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Rapid Detection of Bacteria from Blood with Surface-Enhanced Raman Spectroscopy

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Abstract

Traditional methods for identifying pathogens in bacteremic patients are slow (24–48+ h). This can lead to physicians making treatment decisions based on an incomplete diagnosis and potentially increasing the patient's mortality risk. To decrease time to diagnosis, we have developed a novel technology that can recover viable bacteria directly from whole blood and identify them in less than 7 h. Our technology combines a sample preparation process with surface-enhanced Raman spectroscopy (SERS). The sample preparation process enriches viable microorganisms from 10 mL of whole blood into a 200 μL aliquot. After a short incubation period, SERS is used to identify the microorganisms. We further demonstrated that SERS can be used as a broad detection method, as it identified a model set of 17 clinical blood culture isolates and microbial reference strains with 100% identification agreement. By applying the integrated technology of sample preparation and SERS to spiked whole blood samples, we were able to

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

Supporting Information

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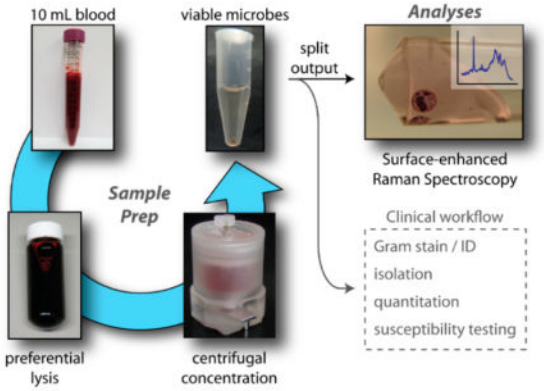
A table consisting of 17 different microorganisms used in the current study and their cultivation conditions (PDF).

correctly identify both *Staphylococcus aureus* and *Escherichia coli* 97% of the time with 97% specificity and 88% sensitivity.

Graphical Abstract



Bacteremia is defined as the presence of bacteria in the blood. Excluding transient bacteremia (e.g., after tooth brushing), blood is sterile; thus, finding live bacteria in the blood is abnormal. Bacteremia can develop from a severe infection at another site (e.g., pneumonia), a surgical wound infection, or contaminated catheters or other implanted prosthetic devices. Bacteremia can lead to serious sequelae, such as infective endocarditis and sepsis. The most common Gram-negative bacteria causing sepsis are *Escherichia coli*, *Klebsiella* species, *Pseudomonas aeruginosa*, and *Enterobacter* species, whereas the most common Gram-positive bacteria include *Staphylococcus aureus*, coagulase-negative *Staphylococci*, *Enterococcus* species, and *Streptococcus* species.[1,2] *Candida* species are also commonly implicated in nosocomial infections.[2] In hospital intensive care units, nearly one out of every 23 patients becomes septic.[3] Sepsis ranks 11th overall among the causes of death in the United States (1.5% of all deaths),[4] with annual estimates of up to 750,000 cases of severe sepsis and mortality rates ranging from 20 to 52%, depending on the facility.[5,6] Additionally, every hour without the appropriate therapy translates to increased mortality.[7] Sepsis is costly; it was the single most expensive condition treated in U.S. hospitals in 2009 (an aggregate cost of $15.4 billion), although it was the sixth most common principal reason for hospitalization.[3] It is predicted that the number of sepsis cases will continue to rise due to the aging American population, spread of antimicrobial resistance, growth of immuno-suppressed populations, and increased use of invasive catheters and prosthetic materials.[8,9]

Currently, the gold standard method used to diagnose bacteremia relies on blood culture. Several draws of 10 mL of blood are diluted into blood culture medium and incubated for >12 h to allow for bacterial replication. Positive cultures provide a rich source of live bacteria for downstream phenotypic- and culture-based testing. The major limitation of the gold standard method is the time to diagnosis, with results taking 24–48 h (or longer for fastidious organisms). Consequently, physicians will typically prescribe an empiric broad-spectrum antimicrobial regimen to kill a large range of different microorganisms until the

results of the blood culture are known. If patients are already on antimicrobial therapy at the time of specimen collection, false negatives can occur as the antimicrobials can inhibit the growth of the microorganisms in culture. Once the microorganism(s) is identified, the antimicrobial treatment can be tailored, and as soon as antibiotic susceptibility testing is complete (which requires an additional 4–24 h),[10] the antimicrobial treatment can be made even more specific. This practice of treating with broad-spectrum antibiotics first, although prudent, undoubtedly contributes to the increasing prevalence of drug-resistant bacteria in hospitals. Given this increase in drug resistance, commonly used broad-spectrum antibiotics can sometimes be ineffective. In an ideal situation, a physician would have near instant identification of the causative bacterium and its antibiotic susceptibility profile, as delays in appropriate antibiotic therapy can increase morbidity and mortality,[11] particularly in cases of serious infection.

Detecting microbes directly from blood has many challenges. These challenges include a low concentration of a variety of infectious microbes (often <10 CFU/mL) and a complex blood matrix containing billions of blood cells per mL and a high protein content. Blood culture overcomes these difficulties by diluting the blood components in the culture medium and significantly increasing the microbe's concentration, though this occurs over many hours. Efforts to separate and detect microbes directly from whole blood (without involving blood culture or microfluidic devices) have included preferential lysis[12,13] and centrifugal gradients and filtration.[14–16] These techniques are limited by poor bacterial recoveries, small blood volumes (less than 5 mL), or additional time requirements for further growth.

Clearly, the need exists for a rapid diagnostic method to identify pathogens in patients who are suspected of having bacteremia while eliminating the time required for culture and addressing the sample preparation problem from whole blood. Many companies have been developing new rapid diagnostic tests directly from positive blood culture, which can reduce the time to get definitive results, although the culture could take upward of 24 h to become positive. Nanosphere's Verigene Test and Alere's BinaxNOW both use positive blood culture for their systems, but with different identification methods. The Verigene Test uses a microfluidic cassette to identify 37 different microorganisms employing nanoparticles[17–23] and BinaxNOW is an immunochromatographic assay for a variety of infectious agents (methicillin-resistant *S. aureus*, *E. coli*, *Streptococcus pneumoniae*, etc.).[24] Both of these methods are destructive, meaning the final product cannot be used for further diagnostic testing. Accelerate Diagnostics also uses positive blood culture for their diagnostic instrument. They take advantage of fluorescence in situ hybridization (FISH) for identification as well as a growth analysis method using automated microscopy.[25,26] Thus, the Accelerate Diagnostics system can provide phenotypic information, which is beneficial since, with the exception of a few particular genes, for example, *mecA* and *vanX*, specific genes inadequately predict functional resistance. Therefore, the vast majority of resistance phenotypes are undetectable by genetic methods (e.g., PCR). Nonetheless, PCR is a commonly used method for identifying microbes in infected blood since there is frequently a low concentration of pathogens in the sample. The FilmArray System (BioFire Diagnostics) is based on PCR performed on positive blood cultures.[27,28] Abbott's IRIDICA assay[29] and T2 Biosystems[30,31] both use PCR as part of their assay starting from whole blood in order to

avoid the positive blood culture requirement and, therefore, significantly reduce time to results.

PCR is a well-established analytical method that efficiently increases the sample's DNA concentration for certain types of analysis. However, this technology is susceptible to false positives due to circulating DNA from contamination (environmental or from the skin during venipuncture) or from pathogens killed by the host's immune system or recently administered antibiotic therapy, since PCR cannot distinguish between DNA in viable bacteria or severely damaged bacteria. It is still an area of active investigation whether circulating pathogenic DNA might be an infection indicator, particularly after antibiotic therapy has been administered.[32] While theoretically circulating pathogenic DNA could be an indication of localized infection, there are reports of poor clinical performance of PCR-based assays that detect all available DNA instead of only those associated with circulating pathogens.[30,33] Moreover, PCR also destroys intact cells (to amplify the contained DNA), producing samples incompatible for other viable cell-based analysis techniques, such as culture. Having viable cells allows the integration of new rapid diagnostic techniques to existing clinical workflows. Additionally, unlike traditional culture, PCR is inherently constrained to diagnose only the pathogens included in the primer set, making deductive information about the sample necessary for positive results. While PCR is a powerful and useful technology in the clinical microbiology laboratory, its weaknesses require complementary phenotypic tests to ensure complete diagnoses.

The ideal tool for the physician would be a diagnostic device that mimics the power of culture-based methods, specifically, the ability to identify a large pool of candidates, without a priori selection, directly from a primary sample in a time frame useful for initial antibiotic drug prescription. Such a tool would be invaluable in cases of serious bloodstream infection, such as bacteremia. To fulfill this demand for a rapid diagnostic method that can analyze samples directly from the patient, we developed a platform that consists of a universal sample preparation system for viable bacteria coupled with a rapid characterization technique, surface-enhanced Raman spectroscopy (SERS) (Figure 1). SERS was discovered more than 30 years ago when it was first observed that the Raman scattering cross-section of analytes close to (<5 nm) a suitably roughened or nanostructured metal (usually Au or Ag) surface increased by more than 10^6 per molecule (ensemble averaged).[34,35] This amplification is primarily due to a resonance effect between the surface plasmons of a nanostructured metal surface and the incident and scattered radiation fields. Consequently, only molecules that are properly oriented and in close proximity to the nanostructured metal surface give rise to signals that appear in SERS spectra. Furthermore, the electromagnetic field enhancement effect is strongly dependent on the metal surface morphology.[36–40] Since each molecule has its own characteristic vibrational modes, this technique has been shown to be a sensitive probe for identifying analytes at low concentrations when combined with library or data-mining multivariate analyses.[41–43]

SERS is a promising technology for the detection and identification of intact bacterial cells since it offers unique advantages compared to other optical and non-optical techniques as a diagnostic method for bacterial infections.[44–56] For example, the enormous Raman signal enhancement permits the acquisition of SERS spectra from bacterial cells on a time scale of

seconds without the need for extrinsic labeling.[57–59] Only low incident laser power (1–5 mW) is required for SERS data acquisition due to the large Raman cross-section enhancement, thus enabling the development of low cost, portable, and eye-safe SERS platforms for point-of-care diagnostics.[60] Furthermore, as for any vibrational spectroscopic-based technique, the narrow Raman spectral features allow multiple markers in a spectrum to identify a given molecular species and different molecular components in complex samples. This multiplexing capability is ideal for studies of biological fluids. SERS spectra of washed, concentrated bacteria excited at 785 nm on Au nanostructured substrates are primarily due to purine degradation products: adenine, hypoxanthine, xanthine, guanine, uric acid, and AMP of nucleic acids and nucleotides.[61] The resulting spectra provide a robust vibrational signature for species and strain level detection and identification when combined with chemometric analysis and a reference library.[58,60,62]

A few groups have investigated using SERS for whole human blood analysis.[60,63,64] Recently, Ziegler's group reported that the age of whole human blood is significant to its 785 nm excited SERS signature.[60] Within 24 h of blood collection, the vibrational signature of hypoxanthine dominates the SERS spectrum of stored whole blood on Au SERS active substrates. This illustrates the importance of using fresh blood for bacterial identification via SERS, as well as SERS' suitability for rapid analysis of fresh whole blood samples. By integrating SERS detection with our universal sample preparation process, the demand for a rapid, sensitive, and specific bacteremia diagnostic method from primary blood samples can be fulfilled.

EXPERIMENTAL SECTION

Overview of Technology Design

A universal sample preparation process was designed that accepts 10 mL of whole human blood, preferentially lyses blood components while maintaining the microorganism's viability (if any micro-organisms are present) and produces an enriched output of viable microorganisms for downstream analysis, specifically by SERS (Figure 1). For simulated bacteremic samples, sterile, human anticoagulated blood was inoculated with log-phase microorganisms and mixed with a proprietary lysis buffer. Following a 3 min lysis treatment, the sample was placed into the bacterial concentrator where the microorganisms were concentrated into a 200 μL aliquot by centrifugation. After the microbes were concentrated and isolated from any culture inhibitors in the blood (e.g., phagocytes, host immune factors, antimicrobials), they were then incubated for 5 h at 37 °C in tryptic soy broth (TSB) with shaking to increase the microorganism's population for SERS detection and identification. An aliquot of the incubated sample was placed on a SERS chip for analysis and identification using our multivariate spectral analysis algorithm. The rest of the incubated sample was available for additional confirmatory testing.

SERS Chip Fabrication

All SERS spectra were obtained using in situ grown, aggregated Au nanoparticle-covered SiO_2 substrates that were previously described.[58] The final product of the fabrication process is about 1 mm^2 SiO_2 substrate coated with 2–15 particle aggregates of

monodispersed ~80 nm Au nanoparticles. The two-stage reduction of a Au ion-doped SiO_2 sol–gel process was optimized for maximum 785 nm excited SERS performance.

Devices

Fraunhofer CMI designed, machined, and tested our microbial concentration devices, shown in Figure 2.[65] Briefly, the device was assembled using a machined acrylic (MSC Industrial Supply Company) body with a screw-on lid, a stainless steel T-shaped slider valve, and a molded poly-propylene funnel (Proto Laboratories). The acrylic body supports the 53 mL funnel containing the processed blood sample which feeds into a collection well in the slider valve during centrifugation. There are two collection wells in the slider valve: one is a 200 μL well for collecting the final sample of concentrated microorganisms, and the other is a 30 μL well, if a smaller output sample volume is desired. Screwing the lid onto the acrylic body pushes the lid against the funnel, creating a seal at the interface between the O-ring at the bottom of the funnel and the slider valve. In Figure 2, the O-rings can be seen as the red ring (silicone) around the lid and the black ring (ethylene propylene diene M-class rubber) at the base of the funnel.

Microorganisms

Logarithmic phase microbial cultures were prepared by inoculating 0.5 mL of an overnight broth culture into 25 mL of the appropriate fresh medium. The culture medium and incubation temperature for each species is shown in Table S1. When the cultures reached an optical density at 600 nm of ~0.5, the culture was diluted to achieve the desired CFU/mL for inoculation into sterile blood. The actual CFU/mL was verified by quantitative plating.

Blood

Whole human blood from three healthy donors with matching blood types was collected in K3EDTA BD Vacutainers and purchased from Biological Specialty Corporation (Colmar, PA) each week. The samples were pooled before processing.

Device Preparation

Prior to experiments, all device components were sterilized with 10% bleach and rinsed with sterile water. The molded funnels were then submerged in a beaker of 0.05% (w/v) Pluronic F-127 (Sigma-Aldrich) to minimize microbial adherence during the procedure.[66,67] The filled beaker was placed in a sonic bath for 10 min to facilitate adsorption of the Pluronic onto the parts' surfaces.

Sample Preparation

A 10 mL aliquot of pooled blood (with log-phase bacteria to simulate a sample from a bacteremic patient) and a proprietary lysis buffer composed of detergents and enzymes were added to a glass vessel. The sealed vessel was lysed for 3 min using a mechanical process. The lysis process was optimized to be selective for the blood components and nondestructive to the microbes.[68] The lysed blood and viable microbes were then transferred to our custom-made microbial concentration devices and spun in a swinging-bucket centrifuge for 20 min

at 3000 × *g*. The 200 μL output containing concentrated, viable microorganisms (and blood debris, if any) was suitable for further processing or for downstream analysis.

Recovery Calculations and Bayesian Analysis for Characterizing Sample Processing

To determine the percentage of viable microorganisms from the initial microbial inoculum, all of the contents of the 200 μL sample well were streaked using 10 μL loops onto agar plates. The plates were incubated at the appropriate conditions overnight (Table S1) for CFU determinations. The percentage of viable micro-organisms recovered was calculated by comparison with the inoculum for each experiment. Using the recovery information (from above), these data were entered into a Bayesian algorithm. Bayesian statistical analysis follows uncertainty by using probability distributions. Subjective probability is utilized to measure the uncertainty regarding the hypothesis. The uncertainty of our hypothesis (that a recovery of ≥40% would occur in a particular 10 mL sample of blood spiked with a known quantity of microorganisms) was determined.

Enrichment of Microorganisms for SERS Analysis

To analyze the samples using SERS, the 200 μL output of the sample preparation process was transferred to a sterile 1.5 mL microcentrifuge tube, rinsed with 200 μL of TSB and spun down. The supernatant was aspirated, and 1 mL of fresh TSB was added. The tubes were left open, but covered with Parafilm M (VWR), and incubated for 5 h at 37 °C on an orbital shaker at 160 rpm. Once the incubation time was complete, the Parafilm was removed and the samples were spun down at 4500 × *g* for 3 min. The supernatant was removed and 1 mL of 1 mM sodium diphosphate buffer (pH 6; Sigma-Aldrich) was used to resuspend the pellets. Sodium diphosphate buffer was found to generate the SERS signal faster for certain species than washing with water.[61] The samples were spun down and the rinsing process was repeated 3×. Upon completion of the third centrifugation step, the supernatant was removed to leave approximately 10 μL of sample for SERS analysis. For samples directly from culture, the log-phase microorganisms were pelleted, washed 4× with deionized Millipore water, and then resuspended in 250 μL of deionized Millipore water.

SERS Analysis

The samples were briefly vortexed and a 1 μL aliquot was placed on the concave side of the SERS chips to dry for approximately 5 min. SERS spectra were obtained immediately and after 60 min to ensure optimal signal-to-noise from the sample. The chips were placed on the micro-manipulator stage of the RM-2000 Renishaw Raman microscope using a 50× objective. To obtain the spectra, 785 nm excitation was employed. SERS spectra typically resulted from <2 mW of incident laser power in about 10 s of illumination time. Approximately 10 microorganisms could be visualized in the 100 μm^2 field, and the 520 cm^{-1} band of a silicon wafer was used for frequency calibration.

Data Fitting and Analysis for Algorithm Development

Data processing and analysis were performed using GRAMS/AI Spectroscopy Software. Averaged spectra (~5 spectra per sample) were imported from SPC files then interpolated to 1 wavenumber data point spacing and Fourier filtered to remove high frequency noise

components. The wavenumber range was cut to 500–1700 cm^{-1}, and spectra were normalized to the spectral peak intensity maximum. Spectra were transformed into a “0” or “1” barcode array on the basis of the sign of the second derivative in order to eliminate broad baseline contributions to the multivariate data analysis procedure and reduce the effects of substrate variability.[62] An empirically determined cutoff value, typically at about 10% of the maximum second derivative value, was used as a threshold for the zero-one assignment.

Hierarchical Cluster Analysis (HCA) for Culture-Based Identification

An HCA treatment was used for preliminary grouping of this data set. The data set was analyzed for the similarity between all spectra represented by their barcodes described above. This similarity was expressed by an index representing Euclidean distance between barcode vectors. To build a hierarchy of similarities, we used an agglomerative approach where each spectral vector starts in its own cluster, and pairs of clusters were merged as one moves up the hierarchy. A standard Ward’s algorithm was used for distance similarity linkage.[69]

Partial Least Squares-Discriminant Analysis (PLS-DA) for Identification of Spiked Sample

The data set was first analyzed for separation and classification between positive and negative spiked blood samples. Positive samples were further subjected to subclassification into *E. coli* and *S. aureus* strains, and PLS-DA was used for both classifications.[70] PLS-DA is an algorithm in which partial least-squares regression is used to rotate principal component analysis loading vectors in order to obtain maximum separation between classes. Each sample is assigned a probability of belonging to a particular class. Model training was carried out using k-fold cross validation, where k was determined by the sample size; here, we used a 5-fold cross validation. Cross validation corrects for the training error and derives a more accurate estimate of model prediction performance.

Safety Considerations

All work with blood and micro-organisms was performed in a biosafety cabinet in a BSL-2 laboratory wearing the proper personal protective equipment. Transportation of samples between laboratories was completed by placing the samples in a double containment system.

RESULTS AND DISCUSSION

Pathogens in the blood that are inappropriately treated are an increasing public health problem.[71] The delay in appropriate antibiotic therapy for bacteremic patients causes increased morbidity and mortality, as well as a rise in antibiotic resistance due to widespread and unsuitable prescription of broad-spectrum antibiotics. To combat this trend, new rapid diagnostic methods must be developed to enable physicians to accurately and rapidly treat bacterial pathogens.[72] We developed a system that provides physicians identification of the pathogens directly from whole, uncultured blood within 7 h. Processing whole blood circumvents the need for positive blood cultures, which can take days before a definitive result is obtained. This allows for a much faster analysis of the viable microorganisms for a more rapid diagnosis and treatment.

Microbial Recovery from Sample Preparation Process

The universal sample preparation process was used to remove blood components from 10 mL of whole human blood and provide a 200 μL output of concentrated microorganisms. This process employs a bacterial concentrator (Figure 2), which has been previously described.[65] Compared to the published method, our modified sample preparation process described here boasts reductions in time (entire process takes less than 30 min) and in blood debris residuals (approximately 10 μL) from the lysis process. Using this faster, cleaner sample preparation process, we were able to successfully recover viable micro-organisms from 10 mL of blood spiked with clinically relevant concentrations of microbes (Figure 3). We measured the recoveries of Gram-positive and Gram-negative bacteria, as well as the yeast *Candida albicans*, by standard quantitative plating methods. This panel of microorganisms was chosen since it contains a variety of clinically relevant species that are frequently associated with bacteremia. At initial concentrations of ~2 CFU/mL for 10 mL of blood, all tested microorganisms had mean recoveries ≥55% (Figure 3). At an initial concentration of 11 CFU/mL, the recoveries for all micro-organisms were ≥72%, with the exception of *P. aeruginosa* (39%). *P. aeruginosa* recoveries at this higher concentration of 11 CFU/mL were lower than for the 2 CFU/mL concentration; this could be due to the stochastic sampling when working at such low concentrations of bacteria. Recoveries greater than 100% for *S. aureus* are possible due to disruption of clusters of bacterial cells, as well as microbial growth over the course of an experiment. Since this method relies on a preferential blood cell lysis process and centrifugal forces to concentrate the microorganisms, variations in the specific gravity, cell membrane, and its associated extracellular matrix could negatively impact recoveries of viable micro-organisms. However, these limitations could be overcome with further improvements to the detection technology, such as integrating an automated scanning system to image entire SERS chips. By automatically scanning each chip, our limit of detection would also improve. It has been shown that one CFU can be detected, if the laser beam, with a cross-sectional area of 10 μm by 30 μm, is focused directly on the bacterium.[58] Automatically scanning across the surface of the 1 mm^2 chip would be extremely beneficial in achieving single bacterium detection routinely since manual scanning is time-consuming and labor intensive. In these tests, we manually scanned each chip for less than 5 min; consequently, a relatively high concentration of bacteria was required for ease of locating these bacteria on the surface of the chip.

The goal of the sample preparation optimization was for the recoveries of all the microorganisms to be ≥40%, even at the lowest inoculum (~2 CFU/mL) such that the processed sample would have at least several viable bacteria. The recoveries of the microorganisms were evaluated using Bayesian analysis to determine the probability that a recovery of ≥40% would occur given a 10 mL sample of blood spiked with a known quantity of microorganisms. The Bayesian algorithm concluded that all of the tested microorganisms at both 2 and 11 CFU/mL concentrations had greater than 93% probability that their recovery would be >40%. Given the high probability of this event using contrived samples, it is likely that with patient samples the technology will be able to concentrate live microorganisms, as we demonstrated by quantitative plating. Further, since the output of the bacterial concentrator is composed of viable microbes in a small aliquot isolated from

confounding growth inhibitors (e.g., antibiotics in the patient's blood), the sample preparation process could be combined with a variety of other downstream methods that subsequently cultivate the bacteria. In our approach, we cultured the bacteria for a short time and only used part of that sample for SERS analysis. The rest of the sample was therefore available for subsequent antibiotic susceptibility testing as would be done if this method were to be integrated into an existing clinical laboratory workflow.

Although only contrived samples were surveyed here, we have explored many variables, demonstrating our sample preparation process is a robust technique that would be suitable for clinical samples. We have tested numerous healthy patient samples (>1500 donors) during our process development, and our method is relatively immune to physiological differences among healthy donors. We have also tested freshly drawn human blood with similar results. However, blood samples from bacteremic patients will have a triggered immune response, such that the blood samples will have a large increase in leukocytes, inflammatory mediators, antibodies, and complement proteins, among other complexities, compared to our healthy donor blood. To address whether our process would be likely to work on such samples, it was tested on blood from rodents with experimental *S. aureus* bacteremia[73,74] with good bacterial recoveries, similar to Figure 3 when assessed with quantitative plating. However, due to inherent differences in human and rodent blood, there was a significant increase in residual blood debris from the rodent blood model compared to whole human blood, even when sterile rodent blood was processed. The increase in blood debris seemingly inhibited consistent accurate analysis of the samples by SERS.

Species-Specific SERS Identification from Culture

To assess the utility of the SERS approach for identifying the pathogens implicated in bacteremia, we amassed a library of SERS spectra from 115 microbial strains that included clinical isolates from blood cultures ($n = 68$) and reference bacterial strains ($n = 47$). We chose a subset of 17 of the most clinically relevant bacterial species as a model for developing our classification algorithm. The SERS spectra of the selected strains are shown in Figure 4. The displayed spectra are averages of approximately 5 individual spectra and are normalized to the maximum peak intensity for each spectrum. Based on their SERS spectra, each species was successfully classified as a distinct group by our algorithm (Figure 4). Briefly, a "barcode" procedure was used to reduce the spectra to a series of "0"s or "1"s based on the sign of the second derivative at each spectral frequency.[62] This spectral transformation step has been found to improve classification precision by minimizing effects due to broad variable spectral background and spectral variability resulting from substrate inhomogeneities. A two-stage procedure was developed for robust classification for this data set, and the results are represented in a dendrogram (Figure 4). All spectra were first separated into four groups by a hierarchical cluster analysis (HCA) of the barcoded spectra. A subsequent partial least-squares-discriminant analysis (PLS-DA) then resulted in the demonstrated separation of all organism classes within each of the four color-coded clusters.[70] For each of the HCA clusters, one example of the PLS-DA classification success is shown. Using this procedure, each species was correctly classified using the corresponding 162 SERS spectra. We have previously shown that 785 nm excited bacterial SERS spectra are due to six main nucleotide degradation products: adenine, hypoxanthine,

guanine, xanthine, uric acid, and AMP.[61] The four groupings developed for this classification procedure are found to be correlated with SERS signatures arising from these different components. For example, the blue group is dominated by adenine, the light green group is dominated by vibrational features of hypoxanthine, the dark green group has features from hypoxanthine, xanthine, and some adenine, and the red group shows features of adenine, hypoxanthine, guanine, xanthine, and uric acid. The classification model shown here is not unique, but demonstrates how these SERS signatures can be used for bacterial identification. Although only 17 species were used in this example, we anticipate it should be straightforward to append additional species to the algorithm. Since the microorganisms were grown in ideal conditions (Table S1), it is possible that their SERS spectra will be different than those of microorganisms that are found in the blood of patients. Because SERS spectra result from purine degradation in response to the low nutrient wash before signal acquisition,[61] the characteristic spectral features should still be evident in microorganisms recovered from clinical samples. Additional SERS spectra will need to be obtained to determine the variability, if any, of the SERS spectra for microorganisms in different environmental conditions.

Identification of Bacteria Inoculated into Whole Blood

To demonstrate the effectiveness of the combined sample preparation and SERS detection methodology, we designed a blinded study wherein 10 mL of human blood samples were inoculated with log-phase bacteria (*E. coli* ATCC 25922 or *S. aureus* ATCC 25904) or remained sterile. Since this was a preliminary demonstration of our complete system, we chose a common Gram-negative rod and a Gram-positive coccus to demonstrate our system's utility. Of the 91 samples tested, 59 samples were inoculated with log-phase *E. coli* or *S. aureus*, and the rest of the samples were negatives. These samples were immediately processed using our universal sample preparation process, incubated at 37 °C, analyzed by SERS, and identified using our algorithm, which was optimized for identifying bacteria from blood. At starting concentrations ranging from 10^1–10^4 CFU/mL in blood, we achieved 97% specificity and 88% sensitivity after processing the spiked whole blood and analyzing the results of the SERS spectra using an algorithm based on PLS-DA. Of the spiked samples, both bacteria were also correctly identified 97% of the time (Figure 5). The positive predictive value was 98% and the negative predictive value was 82%. Given these figures of merit and the relatively short processing time, it can be postulated that this process has the potential to be informative to physicians for rapidly identifying pathogens from a bacteremic patient. However, before translation is possible, further validation of the methodology is required on a larger panel of micro-organisms (including clinical isolates), as well as mixtures of microorganisms, to fully optimize the method's performance. The outcome demonstrated here indicates that, from this albeit limited classification result, this approach is promising and future work will be required to extend these blind testing studies to include greater numbers of classification groups. However, the non-blinded results (Figure 4) indicate that such an extension may be possible.

Because only a small aliquot of the sample was used for SERS analysis, the rest of the sample was available for further testing. Thus, our diagnostic test would easily fit as a screening tool in the clinical workflow. Microbial identification in a clinical microbiology

laboratory is historically dependent on a hierarchy of assays: Gram-stain, propagation, metabolic/phenotypic analysis, and finally, antibiotic susceptibility testing.[75] While an aliquot of sample is being screened by SERS for quick identification, the remainder of the sample could be used for traditional techniques that require live cells, as described above. Furthermore, since our sample preparation technique separates the microbes from the blood components and any inhibitory factors in the blood, culturing after isolation could be more robust to the confounding effects of antimicrobials that have been administered to the patient before collection. The integration of our identification technique in the clinical workflow can provide rapid identification information while still maintaining the traditional stages of microbial identification as confirmatory tests.

Currently, the process is somewhat labor intensive (i.e., pipetting and handling of vials and valves). To streamline the process flow, an individual platform will be developed that can combine our lysis process with centrifugation to rapidly isolate microorganisms directly from 10 mL of blood. The entire sample preparation process will occur in a disposable cartridge with minimal sample handling. The cartridge's design will be based on our current concentration device, however, it will be easier to manufacture, as it will be injection-molded instead of machined (Figure 1). The geometries, surface roughness, and surface chemistry coatings of the concentration devices have already been optimized to effectively focus the microorganisms into the sample collection valve. The final result is a <200 μL sample, free of blood components, that is clean enough for the method to be considered a universal sample preparation process.

CONCLUSION

In this paper, we describe a technique to provide species-specific identification of bacteremic samples using SERS. The sample preparation process provides a rapid method that takes 10 mL of whole blood and produces a μL-sized output of viable microorganisms (if there are any present) for downstream processing. SERS was able to accurately identify blinded samples of cultured isolates, as well as blinded samples of *S. aureus*- or *E. coli*-spiked whole blood with excellent specificity and sensitivity. Since only a fraction of the output was used for SERS, the remainder of the sample was available for additional testing, such as Gram staining, culture, or PCR. This illustrates the universality of the sample preparation process as it can be paired with any number of downstream identification technologies, confirmatory tests, or integrated into the clinical workflow. From sample-to-identification, this technology has the ability to revolutionize the way bacteremic samples are screened for pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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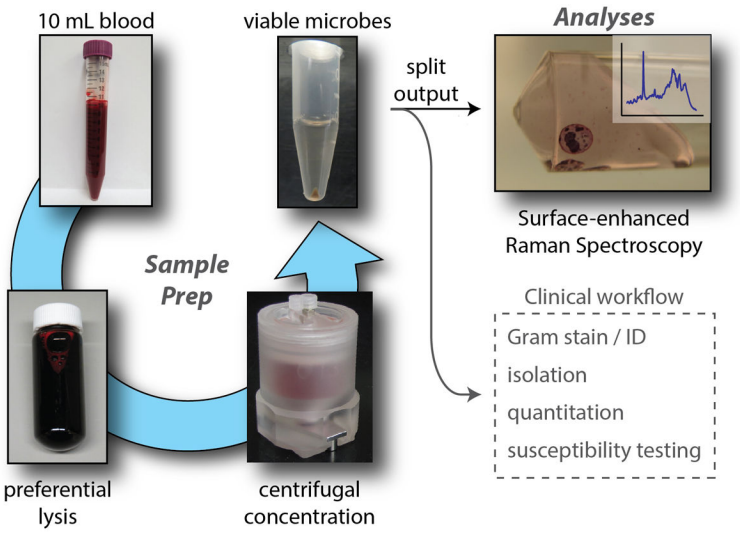

Figure 1.

Technology overview for viable microbe recovery from human blood. A total of 10 mL of whole blood is processed to produce a 200 μL output, which is incubated briefly prior to identification by SERS or for integration into a standard clinical workflow.

Figure 2.
Image of the microbial concentration device. An expanded view of the individual parts of the machined device.

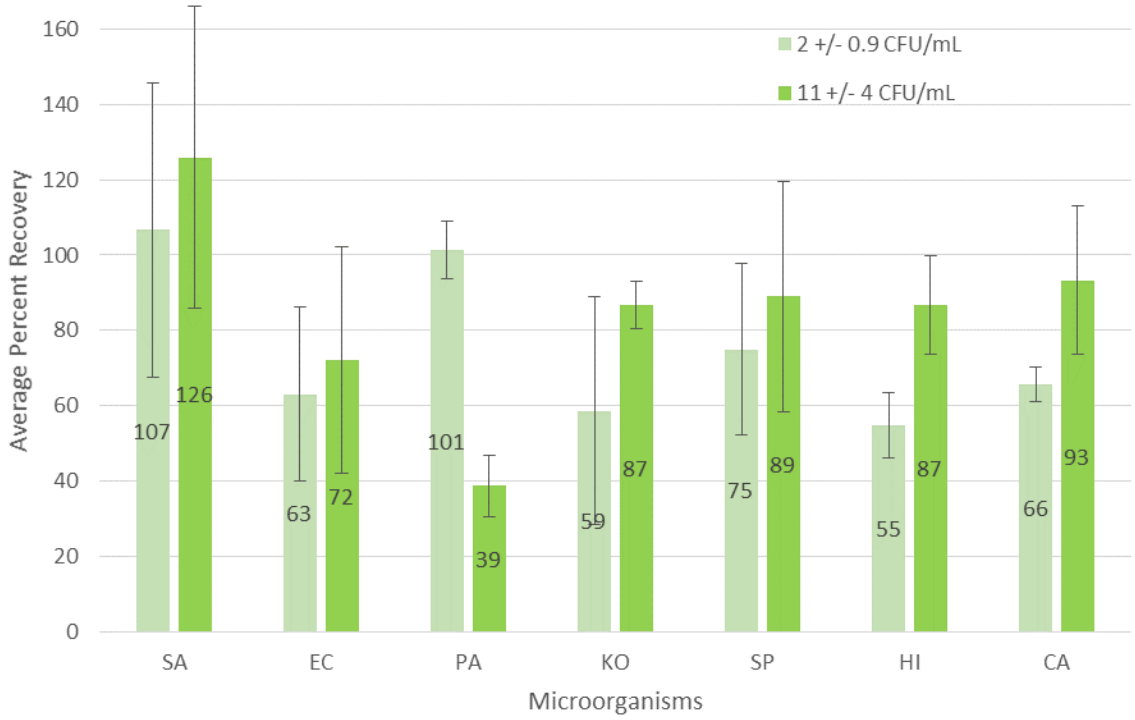

Figure 3.

Graph showing average percent recovery of viable microorganisms following our universal sample preparation process. Microorganisms were inoculated into 10 mL of sterile human blood at a concentration of 2 or 11 CFU/mL. Data are means of $n \geq 3 \pm 1$ SD. SA, *S. aureus* ATCC 25904; EC, *E. coli* ATCC 25922; PA, *P. aeruginosa* ATCC 27853; KO, *K. oxytoca* ATCC 13182; SP, *S. pneumoniae* ATCC 49619; HI, *H. influenzae* ATCC 49247; CA, *C. albicans* ATCC 18804.

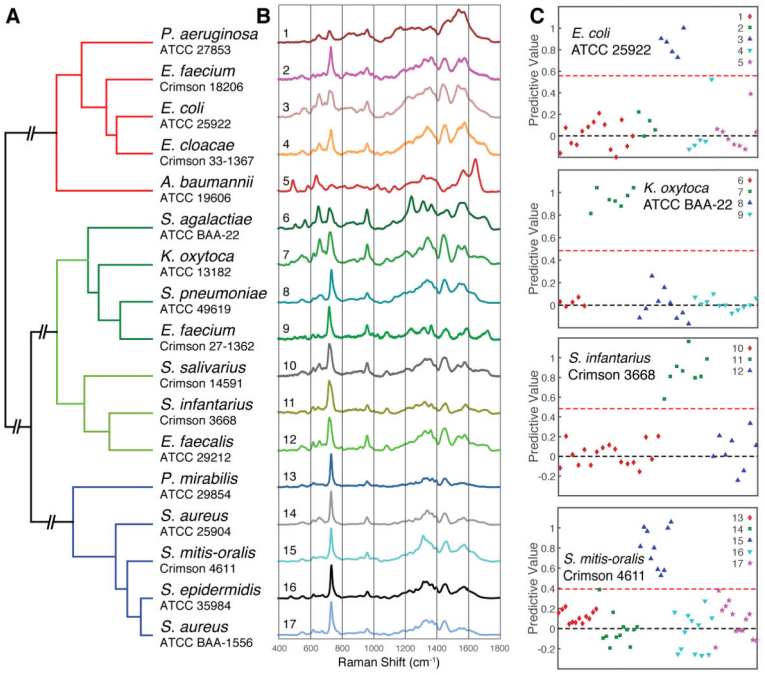

Figure 4.

Dendrogram (A) illustrating the separation of 17 bacterial species from culture using an algorithm based on the barcodes of each of the SERS spectra shown in the center panel (B). Each spectrum is an average of at least five individual spectra that have been baseline corrected and normalized. Examples of the separation between microorganisms in the same cluster are presented in the four boxes on the right (C). In each box, the black dashed line indicates 0 on the *y*-axis and the red dashed line marks the threshold value that best splits the classes with the least probability of false classifications. The threshold is determined by assuming that the predicted values for each class are approximately normally distributed. The point where these two distribution curves intersect is the threshold value.

A

Samples	No bacteria	*E. coli* ATCC 25922	*S. aureus* ATCC 25904
0 CFU Spike	32		
10^2 CFU Spike		5	4
10^3 CFU Spike		10	10
10^4 CFU Spike		10	10
10^5 CFU Spike		5	5
Total	32	30	29

B

Identification	Spiked *S. aureus*	Spiked *E. coli*
$SERS^+$ for *S. aureus*	28	1
$SERS^+$ for *E. coli*	1	29
Correct ID	97% (28/29)	97% (29/30)

C

Detection	Bac+	Bac-	
$SERS^+$	TP: 52	FP: 1	PPV: 0.98
$SERS^-$	FN:7	TN: 31	NPV: 0.82
	SENS: 0.88	SPC: 0.97	ACC: 0.91

Figure 5.

Breakdown of numbers of samples processed and analyzed by SERS (A), identification results for simulated bacteremic blood samples (B), and contingency table for negatives and simulated bacteremic blood samples (C). In the contingency table, Bac+ or Bac− indicates whether bacteria were added to the sample (Bac+) or not (Bac−), and $SERS^+$ and $SERS^-$ indicates whether the algorithm determined if there were bacteria present in the sample ($SERS^+$) or not ($SERS^-$) from the blinded SERS spectra. TP, true positive; FP, false positive; FN, false negative; TN, true negative; PPV, positive predictive value; NPV, negative predictive value; SENS, sensitivity; SPC, specificity; ACC, accuracy.