Ubeda A, Loza A, Martín-Mazuelos E, Blanco A, Jerez V, Ballús J, Alvarez-Rocha L, Utande-Vázquez A, Fariñas O.** 2012. Value of β-D-glucan and *Candida albicans* germ tube antibody for discriminating between *Candida* colonization and invasive candidiasis in patients with severe abdominal conditions. Intensive Care Med. **38:**1315–1325. http://dx.doi.org/10.1007/s00134-012-2616-y.