Development of a Multiplex PCR Platform for the Rapid Detection of Bacteria, Antibiotic Resistance, and Candida in Human Blood Samples

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The diagnosis of bloodstream infections (BSIs) still relies on blood culture (BC), but low turnaround times may hinder the early initiation of an appropriate antimicrobial therapy, thus increasing the risk of infection-related death. We describe a direct and rapid multiplex PCR-based assay capable of detecting and identifying 16 bacterial and four Candida species, as well as three antibiotic-resistance determinants, in uncultured samples. Using whole-blood samples spiked with microorganisms at low densities, we found that the MicrobScan assay had a mean limit of detection of 15.1 ± 3.3 CFU of bacteria/Candida per ml of blood. When applied to positive BC samples, the assay allowed the sensitive and specific detection of BSI pathogens, including blaKPC-, mecA-, or vanA/vanB-positive bacteria. We evaluated the assay using prospectively collected blood samples from patients with suspected BSI. The sensitivity and specificity were 86.4 and 97.0%, respectively, among patients with positive BCs for the microorganisms targeted by the assay or patients fulfilling the criteria for infection. The mean times to positive or negative assay results were 5.3 ± 0.2 and 5.1 ± 0.1 h, respectively. Fifteen of 20 patients with MicrobScan assay-positive/BC-negative samples were receiving antimicrobial therapy. In conclusion, the MicrobScan assay is well suited to complement current diagnostic methods for BSIs.

Keywords: multiplex PCR, bacteria, antibiotic resistance genes, Candida, blood samples, MicrobScan assay

INTRODUCTION

Early diagnosis of bloodstream infections (BSIs), including those caused by bacteria and fungi, is central to reducing drastic infection-related consequences, such as significant risks of morbidity and mortality (Goto and Al-Hasan, 2013; Andes et al., 2016; Seymour et al., 2017). Global estimates show that in high-income countries, the incidence rates for sepsis and severe sepsis cases were 437 and 270 per 100,000 person-years, respectively, with hospital mortality rates of 17.0% for sepsis and 26.0% for severe sepsis during the last decade (Fleischmann et al., 2016). In the United States, the
incidence of patients receiving treatment for septic shock rose from 12.8 to 18.6 cases per 1,000 hospitalizations over 10 years, while mortality declined from 54.9 to 50.7% (Kadri et al., 2017). In a recent study, a delay of 50.0 min for blood culture (BC) or 125.0 min for antibiotic therapy within the 3-h Surviving Sepsis Campaign guideline increased the risk of death in patients with severe sepsis and septic shock (Pruinelli et al., 2018).

The reference standard for the microbiological diagnosis of a BSI is still an automated BC system (Baron et al., 2013). However, despite the good analytical sensitivity of the BC method (limit of detection [LOD], 1–10 CFU/ml) (Yagupsky and Nolte, 1990; Pfeiffer et al., 2011), slow turnaround time (TAT) is the main limitation (Lamy et al., 2016), which necessitates the aggressive empirical usage of antimicrobial agents (Banerjee et al., 2016; Timbrook et al., 2017). Furthermore, delays in optimal antimicrobial therapy may drive poor health care quality as well as increase antimicrobial resistance (Zasowski et al., 2016; Whiles et al., 2017). Early initiation and modification of appropriate antimicrobial therapy depends on the availability of diagnostic methods with rapid TATs, e.g., within 6–8 h of septic patient presentation (Ginn et al., 2017). Molecular detection methods applied to positive BCs or direct blood samples show promise in accelerating microbial identification and predicting antimicrobial susceptibility (Dubourg and Raoult, 2016; Peker et al., 2018). It is imperative that diagnostic tests are tailored to the clinical problem at hand to maximize the cost-effectiveness of clinical decision-making (Pliakos et al., 2018).

Commercially available methods performed directly on whole-blood samples include nucleic amplification-based methods and, only recently, T2 magnetic resonance-based methods (Peker et al., 2018). Alternatively, a plasma metagenomics-sequencing assay (Karius, Redwood City, CA) generates sequences of circulating microbial cell-free DNA to enable the non-invasive diagnosis of infectious diseases (Blauwkamp et al., 2019). Compared to post-culture detection (e.g., by FilmArray® Blood Culture Identification Panel; bioMérieux, Marcy-l’Étoile, France), direct detection (from blood) eludes the “test method-related” shortcomings that may lead to false-negative diagnoses because of slow or not growing microorganisms and cases when the patient has already received antimicrobials (Farrell et al., 2013). For example, the LightCycler® SeptiFast (Roche Molecular System, Switzerland; www.molecular.roche.com) is a Conformité Européenne (CE)-marked multiplex real-time PCR (qPCR) assay that simultaneously detects and identifies DNA from 19 bacterial and 6 fungal species. In a subsequent run, the test assesses the presence of methicillin resistance in *Staphylococcus aureus*-positive samples (Peker et al., 2018). Importantly, the recent finding of significant rates of false positive (up to 20% or higher) and false negative (up to 14%) results has limited the utility of the LightCycler® SeptiFast as a standalone test (Ginn et al., 2017). However, multiplex qPCR assays may offer several advantages (e.g., high-throughput and quantification) (Bustin et al., 2009), making them well suited to the clinical setting (Farrell et al., 2013).

We developed a multiplex qPCR-based detection molecular assay (hereafter designated the MicrobScan assay) that can process a wide range of microorganism densities (CFU/ml) directly from blood samples (Figure 1). Specifically, we tested the general capabilities of this assay using low-density spikes of

![MicrobScan assay workflow](image-url)
phenotypically characterized and culture-quantified bacterial or Candida species in blood. By processing samples from a positive blood culture bottle (PBCB) without measuring the density, we show that the assay allows the sensitive and specific identification of 20 frequently encountered microbial pathogens and relevant antimicrobial resistance genes. Finally, we evaluated the clinical performance of the assay with blinded whole-blood samples from patients with suspected BSI.

MATERIALS AND METHODS

Description of the MicrobScan Assay

We designed the MicrobScan assay to run on a fully automated SepsisScan instrument (Nurex S.r.l., Sassari, Italy), where a robot can handle 24 samples in parallel. The assay requires minimal hands-on time (e.g., typically <15 min) from putting the sample into a microwell strip to loading the strip onto the BioRad CFX Thermal Cycler (BioRad, Hercules, CA, USA), which is a PCR system for gene amplification. We installed the SepsisScan instrument at the clinical microbiology laboratory of the Fondazione Policlinico Universitario A. Gemelli IRCCS of Rome (Italy), where it automatically completed all assay steps in prefilled wells starting from 100 µl of the original sample (i.e., EDTA whole blood). Specifically, the procedural steps that are automated by the SepsisScan instrument are as follows: detergent lysis of blood cells; concentration of cellular debris and microbial cells; extraction of microbial DNA for use as a template in the multiplex qPCRs (see below); and finally bleach decontamination of all liquids on the SepsisScan instrument rack. In particular, DNA extraction is achieved through the sequential action of lytic enzymes (lysozyme, lyticase, chitinase, and proteinase K) and strong detergents (including sodium dodecyl sulfate) and the subsequent purification by binding the extracted DNA to magnetic beads. After alternate guanidine thiocyanate/ethanol washing, microbial DNA is eluted from the beads in 150-µl elution buffer (Tris-EDTA plus 1% Triton X-100), concentrated in a microfluidic extraction cartridge, and immediately dispensed in an 8-well strip containing dried PCR reagents. The entire process enables users to avoid any contamination by hemoglobin, iron, and any blood-cell PCR inhibitor. The assay allows for the simultaneous detection and identification of 20 bacterial/Candida species and 3 antibiotic resistance-associated genes. We chose the 20 microorganisms, with or without any antibiotic-resistance determinant, as molecular targets for the MicrobScan assay based on their prevalence in clinical laboratory BC assays. The MicrobScan assay has recently received CE marking.

Growth and Quantification of Microorganisms

The microorganisms used in this study were well-characterized bacteria and Candida strains obtained from the ATCC® (Manassas, VA, USA) and/or clinical isolates obtained from the aforementioned clinical microbiology laboratory. In addition to clinical isolates (N = 333), the ATCC® strains included Acinetobacter baumannii (19606™), Aspergillus fumigatus (13073™), Bacteroides fragilis (25285™), Burkholderia cepacia (25416™), Candida albicans (10231™), Candida glabrata (2001™), Candida krusei (6258™), Candida orthopsilosis (20503™), Candida parapsilosis (7330™), Candida tropicalis (13803™), Corynebacterium striatum (BA-1293™), Corynebacterium ramosum (25582™), Cryptococcus neoformans (32045™), Enterobacter aerogenes (13048™), Enterobacter cloacae (13047™), Escherichia coli (25922™, BAA-2340™), Enterococcus faecalis (29212™, 51299™), Enterococcus faecium (55593™, 700221™), Haemophilus influenzae (9006™), Klebsiella oxytoca (700324™), Klebsiella ozaenae (25926™), Klebsiella pneumoniae (13883™, BAA-1705™), Legionella pneumophila (33152™), Moraxella catarrhalis (25238™), Neisseria meningitidis (13098™), Pseudomonas aeruginosa (27853™), Proteus mirabilis (7002™), Proteus vulgaris (13315™), Serratia marcescens (8100™), S. aureus (29213™, 4300™), Staphylococcus epidermidis (12228™, 35984™), Staphylococcus haemolyticus (29970™), Staphylococcus hominis (700586™), Stenotrophomonas maltophilia (13637™), Streptococcus agalactiae (12403™), Streptococcus pneumoniae (6305™), Streptococcus pyogenes (12344™), Streptococcus salivarius (25975™). We grew all the microbes on appropriate agar media to harvest single colonies, which were diluted 10-fold at various concentrations in sterile phosphate-buffered saline and then quantified by culture onto the media to estimate the CFU per milliliter.

Primer and Taqman Probe Selection

We detected 20 clinically relevant microorganisms using species- or group-specific target genes as follows: bap gene for A. baumannii (De Gregorio et al., 2015); leuB gene for B. fragilis (Papararaskevas et al., 2013); 5.8S rRNA gene for C. albicans; internal transcribed spacer 1 (ITS1), 5.8S rRNA, and ITS2 genes for C. glabrata and C. krusei (Metwally et al., 2007); 18S rRNA gene for Candida spp.; omp35 gene for E. aerogenes (van der Zee et al., 2016); apbC gene (formerly referred to as unknown gene) for E. cloacae complex (van der Zee et al., 2016); 16S rRNA gene for E. coli (van den Brand et al., 2014); groEL gene for E. faecalis and E. faecium (Fukumoto et al., 2015); rhaA-rhaD operon for Klebsiella spp. (van den Brand et al., 2014); phoE gene for P. aeruginosa (van den Brand et al., 2014); hns gene for P. mirabilis (van der Zee et al., 2016); gyrB gene for S. marcescens (van den Brand et al., 2014); spa gene for S. aureus; tuf gene for Staphylococcus spp. (van den Brand et al., 2014); lytA gene for S. pneumoniae (Gadsby et al., 2015); sdaB gene for S. pyogenes (Fukumoto et al., 2015); and the 16S rRNA gene for Streptococcus species. Additionally, we detected three antibiotic-resistance genes, including the blagPC carbapenemase gene (Zheng et al., 2013), the mecA methicillin resistance gene (Thomas et al., 2007), and the vanA/vanB vancomycin resistance genes. Finally, we used the human RNase gene as an internal control for sample DNA. Unless otherwise specified, we selected primer pairs and Taqman probes from conserved regions of the respective specific sequences using Beacon Designer™ software (Premier Biosoft, Palo Alto, CA, USA). To test for any potential cross-reactivity, we performed in silico specificity studies to compare the oligonucleotide sequences to all known genetic sequences.
Multiplex qPCR System
The MicrobScan assay consists of seven parallel qPCRs in seven reaction wells, including 1 duplex, 3 triplex, and 3 quadruplex reaction wells, which target the 20 microorganisms and 3 antibiotic-resistance determinants per sample. Another well serves as an RNase gene-based control. We optimized the qPCRs by testing different concentrations of each primer pair and Taqman probe, as well as buffer components, and we calculated PCR efficiencies from the slopes of standard curves that were run in triplicate (data not shown). The final 30-μl qPCR mixture contained deoxynucleoside triphosphates (including dUTP) and uracil-N-glycosylase. We used concentrations of 400 nM for all primer pairs and 150 nM for all probes. We carried out qPCRs using the aforementioned BioRad CFX Thermal Cycler using the following thermal cycling conditions: an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 94°C for 15 s and 60°C for 30 s. We ran positive (DNA mixtures of all the targets) and negative (sterile water) PCR controls in qPCRs for each test sample batch. We analyzed qPCR data using proprietary software (Nurex S.r.l.) designed ad hoc to generate a quantification cycle (Cq) (formerly referred to as threshold cycle [Ct]) (Bustin et al., 2009) value for each of the targets. Based on our experiments with spike-in controls, a Cq value of 40 was set up as a cutoff value for positivity, and we considered a sample as positive if the Cq value was ≤40. We based the differentiation between S. aureus and one Staphylococcus species, between S. pneumoniae/S. pyogenes and one Streptococcus species and between C. albicans and one Candida species on the results of two corresponding qPCRs, respectively. If both qPCRs were positive, we identified the microorganism(s) as belonging to the specific species, whereas if only the species-group (i.e., Staphylococcus spp., Streptococcus spp., or Candida spp.) relative qPCR was positive we identified the microorganism(s) as belonging to the specific genus group.

Analytical Studies
We determined the LOD for the assay on isolates from 18 species of bacteria and 6 species of Candida, including species representative of Staphylococcus spp. other than S. aureus, Streptococcus spp. other than S. pneumoniae and S. pyogenes, and Candida spp., using quantified spiked samples. We prepared the sample matrix for spiking experiments using fresh microbial suspensions to the appropriate density (CFU/ml) to obtain replicate samples for testing. Then, we performed a Probit analysis, which transforms the proportions of positive results detected into a “probability unit” (or “probit”), using the Analyze-it statistical analysis software addendum for Microsoft Excel (Analyze-it Software Ltd., Leeds, UK). Briefly, we used several dilutions of the microbial suspensions (CFU/ml) to prepare eight replicates for each density. We plotted the probit (y-axis) against the logarithm of the density (x-axis), and we calculated the 95% LOD value, which was the density of each microbial species in a sample that yielded positive detection 95% of the time. We assessed the assay reproducibility (also known as variability) using the same replicate experiments performed to determine LOD, and we calculated the mean Cq values for both target and internal control, along with the coefficient of variation (% CV) as a variance measure. Finally, we performed a carryover assessment by testing adjacent negative and highly positive samples. Positives consisted of samples spiked with 10⁶ or 10⁷ CFU/ml of either blakPC-positive Klebsiella pneumoniae [KPC], vanA/vanB-positive Enterococcus faecium/Enterococcus faecalis [VRE], or both mecA-positive S. aureus [MRSA] and Candida albicans.

Spiked and Clinical Blood Samples
We collected healthy volunteer blood samples, and we made final dilutions of each of the aforementioned microorganisms for generating blood samples spiked with cultured bacteria/Candida species at a range of densities (10⁻⁷ to 10⁷ CFU/ml). These samples served to either optimize the amount of blood for clinical testing or determine the LOD for each MicrobScan assay target (as specified above), as well as to exclude any between-target cross-reactivity. We used a set of 87 non-consecutive fresh positive BCs (BacT/ALERT® FA, FN, and PF Plus bottles, bioMérieux, Marcy l’Étoile, France) obtained during routine clinical microbiology at the Fondazione Policlinico Universitario A. Gemelli IRCCS of Rome (Italy). These samples were used to determine the analytical sensitivity and specificity of the MicrobScan assay regardless of the density (CFU/ml) of microorganisms cultured from the blood. Additionally, we collected 229 EDTA whole blood (2–3 ml) samples drawn from prospectively consenting patients from September 2017 to August 2018 at the aforementioned Fondazione Policlinico Universitario A. Gemelli IRCCS, using the same venipuncture used for the BC samples. These samples were from patients for whom physicians ordered BCs, as a part of the standard of care per hospital protocol, due to the clinical suspicion of a BSI. Each whole blood sample was used to assess the clinical sensitivity and specificity of the MicroScan assay; all samples were refrigerated at 4°C within 30 min of collection and then frozen until analysis.

Microbiological Methods
We performed culture-based identification and antimicrobial susceptibility testing of BSI isolates according to standard laboratory procedures (Baron et al., 2013). Briefly, we incubated all patient BC bottles that arrived at the clinical microbiology laboratory in the BacT/Alert® Virtuo® system (bioMérieux) at 37°C for up to 5 days or until they signaled a positive result. At the time bottles gave a positive signal or at the end of their incubation period, we performed subcultures of the BC medium on blood (for bacteria) or Sabouraud dextrose (for yeast) agar plates to assess true-positive and true-negative detections. We identified the bacteria and Candida isolates grown in the blood by the MALDI BioTyper® system (Bruker Daltonics, Bremen, Germany) following the manufacturer's instructions. Then, we tested bacterial isolates for antimicrobial susceptibility using the VITEK 2® automated system (bioMérieux), and we used the 2017 EUCAST standards to interpret antimicrobial susceptibility testing (AST) results (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.1_Breakpoint_Tables.pdf). For bacterial isolates found to be phenotypically resistant to extended-spectrum beta-lactamases, oxacillin, or vancomycin, we performed PCR amplification of the isolate DNA as previously described (Fiori et al., 2016) to
confirm the presence of $\text{bla}_{KPC}$, $mecA$, or $\text{van}A/\text{van}B$ genes, respectively, in these isolates.

**Data Analysis**

We initially estimated the diagnostic performance of the MicrobScan assay by calculating sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) compared to the BC method. To resolve discrepancies between the MicrobScan assay and BC results, we used a synopsis of clinical and/or laboratory data to identify the “true-infection” cases equal to those that yielded false-positive MicrobScan assay results. These data included microbiological results of BCs from other BSI episodes and of cultures from other sites of infection that were available within $\pm$ 7 days from the index positive BC and consultation with the treating physician. Based on this composite diagnostic criterion, we calculated the overall agreement between the MicrobScan assay and BC results. Finally, we compared the time to positive detection by the MicrobScan assay with the time to culture-based species identification (i.e., the time to BC positivity plus the time to result by standard identification). Additionally, we used antimicrobial therapy information to assess the potential influence of prior antimicrobial use on the MicrobScan assay performance as well as the potential impact of the MicrobScan assay results on the antimicrobial treatment changes (i.e., escalation or de-escalation) (Murri et al., 2018) in patient subgroups. We performed statistical analyses using IBM SPSS software version 24.0 (Armonk, NY, USA), and we compared continuous variables (expressed as the means $\pm$ SD) using Student’s t-test and categorical variables (expressed as counts and percentages) using Fisher’s exact test. Differences were considered to be statistically significant at $P < 0.05$.

**RESULTS**

**Analytical Sensitivity**

We used qPCR primers designed to amplify unique sequences in species-specific bacteria or $Candida$ spp. and in $\text{bla}_{KPC}$, $mecA$, or $\text{van}A/\text{van}B$ antibiotic resistance genes representing 23 molecular targets in the MicrobScan assay (Table 1). We combined multiple primers for the simultaneous recognition of two, three, or four of the 23 targets in seven single-reaction (one duplex, three triplex, and three quadruplex) wells. This in silico-generated format allowed multiple fluorescently labeled TaqMan probes to react with each of the corresponding PCR amplicons in the well. First, we measured the LOD of the MicrobScan assay with all targeted microorganisms (including KPC, MRSA, and VRE) in both sterile phosphate-buffered saline and whole blood matrices by means of singleplex qPCRs. All microorganism LODs were similar between these two matrices, and the PCR efficiency was $>97\%$ for each microorganism (see Table S1). The assay had a mean (SD) LOD of 15.6 $\pm$ 3.1 CFU for bacteria (13.3 $\pm$ 1.6 for gram-negative and 18.5 $\pm$ 1.9 for gram-positive) or $Candida$ (16.5 $\pm$ 2.0) per ml of blood, and all targeted microorganisms yielded LODs between 10 and 21 CFU/ml (see Table S1). All the targets amplified with a Cq value between 22 and 38. The calibration curve indicated a linear dynamic range of $10^6$-$10^1$ CFU/ml. The primer pairs and probes in multiple reactions did not significantly affect the sensitivity compared with that of singleplex reactions. The assay variation during the detection and identification of 3 $\times$ LOD spikes of the test microorganisms ranged from $+0.4$ to $+1.1$ Cq values. Next, we characterized the repeatability of the MicrobScan assay by testing microorganisms at either 3 $\times$ LOD or 10 $\times$ LOD concentrations. We spiked all the microorganisms into whole blood, and the observed % CV among replicates was $<30\%$, whereas the mean Cq varied significantly depending on the 3 $\times$ LOD or 10 $\times$ LOD concentration. Finally, among the independent possibilities to observe carryover events based on the assay configuration, we did not observe any carryover event.

**Analytical Specificity**

We evaluated the specificity of the multiplex qPCRs for the identification of 20 microbes at the species level (including $Klebsiella$ spp., $Staphylococcus$ spp., $Streptococcus$ spp., and $Candida$ spp.) and for the detection of three (carbapenem, methicillin, or vancomycin) resistance determinants. As shown in Table S2, each of the six species-specific qPCRs correctly detected DNA from the corresponding microbial species with 100% accuracy; the PCRs did not detect DNA from reference ATCC strains or clinical isolates belonging to the different species targeted or non-targeted by the MicrobScan assay. The latter species comprised $A. fumigatus$, $B. cepacia$, $C. striatum$, $C. ramosum$, $C. neoformans$, $H. influenzae$, $L. pneumophila$, $M. catarrhalis$, $N. meningitidis$, $P. vulgaris$, and $S. maltophilia$. Each resistance determinant-specific qPCR showed positive results only with $K. pneumoniae$ (ATCC® BAA-1705™), $S. aureus$ (ATCC® 43300™), $E. faecium$ (ATCC® 700221™), and $E. faecalis$ (ATCC® 51299™) strains, which harbored the $\text{bla}_{KPC}$, $mecA$, $\text{van}A$, or $\text{van}B$ genes, respectively. We did not observe amplification with human DNA except in the sample control (RNase-specific) well. Based on these findings, the MicrobScan assay developed here had 100% specificity.

**Direct Detection of Microbial Pathogens From PBCB Samples**

In the routine molecular diagnosis of BSIIs, we and other clinical microbiologists currently use PBCBs, as testing these samples shortens the time to microbial species identification (Fiori et al., 2014). We applied the MicrobScan assay to 87 samples consisting of aliquots of PBCBs, which yielded isolates in mono- ($N = 61$) and polymicrobial ($N = 26$) growth (Tables 2, 3). For monomicrobial cultures, we obtained 100% detection matching the species identified by culture, except for one sample positive for $B. cepacia$ that the assay was not designed to detect (Table 2). For the polymicrobial cultures, the presence of multiple microorganisms did not prevent the MicrobScan assay from detecting and identifying at least one of the components of the mixed culture. Except for two samples positive for $C. striatum$ and $C. ramosum$ that the assay was not designed to detect, 18 (75.0%) of 24 samples had the second and two of 18 had the third microorganism detected by the MicrobScan assay (Table 3). While differences in the Cq for single microorganism detection reflected differences in the
### TABLE 1 | Sequences of primers and probes used in the multiplex real-time PCRs.

| Species* | Target gene | Primer name | Primer sequence (5′−3′) | Probe name | Probe sequence (5′-3′)† | Amplicon size (bp) | Reference |
|----------|-------------|-------------|--------------------------|------------|---------------------------|-------------------|-----------|
| **GRAM-NEGATIVE SPECIES** | | | | | | | |
| Acinetobacter baumannii | bap | Aba-f | CGCCTGACGATCAAATCTG | Aba-Pro | Cy5-AGCACCTGCTGACACCCTCCACCAA-BHQ2 | 205 | De Gregorio et al., 2015 |
| Bacteroides fragilis | leuB | Baf-f | CCTTGAACAGTTGTTGATGATAAAGC | Baf-Pro | FAM-TGTGCTTGCTCCAGTCGCTCATTG | 135 | Papapanouskas et al., 2013 |
| Enterobacter aerogenes | omp35 | Ena-f | CCACGCTTTCAATCTTGTCA | Ena-Pro | TqRd-CGGTGCGCGTACGTTTCTGGTCA | 74 | van der Zee et al., 2016 |
| Enterobacter cloacae complex | apbC | Enc-f | ACAAGGAGTGGGGATGACGTAGTCGTTTATG | Enc-Pro | FAM-CAATCAGGGCAATATGAACG-BHQ1 | 65 | van der Zee et al., 2016 |
| Escherichia coli | 16S rRNA | Eco-f | CATGCCGCGTGTATGAAAGA | Eco-Pro | FAM-TATTAACTTTACTCCCTTCCCGCTGAA-BHQ1 | 96 | van den Brand et al., 2014 |
| Klebsiella spp. | rhaA–rhaD operon | Kle-f | AACCACGCGTCCGATA | Kle-Pro | HEX-CGACAACCGCAAGGAAGCCGA-BHQ1 | 107 | van den Brand et al., 2014 |
| Proteus mirabilis | hns | Pmi-f | TGGTACAGCTCAGATGCTGTTT | Pmi-Pro | TqRd-CGGTGCGCGTACGTTTCTGGTCA | 71 | van den Brand et al., 2014 |
| Pseudomonas aeruginosa | phzE | Pae-f | GCCGAGGTCATGGAATTC | Pae-Pro | HEX-CGACAACCGCAAGGAAGCCGA-BHQ1 | 89 | van den Brand et al., 2014 |
| Serratia marcescens | gyrB | Sma-f | GACCGTGAAGACCACTTCCATT | Sma-Pro | FAM-AGCTRAGGCTGTTACCTCTTC- | 125 | van den Brand et al., 2014 |
| **GRAM-POSITIVE SPECIES** | | | | | | | |
| Enterococcus faecalis | groESL | Efs-f | TATGCACACCGGTTCACATT | Efs-Pro | Cy5-AACACAAATGCGGGAAACAGACGTCG- | 77 | Fukumoto et al., 2015 |
| Enterococcus faecium | groESL | Efm-f | GTTCAAGATCCTGTTGCTGTTT | Efm-Pro | TqRd-AAGAAAGAATTCCTGCAATACTCG- | 74 | Fukumoto et al., 2015 |
| Staphylococcus aureus | spa | Sau-f | CACGCCACGGCAGCTGCTG | Sau-Pro | FAM-AAAAGCTCAAGATTACAAAGACTG- | 101 | This study |
| Staphylococcus tuf spp. | Staf-f | Sta-f | CACACCACGCAAGTGATYGTGCTG | Sta-Pro | HEX-AACAGGCCGTGTT AACGCGCAAAATCAACGTCG- | 222 | van den Brand et al., 2014 |
| Streptococcus pneumoniae | tlyA | Spn-f | AACGAAATCTGAGATGAGGAGCA | Spn-Pro | HEX-TGGCGAAACACGGCTTGTACAGGAG- | 74 | Gadsby et al., 2015 |
| Streptococcus pyogenes | sdaB | Spy-f | GCCGCCGCAAGCAGCTGCTG | Spy-Pro | TqRd-CGGTGCGCGTACGTTTCTGGTCA | 73 | Fukumoto et al., 2015 |
| Streptococcus spp. 16S rRNA | Str-f | Str-f | ATGTCWCTAAGAAGAAGG | Str-Pro | FAM-CTCACCTTTCCAACACTACCAGCG-BHQ1 | 117 | This study |
| **FUNGAL SPECIES** | | | | | | | |
| Candida albicans | 5.8S rRNA | Calb-f | GGTTCGTTAAGAAGAAGCAGGA | Calb-Pro | Cy5-TTACOCCCGCGCAAGACTTGTG | 109 | This study |
| Candida krusei | IT31, 5.8S rRNA, ITS2 | Ckru-f | CCGTTTGAGGCCGCTATTCC | Ckru-Pro | HEX-A0CTGGCGCGCAAGAATCTAGACTTTT- | 219 | Metwally et al., 2007 |
| Candida glabrata | IT31, 5.8S rRNA, ITS2 | Cgba-f | CCGTTTGAGGCCGCTATTCC | Cgba-Pro | FAM-TAGTGGTTTACACCTGGTGTG- | 229 | Metwally et al., 2007 |
| Candida spp. 18S rRNA | Can-f | Can-f | TGGTCGTTAAGAAGAAGCAGGA | Can-Pro | TqRd-AACCTACTAAATAGTGCTGCTAGCCAT | 159 | This study |
| **ANTIMICROBIAL-RESISTANT SPECIES** | | | | | | | |
| Carbapenem-resistant | blaPC | Kpc-f | CDCACAATGTAAGAAGCAGGA | Kpc-Pro | Cy5-CCACTGTCGAGCTCATTCAAGAG- | 187 | Zheng et al., 2013 |

(Continued)
TABLE 1 | Continued

| Species* | Target gene | Primer name | Primer sequence (5′–3′) | Probe name | Probe sequence (5′-3′)† | Amplicon size (bp) | Reference |
|----------|-------------|-------------|-------------------------|------------|-------------------------|-------------------|-----------|
| Methicillin-resistant | mecA | Mec-f | AAAGAACCCTCTGCTCAACCAAGT | Mec-Pro | HEXOCGAATTACACTTCCAGGGTTCACT-BHQ1 | 88 | Thomas et al., 2007 |
| Vancomycin-resistant | vanA | VanA-f | TTCTAAATGAAAGGCTGTT | VanA-Pro | FAM-TACGCCACACGCGCTCACA-BHQ1 | 82 | This study |
| | vanB | VanB-f | GATAGAAAGAGGACGAGGAT | VanB-Pro | FAM-CGAGCGCGCTCAGCAG-BHQ1 | 109 | This study |

†Duplex, triplex, or quadruplex reactions were optimized to include Taqman probes, together with each relative primer (forward [f] and reverse [r]) pair, in single wells, which allowed the detection of the DNA from two, three, or four of the 23 listed molecular targets. The best combinations were Staphylococcus aureus and Staphylococcus spp.; blaKPC, mecA, and vanA/vanB; Klebsiella aerogenes, Enterobacter cloacae complex, and Klebsiella spp.; Enterococcus faecalis, Enterococcus faecium, and Streptococcus spp.; Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa, and Proteus mirabilis; Bacteroides fragilis, Serratia marcescens, Streptococcus pyogenes, and Streptococcus pneumoniae; Candida albicans, Candida glabrata, Candida krusei, and Candida spp. As a result, the MicrobScan assay consisted of eight distinct wells (seven with multiplex organism-specific reactions and one with a human RNase-coding gene-based control) per sample. Including Klebsiella pneumoniae, Klebsiella oxytoca, and Klebsiella ozaenae (Klebsiella spp.); S. aureus, Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus haemolyticus, Staphylococcus lugdunensis, and Staphylococcus warneri (Staphylococcus spp.); Streptococcus agalactiae, Streptococcus anginosus, Streptococcus galactolyticus, Streptococcus gordonii, Streptococcus oralis, Streptococcus mitis, Streptococcus parasanguinis, Streptococcus pneumoniae, and Streptococcus pyogenes (Streptococcus spp.); and Candida albicans, Candida parapsilosis, Candida orthopsilosis, and Candida tropicalis (Candida spp.).

From Blood Samples

Direct Detection of Microbial Pathogens From Blood Samples

We conducted a clinical evaluation of the MicroScan assay using whole-blood samples from patients with a suspected BSI who were hospitalized at the Fondazione Policlinico Universitario A. Gemelli IRCCS of Rome, Italy, during September 2017–August 2018. To evaluate the diagnostic...
TABLE 3 | Results for 26 positive blood culture broth (PBCB) samples with polymicrobial growth tested by the MicrobScan assay.

| Species identified | Microbial concentration (CFU/ml) | Positive detection for indicated species | Qc for single detection |
|--------------------|---------------------------------|----------------------------------------|------------------------|
|                    | Species 1 | Species 2 | Species 3 | Species 1 | Species 2 | Species 3 |
| A. baumannii/P. aeruginosa | $1.3 \times 10^9$ | $1.1 \times 10^7$ | – | A. baumannii | 17.89 | – | – |
| A. baumannii/P. aeruginosa | $2.0 \times 10^9$ | $1.0 \times 10^6$ | – | A. baumannii | 17.02 | – | – |
| A. baumannii/S. epidermidis | $1.2 \times 10^8$ | $1.2 \times 10^6$ | – | A. baumannii/Staphylococcus spp. | 17.11 | 17.21 | – |
| A. baumannii/S. epidermidis | $1.7 \times 10^8$ | $1.1 \times 10^6$ | – | A. baumannii/Staphylococcus spp. | 17.50 | 17.98 | – |
| C. albicans/K. oxytoca/E. cloacae | $3.0 \times 10^9$ | $1.5 \times 10^6$ | $1.2 \times 10^8$ | C. albicans/Klebsiella spp./E. cloacae complex | 18.46 | 22.42 | 23.71 |
| C. albicans/S. epidermidis | $1.2 \times 10^9$ | $1.2 \times 10^6$ | – | C. albicans/Staphylococcus spp. | 17.95 | 22.11 | – |
| C. parapsilosis/S. capitis | $1.4 \times 10^9$ | $1.2 \times 10^6$ | – | Candida spp./Staphylococcus spp. | 18.94 | 19.31 | – |
| E. faecalis/A. baumannii | $1.3 \times 10^9$ | $3.0 \times 10^6$ | – | E. faecalis/A. baumannii | 16.40 | 22.90 | – |
| E. faecalis/C. albicans | $1.2 \times 10^8$ | $1.2 \times 10^6$ | – | E. faecalis/C. albicans | 17.97 | 21.20 | – |
| E. faecalis/S. aureus | $1.3 \times 10^9$ | $1.2 \times 10^6$ | – | E. faecalis/S. aureus | 14.88 | 17.02 | – |
| E. faecium/K. oxytoca | $1.3 \times 10^9$ | $1.5 \times 10^6$ | – | E. faecium/Klebsiella spp. | 17.94 | 20.63 | – |
| E. faecium/S. aureus/S. capitis | $1.7 \times 10^9$ | $1.9 \times 10^6$ | $1.2 \times 10^8$ | E. faecium/S. aureus/Staphylococcus spp. | 14.60 | 17.29 | 20.34 |
| E. coli/K. pneumoniae/P. aeruginosa | $1.4 \times 10^9$ | $1.6 \times 10^7$ | $1.2 \times 10^8$ | Escherichia coli/Klebsiella spp. | 16.28 | 20.77 | – |
| K. pneumoniae/E. cloacae | $2.1 \times 10^9$ | $1.4 \times 10^8$ | – | Klebsiella spp. | 17.32 | – | – |
| K. pneumoniae/E. coli | $3.0 \times 10^9$ | $1.7 \times 10^6$ | – | Klebsiella spp. | 18.74 | – | – |
| K. pneumoniae/P. aeruginosa | $2.1 \times 10^9$ | $1.9 \times 10^7$ | – | Klebsiella spp./P. aeruginosa | 18.25 | 19.22 | – |
| K. pneumoniae/S. hominis | $2.1 \times 10^9$ | $1.5 \times 10^7$ | – | Klebsiella spp./Staphylococcus spp. | 18.66 | 19.02 | – |
| P. mirabilis/S. aureus | $1.3 \times 10^9$ | $1.0 \times 10^7$ | – | P. mirabilis/S. aureus | 18.07 | 20.27 | – |
| P. aeruginosa/C. striatum* | $1.1 \times 10^9$ | $2.0 \times 10^6$ | – | P. aeruginosa | 18.04 | – | – |
| S. aureus/P. aeruginosa | $2.8 \times 10^8$ | $1.7 \times 10^6$ | – | S. aureus/P. aeruginosa | 21.15 | 21.35 | – |
| S. aureus/S. salivarius | $1.3 \times 10^9$ | $2.5 \times 10^6$ | – | S. aureus/Streptococcus spp. | 18.56 | 23.38 | – |
| S. aureus/S. anginosus | $1.3 \times 10^9$ | $1.8 \times 10^7$ | – | S. aureus/Streptococcus spp. | 20.24 | 21.28 | – |
| S. epidermidis/A. baumannii | $1.5 \times 10^9$ | $1.2 \times 10^6$ | – | Staphylococcus spp. | 17.59 | – | – |
| S. epidermidis/S. aureus | $1.7 \times 10^8$ | $2.0 \times 10^7$ | – | Staphylococcus spp./S. aureus | 19.65 | 20.83 | – |
| S. hominis/S. mitis | $1.3 \times 10^9$ | $1.2 \times 10^6$ | – | Staphylococcus spp. | 17.72 | – | – |
| S. anginosus/C. ramosum* | $1.4 \times 10^8$ | $1.5 \times 10^6$ | – | Streptococcus spp. | 17.46 | – | – |

*Not included in the target species panel.

accuracy of the assay, we compared the MicroScan assay results with those of the BCs simultaneously performed as a standard diagnostic method (Table 4). Of the 229 samples tested, four samples were positive in culture for microorganisms not targeted by the MicroScan assay (i.e., Morganella morganii, N. meningitidis, Bacteroides thetaiotaomicron, and Listeria monocytogenes). Overall, sensitivity and specificity were 72.1 and 86.6%, respectively. The remaining 225 samples (133 from adult patients and 92 from pediatric patients) yielded 56 organism detections (52 single and 4 multiple) by the assay, whereas BCs yielded 39 (35 monomicrobial and 4 polymicrobial). The polymicrobial BCs consisted of three cultures with two microorganisms and one culture with three microorganisms. The MicroScan assay matched 31 of the 39 positive detections obtained by culture (79.5% agreement) and detected an additional 25 microorganisms in culture-negative samples (15 from pediatric patients and 10 from adult patients) (Table 4). The MicroScan assay-positive, culture-negative detections regarded species such as E. coli (N = 8), S. aureus (N = 6), and S. pneumoniae (N = 4) in most cases, or species such as C. glabrata (N = 1) and E. aerogenes (N = 1). In addition with meca-positive microorganism detection (4 S. aureus and 2 S. epidermidis), the results from the MicroScan assay were concordant with the results obtained by the laboratory reference method. To support the MicroScan assay-positive, culture-negative detection results, we used “clinical indications” of infection, which consisted of detecting the same microorganisms and identifying them as causal pathogens in concomitant analyses of sterile fluids other than blood, respiratory tract fluids or aspirates, and/or urinary tract samples from the same patients. By applying this criterion, the sensitivity and specificity increased to 86.4 and 97.0%, respectively, suggesting that the discordant results for 20 MicroScan assay-positive/BC-negative samples (13 from pediatric patients and 7 from adult patients) were true positives (Table 4). Notably, 11 (84.6%) of 13 and 4 (57.1%) of 7, accounting for a total of 75.0% (15/20), of the patients were receiving antimicrobial therapy. Overall, 13 MicroScan assay results remained discordant, five of which represented overdetection (2 E. coli, 1 E. faecalis, 1 C. albicans, and 1 C. glabrata) and eight missed detections (2 E. faecalis, 1 K. pneumoniae, 1 S. aureus, 1 S. parasanguinis, and 3 Staphylococcus species other than S. aureus). In addition
to *S. parasanguinis* and one *E. faecalis* grown in pediatric bottles, the missed microorganisms included three coagulase-negative staphylococci, which were likely contaminants, and one *E. faecalis* (vancomycin susceptible), which was detected after 47.19 h of incubation and for which the clinical relevance could not be determined from the patient's clinical course. Two other microorganisms, one *K. pneumoniae* (carbapenem susceptible) and one *S. aureus* (methicillin susceptible), represented missed detections that were not explainable as contaminants or not clinically relevant. Finally, the mean (±SD) times to positive results by the MicrobScan assay and the BC method (including species identification) were 5.3 ± 0.2 h and 17.8 ± 12.1 h for bacterial species and 5.3 ± 0.2 h and 22.7 ± 10.1 h for *Candida* species, respectively (*P* < 0.001, for all comparisons). The mean (±SD) times to negative results by the MicrobScan assay and the BC method were 5.1 ± 0.1 h and 120.0 ± 0.0 h, respectively (*P* < 0.001).

### Clinical Value Assessment

We assessed the MicrobScan assay from a strictly clinical standpoint by reviewing the records of the 31 BSI patients with positive detections by both the MicrobScan assay and the BC method. Based on the BC results, the physicians decided to change the antimicrobial therapy initiated at the BSI onset in 20 (64.5%) patients [mean (±SD) time to change, 21.3 ± 13.2 h]. Five patients had their antimicrobial therapy changed the same day (9.8 h ± 5.0 h), and 15 had their antimicrobial therapy changed on the subsequent days (25.1 ± 12.9 h) of BC collection. The initial antimicrobial therapy was escalated in 12 patients and de-escalated in eight patients. In the remaining 11 (35.5%) patients, the initial antimicrobial therapy was unchanged. Based on the MicrobScan assay results, the physicians could have escalated or de-escalated the antimicrobial therapy initiated at the BSI onset in the 20 patients with a mean (±SD) time of 11.6 ± 5.2 h. Furthermore, we assessed the impact of prior antimicrobial use on the MicrobScan assay performance in comparison with the BC method for all 225 cases (88 with and 137 without antimicrobials) studied. The subgroup analysis of the BC results showed that prior antimicrobial use was significantly associated with culture-negative but MicrobScan-positive cases compared with cases in which both culture and MicrobScan results were negative [72.0% (18/25) and 37.3% (60/161), respectively; *P* < 0.002]. Consistently, the subgroup analysis of the MicrobScan results showed that a previous antimicrobial use was significantly associated with MicrobScan-positive but culture-negative cases compared with cases in which both culture and MicrobScan results were positive [72.0% (18/25) and 29.0% (9/31), *P* < 0.003].

### DISCUSSION

We show that the MicrobScan assay is capable of reliably identifying bacterial and fungal (*Candida*) pathogens directly in whole-blood samples from patients with a suspected BSI. The assay combines automated sample preparation with multiplex qPCR for selected targets within single-reaction wells, and detects 16 bacteria and 4 fungi as well as 3 antibiotic resistance determinants in ∼5 h. Interestingly, in 31 (79.5%) of the 39 culture-documented BSI cases with concordant positive MicrobScan assay results, 20 patients could have benefited from timely appropriate antimicrobial therapy. In these patients, physicians changed the initial antimicrobial treatment at 21.3 ± 13.2 h following the notification of a BC result. These changes could have occurred 9.7 ± 8.0 h earlier in the case of a MicrobScan assay result notification. Nevertheless, assay failures occurred in eight of 39 (20.5%) samples, which is of concern. We hypothesized possible reasons for this lack of success, such as the uneven distribution of bacterial/*Candida* species within clinical samples or the errors relating to the sensitivity and specificity of qPCRs. Clinically appropriate management of sepsis and septic shock includes performing BCs before initiating antimicrobial therapy, with at least two BC pairs (using both aerobic and anaerobic bottles) collected per BSI episode (Rhodes et al., 2017). Among the eight cases with MicrobScan assay-negative results, we detected microorganisms (all in monomicrobial growth) in only one of the BC pairs obtained from the patients. Considering the clinical significance of four of these organisms questionable, the rate of false-negative results by the assay decreased to 10.2% (4/39), which is of less concern.

We sought to rule out any impairment in the accurate quantification of multiple targets in a single reaction by demonstrating that the assay efficiency and the LOD were the same as when the reactions ran in a singleplex manner. This issue is particularly crucial when amplifying targets of low relative abundance together with targets of high relative abundance (Bustin et al., 2009). We tested the MicrobScan assay on polymicrobial PBCB samples before blindly using it directly on whole-blood samples. Expectedly, the qPCR detection efficiency
was good in all 24 of the evaluable 26 polymicrobial PBCBs. Two PBCBs grew, as a second microorganism, species (i.e., C. striatum and C. ramosum) not included in the target species panel of the MicrobScan assay. Failure to detect the second microorganism occurred in six samples and, surprisingly, two of undetected microorganisms were P. aeruginosa. This might be attributed to quantitative differences between the two microorganisms present in the same PBCB sample, especially when the two microorganisms had to be detected in the same reaction. Nevertheless, it is improbable that such high concentrations of qPCR template represent the microbial density levels in infected patient’s blood samples before their “amplification” in culture, as in PBCBs. The microbial density in blood is generally very low, particularly for cases of invasive candidiasis that encompass candidemia and deep-seated candidiasis (Clancy and Nguyen, 2013). Indeed, the LOD for BCs is comparable to that of PCR-based methods, at least in invasive Candida infections (Arvanitis et al., 2014).

Using BCs as a comparator may not be appropriate due to limitations including sensitivity. We know that a number of factors can influence the diagnostic accuracy of BCs, and antimicrobial therapy prior to blood sampling is one such factor (among others) (Lamy et al., 2018). However, PCR-based detection of bacteria and fungi in culture-negative clinical samples, especially obtained from patients on antimicrobial therapy, continues to create a clinical interpretation challenge (Farrell et al., 2013). MicroScan assay-positive/culture-negative results may promote the inappropriate antimicrobial treatment of patients who are unlikely to have bacteremia or candidemia, which would inadvertently lead to unintended consequences for institutional efforts to optimize antimicrobial prescription practices (Murri et al., 2018). Among the 225 patients studied, 88 (39.1%) were under antimicrobial treatment at the time a blood sample was collected for testing with the MicroScan assay. We showed that prior antimicrobial exposure did not adversely affect the performance of the MicroScan assay. Eighteen (23.1%) of 78 patients with a negative BC result and nine (90.0%) of 10 patients with a positive BC result had positive detection results in the MicroScan assay.

We performed an in-depth culture-based analysis of the 25 patient cases where the MicroScan assay identified additional microorganisms. We found that 20 of 25 microorganisms were isolated and identified as causal pathogens from the primary cultures of clinical specimens obtained from the same patients almost concomitantly with the blood samples for MicroScan assay testing. In all 20 cases, the microorganism was the same, thus reflecting the source of infection, and interestingly, 15 (75.0%) of the 20 patients were receiving antimicrobial therapy. More interestingly, the MicroScan assay mirrors what seen with the T2 magnetic resonance-based method, from which it differs technologically (T2 Biosystems, Lexington, Massachusetts, USA; www.t2biosystems.com). A recent multicenter study by Nguyen et al. (2019) showed that 13.0% of the patients studied had BC and T2Bacteria results positive for the six T2Bacteria-targeted bacterial species. Among negative BCs with a positive T2Bacteria result, 60.0% of them were associated with probable or possible BSIs (Nguyen et al., 2019). In our previous evaluation of the T2Bacteria Panel (De Angelis et al., 2018), we found 89.0% sensitivity and 98.0% specificity among patients with positive BCs for the 6 bacterial species or fulfilling the criteria for infection (named CI, but similar to those specified above). Consistent with the present study, T2 Bacteria-positive/BC-negative results were significantly more likely to occur among patients receiving antimicrobial therapy (P < 0.001). Taken together, these findings show that the MicroScan assay may be particularly worth using in conjunction with cultures and during antimicrobial therapy.

In summary, the MicroScan assay has the potential to rapidly exclude and identify diverse bacterial and Candida BSIs, including cases that the standard BCs may not detect. The spectrum of molecular targets is restricted to common infecting pathogens and antimicrobial resistance-associated genes. However, we noticed that the MicroScan assay targets cover ~92% of the BSIs diagnosed in our clinical microbiology laboratory (Fiori et al., 2016), which yet excludes clinically relevant species such as N. meningitidis or H. influenzae. Theoretically, small laboratories without experience in molecular detection platforms and techniques may be suitable users. In practice, it is mandatory that clinical microbiologists interpret the MicroScan assay results in the context of clinical and other laboratory findings, as with current culture-based identifications. Thus, we believe that large laboratories can better supply both expertise and additional information. Carefully designed clinical studies are therefore necessary to establish the role that the MicroScan assay may play in the future microbiology laboratory.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed by the Ethics Committee of the Fondazione Policlinico Universitario A. Gemelli IRCCS of Rome (Italy), which approved the study protocol (Approval No. 0023266/17) and required informed consent be obtained from all participants and/or their legal guardian(s). All the methods described in the study were in accordance with the Declaration of Helsinki and national and international standards. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FL performed experiments for the clinical validation of the MicroScan assay. BP, FT, and MS designed the study and planned the experiments. GD and GM collected clinical samples and performed the analysis of the data. FM, FC, AP, and PF performed experiments for the creation and establishment of the MicroScan assay. TS and MS supervised the study. BP wrote the
first draft of the article and prepared the figures and tables. All authors wrote and revised the manuscript and approved the final version of the manuscript.

ACKNOWLEDGMENTS

The Horizon 2020—PON 2014/2020 grants to FT, MS, and PF (All partners in the project Nuovo dispositivo per la diagnostica microbiologica rapida nella sepsi) supported this study. We are grateful to Patrizia Posteraro for critical reading of the manuscript. We thank the American Journal Experts staff for English language editing.

SUPPLEMENTARY MATERIAL

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00389/full#supplementary-material

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**Conflict of Interest:** FM and FC are employed by the company Nurex S.r.l.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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