Rapid Intrinsic Fluorescence Method for Direct Identification of Pathogens in Blood Cultures

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ABSTRACT A positive blood culture is a critical result that requires prompt identification of the causative agent. This article describes a simple method to identify microorganisms from positive blood culture broth within the time taken to perform a Gram stain (<20 min). The method is based on intrinsic fluorescence spectroscopy (IFS) of whole cells and required development of a selective lysis buffer, aqueous density cushion, optical microcentrifuge tube, and reference database. A total of 1,121 monomicrobial-positive broth samples from 751 strains were analyzed to build a database representing 37 of the most commonly encountered species in bloodstream infections or present as contaminants. A multistage algorithm correctly classified 99.6% of unknown samples to the Gram level, 99.3% to the family level, and 96.5% to the species level. There were no incorrect results given at the Gram or family classification levels, while 0.8% of results were discordant at the species level. In 8/9 incorrect species results, the misidentified isolate was assigned to a species of the same genus. This unique combination of selective lysis, density centrifugation, and IFS can rapidly identify the most common microbial species present in positive blood cultures. Faster identification of the etiologic agent may benefit the clinical management of sepsis. Further evaluation is now warranted to determine the performance of the method using clinical blood culture specimens.

IMPORTANCE Physicians often require the identity of the infective agent in order to make life-saving adjustments to empirical therapy or to switch to less expensive and/or more targeted antimicrobials. However, standard identification procedures take up to 2 days after a blood culture is signaled positive, and even most rapid molecular techniques take several hours to provide a result. Other techniques are faster (e.g., matrix-assisted laser desorption ionization—time of flight [MALDI-TOF] mass spectrometry) but require time-consuming manual processing steps and expensive equipment. There remains a clear need for a simple, inexpensive method to rapidly identify microorganisms directly from positive blood cultures. The promising new method described in this research article can identify microorganisms in minutes by optical spectroscopy, thus permitting the lab to simultaneously report the presence of a positive blood culture and the organism’s identity.

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Bacteremia and fungemia are potentially life-threatening medical conditions that require aggressive management. Typically, broad-spectrum multidrug therapy is initiated based on clinical symptoms, patient demographics, and clinical guidelines or antimicrobial stewardship programs. Yet despite the best intentions, empirical therapy is not universally effective, and a delay in receiving appropriate therapy is associated with increased mortality (1, 2), particularly for patients in septic shock (3).

Gram staining of a positive blood culture provides the first laboratory evidence of a possible bloodstream infection and influences the selection of antimicrobial therapy more than receipt of full antimicrobial susceptibility testing (AST) results (4). Additionally, prompt reporting of Gram stain results has been shown to reduce patient mortality (5). However, the ability to quickly identify the etiologic agent to the species level would also help establish the clinical relevance of the organism, enable empirical therapy to be more specifically tailored (targeted, safer, or less expensive agents), and reduce the risk of contributing to the development of resistance against broad-spectrum antibiotics, all central tenets of antibiotic stewardship programs (6). For example, early identification of an isolate as “Pseudomonas aeruginosa” rather than a “Gram-negative rod” may reduce the indiscriminate
use of carbapenems and lower (7) or at least prevent further increase in (8) the level of carbapenem-resistant isolates. In a recent blood culture study, early reporting of a species identification (ID) resulted in modifications to empirical therapy in 35% of cases, compared to 21% for the Gram stain result alone (9). Finally, while specific outcomes were not always consistent between studies, several groups have reported that earlier provision of clinically relevant blood culture results generated benefits in terms of either patient outcome, length of hospitalization, antimicrobial utilization, or overall cost of medical care (10–14).

Continuously monitored cultivation of microorganisms in blood specimens with liquid media is currently the "gold standard" for the detection of bacteremia/fungemia and/or sepsis, and notification of a positive blood culture demands immediate attention. Standard protocol calls for the preparation of a Gram stain and the subculture of organisms in the broth onto supportive media for subsequent phenotypic, molecular, or mass spectrometry (MS)-based ID and full AST, a process that can take two or more days to complete.

Given that positive blood culture broth contains an abundant population of microorganisms, some laboratories have obtained faster results by using growth-based phenotypic ID systems loaded with microorganisms recovered directly from positive culture bottles (15–17). However, these "direct-from-the-bottle" phenotypic tests require manual manipulation of large volumes of blood culture broth, are not appropriate for all microorganism types, are not validated by the test manufacturers, and generally take 3 to 8 h to generate an ID.

Over the past decade, many molecular tests have become commercially available that decrease the time to identification. These tests are now being used in conjunction with positive blood cultures to identify a limited number of specific pathogens and resistance mechanisms. Examples include probe-based tests, such as peptide nucleic acid fluorescence in situ hybridization (PNA-FISH) (18–20), and nucleic acid amplification-based tests, such as PCR (21–23). The probe-based tests require multiple manual processing steps, while the PCR-based assays are relatively expensive, and most still require 1 to 3 h to perform.

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is fast becoming an established method for the rapid identification of microorganisms, and commercial systems are on the market and in the process of regulatory approval (24). However, blood and culture medium components also interfere with this technology and must be manually removed prior to the direct analysis of positive blood cultures to obtain acceptable sensitivity (9, 25–34). Recently, an evaluation of three processing methods for blood culture media was published (33). While MALDI-TOF MS technology is accurate, fast, and relatively inexpensive, it shares with many other rapid technologies the disadvantage of requiring a significant number of manual processing steps to prepare the sample prior to analysis.

Optical spectroscopy methods, such as Raman spectroscopy, Fourier transform infrared (FTIR) spectroscopy, and intrinsic fluorescence spectroscopy (IFS), can identify microorganisms very quickly. These methods require little preanalytical work, as intact cells are directly measured. Prior extraction of proteins or nucleic acids is not required. Both Raman spectroscopy and FTIR spectroscopy have been used to identify microcolonies (35) and even single microbial cells (36–38). IFS measures naturally occurring cell components that are capable of fluorescence, such as tryptophan, NADH, flavin adenine dinucleotide (FAD), and porphyrins. Methods that identify microorganisms based on analysis of their intrinsic fluorescence have been described in the literature for over 25 years (39–45). Recently, the potential of IFS to identify colonies growing on agar has been demonstrated (46, 47). However, all optical spectroscopy methods also encounter interference from the highly fluorescent and absorptive compounds present in liquid microbiological culture media, in container materials, and in blood-containing clinical samples.

Simple, safe, and reliable methods are needed in the microbiology lab to isolate microorganisms present in blood culture broth that are free of interfering materials and suitable for rapid identification technologies. In addition, fast and inexpensive technologies are urgently needed to identify the isolate within the time frame for performing a Gram stain. This article describes a novel method fulfilling both needs, combining a one-step purification of microorganisms with rapid identification using in situ measurements of microbial intrinsic fluorescence.

RESULTS

Positive blood culture broth samples from 751 isolates representing 37 of the most commonly isolated microbial species from blood cultures were tested. A list of species present in the database and their relative abundance are shown in Table 1. These species represent >90% of positive blood cultures found globally (48).

A summary of the cross-validated, multilevel classification algorithm results is given in Table 2. In brief, when an identification result was given, 100% were correct to the Gram and family classification levels, and 99.2% were correct to the species level. Of the 1,121 tests completed, 99.6% were assigned a correct, one-choice Gram result. Of the 1,117 tests correctly identifying the isolate to the Gram level, 99.3% were correctly assigned a one-choice family result. Similarly, of the 1,109 tests correctly identifying the isolate to the family level, 1,080 (97.4%) were correctly assigned to the species level. Compared to the number of initial tests (1,121), 96.5% of samples gave correct species-level identification, 2.7% gave no identification, and 0.8% gave a discordant result.

The species-level results are shown in Table 3. There were 1,048 one-choice (94.5%) and 32 low-discrimination (2.9%) species-level results, for an overall accuracy of 97.4%. Excellent results were obtained for all three major Gram groups: 96.3% of 519 Gram-negative broth samples, 97.7% of 430 Gram-positive broth samples, and 100% of 160 yeast-containing broth samples were assigned to the correct species. Many of the low-discrimination calls were to species of the same genus. Five of these cases were Klebsiella pneumoniae-Klebsiella oxytoca pairings, four were Enterobacter cloacae Cpx–Enterobacter aerogenes, two were Proteus mirabilis-Proteus vulgaris, and the final case was Staphylococcus warneri-Staphylococcus capitis. There were 20 test samples that were not identified at the species level (1.8%); however, all were correctly identified at the family level. Sixteen were identified as members of the Enterobacteriaceae family (5 as Providencia stuartii , 1 as Proteus mirabilis, 2 as Morganella morganii, 3 as Klebsiella pneumoniae, 1 as Escherichia coli, 2 as Enterobacter cloacae Cpx, and 2 as Citrobacter freundii), and the remaining four were identified as members of the Staphylococcaceae family (1 each as Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus hominis, and Staphylococcus capitis). Nine isolates were incorrectly identified. Apart from an Escherichia coli isolate that was classified as an Enterobacter cloacae Cpx, the remaining eight misidentified...
TABLE 2 Multilevel microbial classification using intrinsic fluorescence

| Level | Microbial group | No. (%) of tests | One-choice agreement | Low-discrimination agreement | Incorrect ID | No ID |
|-------|-----------------|------------------|----------------------|-----------------------------|--------------|-------|
| 1     | Gram type       | 1,121            | 1,117 (99.6)         | 0 (0)                       | 0 (0)        | 4 (0.4) |
| 2     | Family          | 1,117            | 1,109 (99.3)         | 2 (0.2)                     | 0 (0)        | 6 (0.5) |
| 3     | Species         | 1,109            | 1,048 (94.5)         | 32 (2.9)                    | 9 (0.8)      | 20 (1.8) |

*Species in model Total no. of strains No. of known blood culture isolatesa

| Species in model | Total no. of strains | No. of known blood culture isolates |
|------------------|----------------------|-------------------------------------|
| Staphylococcus aureus | 55                   | 11                                  |
| Escherichia coli   | 53                   | 4                                   |
| Staphylococcus epidermidis | 42                 | 12                                  |
| Streptococcus pneumoniae | 39               | 6                                   |
| Klebsiella pneumoniae | 38                 | 6                                   |
| Enterococcus faecalis | 31                  | 3                                   |
| Candida albicans   | 30                   | 0                                   |
| Candida tropicalis | 29                   | 0                                   |
| Streptococcus mitis/Streptococcus oralis | 28 | 0 |
| Pseudomonas aeruginosa | 27               | 0                                   |
| Candida parapsilosis | 27              | 0                                   |
| Enterococcus faecium | 26                | 2                                   |
| Enterobacter cloacae Cpx | 25            | 3                                   |
| Enterobacter aerogenes | 24             | 1                                   |
| Citrobacter freundii | 23                | 0                                   |
| Salmonella enterica serovar Enteritidis | 20 | 0 |
| Providencia stuartii | 18                 | 2                                   |
| Staphylococcus lugdunensis | 15               | 1                                   |
| Stenotrophomonas maltophilia | 14          | 1                                   |
| Staphylococcus hominis | 14                | 4                                   |
| Haemophilus influenzae | 12              | 0                                   |
| Klebsiella oxytoca | 12                  | 0                                   |
| Proteus mirabilis   | 12                   | 1                                   |
| Listeria monocytogenes | 12              | 0                                   |
| Candida krusei      | 12                   | 0                                   |
| Morganella morganii | 11                  | 0                                   |
| Neisseria meningitidis | 11             | 0                                   |
| Proteus vulgaris    | 11                   | 0                                   |
| Streptococcus pyogenes | 11            | 0                                   |
| Acinetobacter baumannii | 10           | 0                                   |
| Staphylococcus capitis | 10              | 0                                   |
| Staphylococcus warneri | 10              | 0                                   |
| Streptococcus agalactiae | 10             | 0                                   |
| Streptococcus infantarius subsp. coli | 10          | 0                                   |
| Candida glabrata    | 10                   | 0                                   |
| Serratia marcescens | 9                    | 0                                   |

*No. of strains isolated from clinical blood cultures.

The immediate isolation of purified microorganisms from positive blood cultures is not a trivial task. Commonly employed methods for recovering microorganisms directly from blood culture broth include differential centrifugation (15, 25, 31), centrifugation within a serum separator tube (16, 17), or a combination of the two (26, 53). For some direct testing applications, mild (27) or harsh (28, 30, 32) cell lytic agents are used at neutral pH, followed by several washing steps. Others have used hypotonic solutions in combination with washing (29). However, all of these methods have drawbacks. The resultant microbial preparations often contain contaminating red blood cells, incompletely solubilized blood cell membranes, precipitated proteins, platelets, lipid particles, plasma enzymes, and hemoglobin, which can lead to poor results. Some of these processing methods use harsh conditions that may damage microorganisms. They are also very labor-intensive and potentially unsafe due to steps that can result in aerosol exposure to lab technicians.

In recent years, rapid microbial identification obtained by the processing of positive blood cultures for use with MALDI-TOF MS systems has been more successful with samples containing Gram-negative rather than Gram-positive microorganisms. Typically, the species-level identification rate is 20 to 50% lower for the Gram-positive group (25, 29–31, 33, 34). We believe this is partly related to the significantly higher cell concentration of the most common Gram-negative species found in positive blood cultures (unpublished data), which results in further dilution of contaminating nonmicrobial debris, relative to the microbial cells, in the suspensions used for analysis. Additionally, the sample pro-
cessing conditions also play a role in determining the final outcome. The brief alkaline detergent lysis method described here reduces the level of nonmicrobial debris effectively enough to allow unencumbered measurement of intact cell fluorescence and thereby generate a highly accurate species ID for both cultures containing Gram-positive organisms (97.7%) as well as those containing Gram-negative organisms (96.7%).

The novel lysis buffer of this method combines a high-pH CAPS (N-cyclohexyl-3-amino-2-propanesulfonic acid) buffer with a mild nonionic poloxyl-ethylene class detergent (Brij-97) to rapidly solubilize human blood cells while leaving microorganisms intact. The selective lysis conditions, including detergent, buffer, sample dilution, temperature, and contact time, were chosen to minimize any alteration in intrinsic fluorophores and maximize viability of the most common microorganisms found in blood cultures.

The salt-based aqueous density cushion provides two important functions. First, passage of microorganisms through the high-density salt solution is a very efficient wash step, removing interfering plasma and fluorescent or fluorescence-attenuating medium-derived compounds while rapidly transferring the cells from an alkaline to a physiological pH environment. Second, the high ionic strength of this solution causes microbial cells to shrink, increase in buoyant density, and sediment more rapidly to the base of the optical centrifuge tube, where they can be interrogated by front-face IFS. Such a mechanism has been described for some Gram-negative rods.

Simplicity and enhanced safety are other features of the method. Once the lysate has been prepared and loaded into the optical centrifuge tube, the remaining steps of the method can be

| Species                     | No. (%) of tests | One-choice ID | Low-discrimination ID | Incorrect ID* | No ID |
|-----------------------------|------------------|---------------|-----------------------|---------------|-------|
| **Total**                   |                  |               |                       |               |       |
| **Gram positive**           |                  |               |                       |               |       |
| Enterococcus faecalis       | 45               | 45 (100)      | 0 (0)                 | 0 (0)         | 0 (0) |
| Enterococcus faecium        | 39               | 39 (100)      | 0 (0)                 | 0 (0)         | 0 (0) |
| Listeria monocytogenes      | 12               | 12 (100)      | 0 (0)                 | 0 (0)         | 0 (0) |
| Staphylococcus aureus       | 85               | 82 (96.5)     | 1 (1.2)               | 1 (1.2)       | 1 (1.2) |
| Staphylococcus capitis      | 10               | 9 (90)        | 0 (0)                 | 0 (0)         | 1 (10.0) |
| Staphylococcus epidermidis  | 65               | 63 (96.9)     | 0 (0)                 | 1 (1.5)       | 1 (1.5) |
| Staphylococcus hominis      | 14               | 13 (92.9)     | 0 (0)                 | 0 (0)         | 1 (7.1) |
| Staphylococcus lugdunensis  | 15               | 15 (100)      | 0 (0)                 | 0 (0)         | 0 (0) |
| Staphylococcus warneri      | 9                | 7 (77.8)      | 2 (22.2)              | 0 (0)         | 0 (0) |
| Streptococcus agalactiae    | 20               | 20 (100)      | 0 (0)                 | 0 (0)         | 0 (0) |
| Streptococcus infantarius subsp. coli | 10 | 7 (70) | 1 (10.0) | 2 (20) | 0 (0) |
| Streptococcus mitis-Streptococcus oralis | 35 | 35 (100) | 0 (0) | 0 (0) | 0 (0) |
| Streptococcus pneumoniae    | 49               | 47 (95.9)     | 0 (0)                 | 2 (4.1)       | 0 (0) |
| Streptococcus pyogenes      | 22               | 22 (100)      | 0 (0)                 | 0 (0)         | 0 (0) |
| **Total**                   | 430              | 416 (96.7)    | 4 (0.9)               | 6 (1.4)       | 4 (0.9) |
| **Gram negative**           |                  |               |                       |               |       |
| Citrobacter freundii        | 33               | 30 (90.9)     | 1 (3.0)               | 0 (0)         | 2 (6.1) |
| Enterobacter aerogenes      | 34               | 27 (79.4)     | 7 (20.6)              | 0 (0)         | 0 (0) |
| Enterobacter cloacae Cpx    | 35               | 28 (80.0)     | 3 (8.6)               | 2 (5.7)       | 2 (5.7) |
| Escherichia coli            | 76               | 71 (93.4)     | 3 (3.9)               | 1 (1.3)       | 1 (1.3) |
| Klebsiella oxytoca          | 24               | 22 (91.7)     | 2 (8.3)               | 0 (0)         | 0 (0) |
| Klebsiella pneumoniae       | 53               | 43 (81.1)     | 7 (13.2)              | 0 (0)         | 3 (5.7) |
| Morganella morganii         | 22               | 20 (90.9)     | 0 (0)                 | 0 (0)         | 2 (9.1) |
| Proteus mirabilis           | 23               | 19 (82.6)     | 3 (13.0)              | 0 (0)         | 1 (4.3) |
| Proteus vulgaris            | 20               | 20 (100)      | 0 (0)                 | 0 (0)         | 0 (0) |
| Providencia stuartii        | 18               | 13 (72.2)     | 0 (0)                 | 0 (0)         | 5 (27.8) |
| Salmonella enterica serovar Enteritidis | 31 | 29 (93.5) | 2 (6.5) | 0 (0) | 0 (0) |
| Serratia marcescens         | 18               | 18 (100)      | 0 (0)                 | 0 (0)         | 0 (0) |
| Acinetobacter baumannii     | 20               | 20 (100)      | 0 (0)                 | 0 (0)         | 0 (0) |
| Neisseria meningitidis      | 22               | 22 (100)      | 0 (0)                 | 0 (0)         | 0 (0) |
| Haemophilus influenzae      | 21               | 21 (100)      | 0 (0)                 | 0 (0)         | 0 (0) |
| Pseudomonas aeruginosa      | 47               | 47 (100)      | 0 (0)                 | 0 (0)         | 0 (0) |
| Stenotrophomonas maltophilia | 22      | 22 (100)     | 0 (0)                 | 0 (0)         | 0 (0) |
| **Total**                   | 519              | 472 (90.9)    | 28 (5.4)              | 3 (0.6)       | 16 (3.1) |
| **Species**                 |                  |               |                       |               |       |
| **No. ID**                  |                  |               |                       |               |       |

*Two E. cloacae Cpx isolates were misidentified as E. aerogenes, one E. coli isolate as E. cloacae Cpx, one S. aureus isolate as S. lugdunensis, one S. epidermidis isolate as S. warneri, two S. pneumoniae isolates as S. mitis-S. oralis and S. agalactiae, and two S. infantarius subsp. coli isolates as S. mitis-S. oralis and S. agalactiae.

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performed using general lab equipment without the risk of aerosol generation. The sealed tube is centrifuged, transferred to a spectrofluorimeter for reading, and then disposed of. No manual suspension, washing, or extraction of microbial cell pellets is required, rendering the method potentially useful for the direct and safe identification of mycobacteria as well.

The use of intrinsic fluorescence for the identification of microorganisms has been described in the literature for many years, and there is renewed interest in the applications of fluorescence spectroscopy in the field of medical microbiology (55). These studies employed either time-resolved fluorescence (56), steady-state fluorescence (39–47), or steady-state fluorescence in combination with specific ligands (57). Although promising, they lacked the strain numbers or species diversity to draw any firm conclusions about the potential of IFS to become a robust ID method for clinical use. During our initial evaluation of IFS using a diverse range of clinically and industrially relevant microorganisms (J. Hyman and J. Walsh, unpublished data), like other investigators, we found that when suspensions of microbial cells were examined by standard right-angle fluorescence optics, tryptophan was the predominant fluorophore measured. Surprisingly, when microbial cells were firmly packed into the UV-transparent centrifuge tubes used in this study and then examined by front-face fluorescence, many additional cellular fluorophores were evident, and more importantly, the spectral distribution of these fluorophores enabled a significant improvement in classification accuracy.

We chose to use a nontargeted approach in analyzing the EEM data presented in this report and found that the species-resolving power of intrinsic fluorescence measurements of intact microbial cells was not limited to the analysis of a few well-known fluorophores, such as tryptophan or NADH, but rather to a combination of fluorophores existing in multiple physical states and environments within viable, metabolically active cells. This finding is hardly surprising as the fluorescence of many intrinsic fluorophores is dependent upon their physical environment within a cell, and it is well known that the interaction of cell fluorophores and proteins alters their fluorescence properties (58). For example, the fluorescence intensity of NADH increases when bound to cellular proteins, and the excitation and emission maxima are blue-shifted (59). The emission maximum of another intrinsic fluorophore, pyridoxal, is also blue-shifted when bound to proteins such as phosphorylase (58). Furthermore, both NADH and FAD act as electron donors and acceptors and play critical roles in cell energy metabolism (60), and their relative levels provide an estimate of the redox state of a cell (61). Additional cellular fluorophores include a variety of porphyrins (intermediates in heme biosynthesis), riboflavin, pterins, lipofuscin, pyridoxamine, and structural compounds, such as elastin and collagen (49). ATP has also been shown to autofluoresce, albeit under specific conditions (62). Furthermore, some fluorophores are produced only by microorganisms. For example, *P. aeruginosa* cells contain a characteristic yellow-green fluorescent siderophore called pyoverdin.

Many rapid identification methods measure physical properties of unknown cells, such as nucleic acid sequences, protein masses, or the vibration of various chemical bonds. However, a nontargeted analysis of intact microbial cell intrinsic fluorescence provides a snapshot of both structural and metabolic components of viable cells, without the use of specific or expensive reagents. This method has the potential to provide species-level identification for the most commonly encountered sepsis-associated organisms. The availability of an ID result within 20 min of detection of a positive blood culture would result in a single call being made to report both a positive culture and the organism’s identity, something not always possible with other rapid technologies. The more concise reporting provided by this method will result in improved efficiency, more timely management of empirical therapy, a re-
duction in laboratory and hospital costs, and, potentially, a reduction in patient mortality.

MATERIALS AND METHODS

Material. BacT/ALERT SA blood culture bottles, tryptic soy broth (TSB), tryptic soy agar with 5% sheep blood, and Sabouraud dextrose agar were from bioMérieux, Inc. (Durham, NC). CAPS buffer (N-cyclohexyl-3-aminopropanesulfonic acid) was purchased from Alpha Aesar, HEPES buffer from JT Baker, Brij-O10 (formerly Brij-97) detergent from Sigma-Aldrich, Pluronic F-108 from BASF, cesium chloride from MP Biomedicals, and polypropylene balls from CIC, Ball Company. The optical microcentrifuge tube used for this study was designed by bioMérieux and built under contract by an external manufacturer. This screw-cap tube was constructed of a low-fluorescence, UV-transparent acrylic (Acrylite H15-012; Cyro Industries). The tube was centrifuged in an Eppendorf 5417R microcentrifuge with a standard A-8-11 swing-out rotor and buckets. Small inserts were placed in the buckets to support the base of the optical tube during centrifugation. The fluorescence spectrophotometer used was a FluoroLog-3 2T2 system, built by Horiba-Jobin Yvon. The fiber optic probe (6 excitation around 1 emission bundle of 300-μm diameter core fibers) was supplied by Ocean Optics.

Blood cultures. Samples of positive blood culture broth were generated in the following manner. BacT/ALERT SA culture bottles were inoculated with 10 ml of freshly collected sodium polyanethol sulfonate (SPS)-anticoagulated human blood from over 50 healthy donors, followed by 50 to 500 CFU of each test strain suspended in approximately 0.5 ml TSB. Inoculated bottles were then incubated at 35 to 37°C in the BacT/ALERT 3D microbial detection system.

A total of 751 bacterial and fungal strains were tested in this study. Ninety-two percent of the strains were selected from bioMérieux’s culture collection in St. Louis, while the remaining 8% were known blood culture isolates obtained from deidentified clinical blood cultures collected in 2009. Of the culture collection isolates with known sources of origin (80%), 60% were collected from multiple clinical institutions within the United States, 27% were obtained from other countries, and 13% were stock strains from ATCC or the CDC. Where possible, ATCC type strains from each species were included. Furthermore, the strains selected were deposited into the culture collection spanning the period 1984 to 2009. The list of species and their relative abundance in the database are shown in Table 1.

Test strains were recovered from frozen stock and subcultured three times on the appropriate solid media before use. The identity of each strain was confirmed with Vitek 2 cards and MALDI-TOF MS using the Axima assurance system (Shimadzu Corp.) and SARAMIS database (bioMérieux). To ensure that isolated microorganisms were always in the logarithmic growth phase, bottles were removed from the BacT/ALERT 3D microbial detection system within 5 min of generation of a positive signal and processed within 15 min of removal, unless otherwise specified.

Rapid ID procedure. An overall schematic of the simple three-step process (lyse-spin-read) is given in Fig. 1. Briefly, a 2.0-ml sample of warm (35 to 37°C) positive broth is removed from the test blood culture bottle and added to 1.0 ml of warm (35 to 37°C) selective lysis buffer (0.45% [wt/vol] Brij-O10 in 0.3 M CAPS [pH 11.7]) contained in a 15-ml screw-cap polypropylene tube. The mixture was vortexed for 5 s at maximum speed and then placed in a 35 to 37°C water bath for 60 s. After an additional 1 to 2 s vortex, 1.0 ml of the lysate was removed and layered onto a single 5/16-in.-diameter polypropylene ball (CIC, Ball Co.) floating on the surface of 0.5 ml of a solution of 24% (wt/vol) cesium chloride plus...
0.005% (wt/vol) Pluronic F-108 plus 10 mM HEPES (pH 7.4) contained within an optical microcentrifuge tube. The polypropylene ball was used to control the layering process and create an undisturbed interface. The tube was sealed with a screw cap and centrifuged for 2 min at 10,000 rpm at 20 to 25°C in a microcentrifuge with an A-8-11 swing-out rotor. Photographs of a tube loaded with \textit{S. lugdunensis}-positive broth taken before and after the centrifugation step are shown in Fig. 2. Following centrifugation, the tube was seated upon the end of the fiber optic probe in a custom-built holder (Fig. 3A) within a light-tight sample compartment. Fluorescence of the pelleted microorganisms was then collected through the 0.5-mm-thick bottom section of the tube using the FluoroLog-3 system with the following excitation-emission matrix (EEM) parameters. The excitation scan range was every 5 nm from 260 to 800 nm (109 discrete wavelengths), with a slit width of 5 nm. Emission was collected every 5 nm from 260 to 1,100 nm, with a slit width of 5 nm. The run time for a full EEM scan was 13.6 min per sample, however, fewer than 10% of the excitation wavelengths measured were required for the classification model. A 450-W xenon lamp and double-grating monochromator provided the specific excitation energy. Fluorescence was collected using an F3000 mirror adapter connected to the 300-μm-diameter fiber optic probe and measured using a Triax 320 spectrometer with a 600-line/mm grating (500-nm blaze) and a Symphony charge-coupled device (CCD) array. A schematic of the fiber optic probe interface to the spectrofluorimeter is given in Fig. 3B. Fluorescence signals were normalized for lamp intensity (S/R) and output to Excel spreadsheets for analysis (8,945 data points per sample). Characteristic EEMs from \textit{S. aureus}, \textit{S. epidermidis}, \textit{E. coli}, and \textit{Candida tropicalis} recovered directly from positive blood cultures are shown in Fig. 4A to D, respectively, along with the location of some known cellular fluorophores (49). Following the scan, the optical microcentrifuge tubes containing purified microbial pellets were stored frozen at −70°C for later assessment of discrepant or unidentified test results. The purity of the test broth sample was also confirmed by subculture. To better understand the stability of intrinsic microbial fluorophores to low-temperature storage conditions, 5-ml samples of positive broth from 370 of the 751 isolates were stored at 2 to 8°C for 4 to 6 h. Broth was warmed prior to testing by placing the tube in a 35 to 37°C water bath for 5 min.
Data analysis. A classification database was built using the EEMs of both fresh and stored positive broth samples, for a total of 1,121 scans. The classification method was based on a mathematical distance calculation. For a given scan from the test set, the distance from the centroid was calculated for each possible classification group. If a distance was within a prespecified percentage of the shortest distance, the test result was considered a low-discrimination agreement between the two classification groups. However, if a second distance was not within this prespecified percentage, the group associated with the shortest distance was called a “one-choice agreement.” If none of the distances were less than a maximum distance, the test was given a “no identification” result. An incorrect result was obtained if a test scan was classified into the wrong group. Data were transformed by first converting fluorescence signals to a natural log and then calculating the first derivative. A limited set of nine excitation wavelengths (285, 300, 315, 330, 345, 400, 415, 430, and 445 nm) were selected for the ability to discriminate between the species represented in the model and were not limited to an evaluation of known fluorophores. Classification was performed using a multilevel algorithm that assigned results to the Gram, family, and then species level. For the Gram level to be considered correct, independent analyses to the Gram and family group levels had to agree. Data with confident Gram classification (1st level) were then further analyzed into family-level groups (2nd level) and finally to the species level (3rd level). All results were cross-validated using a 10-fold venetian blind approach. In this method, 9/10 of the data were used to train the model, and the remaining set of data was used as a test or challenge set. Each combination of groups was analyzed, and the results were pooled to provide an overall estimate of performance.

Instrument QC. As a daily internal quality control (QC) for the reproducibility of the custom optical separation tube and the fiber optic interface with the spectrofluorimeter, positive broth from a BacT/ALERT SA bottle inoculated with 400 CFU of E. coli ATCC 8739 (no blood) was overlaid directly onto the cesium chloride density cushion and spun, and the EEMs were collected.

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