NAVAL MEDICAL RESEARCH UNIT SAN ANTONIO

SURFACE ENHANCED RAMAN SPECTROSCOPY FOR THE RAPID DETECTION AND IDENTIFICATION OF MICROBIAL PATHOGENS IN HUMAN SERUM

CHRISTIAN N. KOTANEN, PHD; LUIS MARTINEZ, PHD; RENE ALVAREZ, PHD

IMMUNODIAGNOSTICS AND BIOASSAY DEVELOPMENT DEPARTMENT
COMBAT CASUALTY CARE AND OPERATIONAL MEDICINE DIRECTORATE

NAMRU-SA REPORT # 2015-15
Approved for public release; distribution is unlimited
DECLARATION OF INTEREST

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government. This work was funded by work unit number G1211. The authors are employees of the US Government. This work was prepared as part of their official duties. Title 17 USC §105 provides that ‘copyright protection under this title is not available for any work of the US Government.’ Title 17 USC §101 defines a US Government work as a work prepared by a military service member or employee of the US Government as part of that person’s official duties.
Reviewed and Approved by:

John Simecek, DDS, MPH
Director, Combat Casualty Care & Operational Medicine
Chair, Scientific Review Board
Naval Medical Research Unit San Antonio
3650 Chambers Pass, BLDG 3610
Fort Sam Houston, TX 78234-6315

Date

CAPT Rita Simmons, MSC, USN
Commanding Officer
Naval Medical Research Unit San Antonio
3650 Chambers Pass, BLDG 3610
Fort Sam Houston, TX 78234-6315

Date
# Table of Contents

Abbreviations ........................................................................................................................................ 4
Executive Summary .............................................................................................................................. 5
Introduction .......................................................................................................................................... 6
Materials and Methods ....................................................................................................................... 8
Results ................................................................................................................................................ 12
Discussion ......................................................................................................................................... 21
Conclusions ....................................................................................................................................... 26
Military Significance .......................................................................................................................... 27
Supplemental Figures .......................................................................................................................... 31
ABBREVIATIONS

%CV            Coefficient of variation  
AgNR           Silver nanorod          
*A. baumannii*  *Acinetobacter baumannii*  
ADP            Adenosine diphosphate   
ATP            Adenosine triphosphate  
CCC            Combat Casualty Care    
CFU            Colony forming units    
ddH₂O           Ultrapure, distilled, deionized water  
e-beam          Electron beam          
*E. coli*      *Escherichia coli*     
FAD            Flavine adenine dinucleotide 
FYSA           For your situational awareness 
ICU            Intensive care unit     
*K. pneumoniae*  *Klebsiella pneumoniae*  
NAD            Nicotinamide adenine dinucleotide 
OD             Optical density        
P. aeruginosa   *Pseudomonas aeruginosa*  
PC             Principal component      
PCA            Principal component analysis 
PCR            Polymerase chain reaction 
PDMS           Polydimethylsiloxane    
PLSDA          Partial least squares-discriminant analysis 
PRELOADED      Fresh pure cultured bacteria suspended in ddH₂O   
RECOVERED      Bacteria that underwent lysis filtration and resuspension in ddH₂O  
*S. aureus*    *Staphylococcus aureus*   
SERS           Surface Enhanced Raman Scattering  
STDEV          Standard deviation      
TSB            Tryptic soy broth      

EXECUTIVE SUMMARY

Background: Bacterial diagnostic techniques take between 24 to 48 hours and require plating, growth and examination of colony morphology or color for identification. Biosensors based on Surface Enhanced Raman Scattering (SERS) spectroscopy hold great promise as a platform for rapid and sensitive detection of bacterial pathogens by decreasing time of diagnosis and preventing infection related mortality and morbidity.

Objective: The objective of this study was to characterize and evaluate a SERS-based diagnostic system for the detection and identification of bacteria in pooled human sera.

Methods: Species of Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, and Pseudomonas aeruginosa were individually inoculated into pooled human serum samples. Samples were processed by lysis filtration to separate and isolate bacteria. Processed bacterial samples were incubated onto silver nanorod substrates at 60 °C for three hours. Measurement of bacterial SERS spectra was carried out utilizing a handheld Raman spectrometer having a standard fixed wavelength of 785 nm, a spot size of 100 µm in diameter, power of 75 mW, and measurement time of 500 ms. Spectra of bacteria recovered from serum were compared to spectra of pure culture bacteria. Principal Component Analysis and Partial Least Squares Differential Analysis were performed to determine uniqueness and commonalities of measured spectra.

Results: The successful detection, identification, and classification of bacteria from human serum using a hand-held Raman spectrometer were demonstrated. Pure culture bacteria were readily identifiable and distinguishable by their SERS-based “molecular fingerprints” at the species level. Hydrophilic bacteria were readily detected and identified from serum samples without significant changes occurring to their spectra due to sample processing. Shifts in relative peak intensities of SERS spectra were observed primarily for hydrophobic bacteria after recovery from serum.

Conclusions: Hydrophilic bacteria recovered from serum using lysis filtration can be readily identified by SERS. However, bacteria sensitive to lysis filtration will require additional reference criteria for SERS identification.
INTRODUCTION

Bacterial infection is a frequent complication among trauma and surgical patients, both civilian and military. Damage to soft tissues and organs with accompanying immune system suppression enables opportunistic pathogens to colonize and infect [1]. Patients with infection are more likely to be admitted to an ICU, require extensive debridement, have longer lengths of stay in hospital, and have a higher risk of early mortality [2-4]. Adverse outcomes within one year after injury with accompanying infection include increased risk of death and repeat hospitalization, greater dependence on healthcare, decreased likelihood to return to work, and diminished overall function [5]. According to the World Health Organization, the prevalence of all healthcare associated infections is 4.5% in the United States, 7.1% in Europe, and 15.5% in underdeveloped countries [6]. The rate of infection in the United States military is approximately 25% for combat casualties, with the rate approaching 50% in wounded warriors requiring intensive care treatment (ICU).

The significant limitations of current standards of diagnosis can lead to delayed interventions and poor prognosis. Military trauma patients in transit to higher echelons of care presents a challenge to healthcare providers due to the disparity in technology available to caregivers outside the hospital setting [7]. Colonization of combat casualties by infectious pathogens increase during transit through evacuation chains [8] and require reevaluation at each level of care. Culture-based techniques for standard diagnosis take between 24 to 48 h and require plating, growth and examination of colony morphology or color to identify bacteria [9]. The exceedingly long turnaround times required for culture-based methods led to the development of DNA- and enzyme-based assays utilizing nucleic acid and antibody probes for identification and quantification of target bacterial cells. Diagnosis using traditional polymerase chain reaction (PCR) requires technical expertise and sophisticated laboratory equipment. Furthermore, assays with PCR typically require species and/or strain specific probes that may or may not be available for a particular organism. The instability of DNA and antibodies in harsh environments can limit their applicability for use outside of controlled settings.

Advancement in diagnostic capabilities is an important step to improving outcomes of trauma-related infections. Rapid diagnosis, treatment, and control of bacterial infections are necessary to reduce morbidity and mortality. Raman spectroscopy combined with
nanotechnology is a potential platform upon which an effective novel diagnostic system can be developed. Spectroscopic techniques are fast, efficient, and have the potential to reduce the overall time of diagnosis while requiring little to no technical expertise and additional equipment. Raman spectroscopy is a vibrational spectroscopic technique for ‘biomolecular fingerprinting’ of tissues, cells, proteins, nucleic acids, and other small organic and inorganic compounds. The technique boasts applications in several fields of study including archaeology, arts, agriculture, environmental science, geosciences, astrobiology, forensics, and material science. In the medical field, Raman spectroscopy is under investigation for use in prediction of preterm birth [10], identification of basal cell carcinoma [11], identification of leukemia cells [12], diagnosis of dysplasia in Barrett’s esophagus [13], and identification of prostate cancer [14].

The addition of nanotechnology further improves the capabilities of Raman spectroscopy. Surface Enhanced Raman Scattering (SERS) was developed by utilizing nanoparticles as enhancing substrates to improve overall Raman signal intensity, resolution and limits of detection. Silver nanorod (AgNR) substrates for example have recently been developed for detection and identification of viral and bacterial pathogens [15] such as E. coli [16], pathogenic mycoplasmas [17], and highly infectious viral pathogens such as the human immunodeficiency virus [18]. Enhancement factors of up to $5 \times 10^8$ have been reported with the length of nanorods having a direct influence on the measured SERS signal intensity [15, 19]. Identification and discrimination of bacteria at the species and strain level using AgNR substrates has been demonstrated [20]. The rapid measurement time, ease of operation, and potential field deployability makes this technology a promising alternative infection diagnostic tool for military and civilian caregivers alike. The objective of this research was to characterize and evaluate a SERS diagnostic system consisting of a handheld Raman spectrometer and multiwell AgNR substrates for detection and identification of five different species of pathogenic bacteria recovered from human serum.
MATERIALS AND METHODS

Chemicals and reagents

Reagents, common buffers and chromatography grade solvents were purchased from Sigma-Aldrich (St. Louis, MO). UltraPure distilled water (ddH₂O) was purchased from Life Technologies (Grand Island, NY), nutrient agar was purchased from BD Biosciences (San Jose, CA), Tryptic soy broth (TSB) was acquired from BD (Franklin Lakes, NJ), Luria Bertani broth was acquired from Amresco (Solon OH) and nutrient broth was acquired from Sigma-Aldrich. Filters of 0.45 µm pore diameter were purchased from EMD Millipore (Billerica, MA). Lysis buffer of 0.6% polyoxyethylene-(10)-oleoyl ether in 0.4 M 3-(cyclohexylamino)-1-propane sulfonic acid at pH = 11.7 was prepared followed by filtration through a 0.2 µm filter. Human serum was obtained from Bioreclamation and pooled.

Silver nanorod substrate preparation

Silver nanorod substrates were acquired from Argent Diagnostics Inc. (Athens, GA). Substrates were prepared by oblique angle deposition technique using an electron beam (e-beam) evaporation system [15, 16, 21]. Briefly, glass microscope slides were cleaned with piranha solution (80% sulfuric acid and 20 % hydrogen peroxide by volume) for 10 min followed by rinsing with deionized water and drying with nitrogen gas. Glass slides were loaded into the e-beam deposition system with the substrate surface perpendicular to the incident vapor direction. A film of 20 nm Ti and followed by 200 nm Ag were evaporated onto glass slides at a rate of 0.2 nm s⁻¹ and 0.3 nm s⁻¹, respectively. Substrates were rotated 86° with respect to the incident vapor and silver nanorods were deposited at a rate of 0.3 nm s⁻¹. Thickness was measured using a quartz crystal microbalance. Substrates with nanorod length of 2000 nm were fabricated. A patterning plate mold was applied to the substrate surface and filled with polydimethylsiloxane to form a 4x10 array of wells having 3 mm diameter and 1 mm depth.

Bacterial culture and cell count determination

Bacterial species of Acinetobacter baumannii (A. baumannii, ST-3), Escherichia coli (E. coli, ST-11), Klebsiella pneumoniae (K. pneumoniae, ST-9), Pseudomonas aeruginosa (P. aeruginosa, ST-14) and Staphylococcus aureus (S. aureus, ST-19) were obtained from
Bioreclamation (Westbury, NY). Bacterial cells were grown overnight in TSB media at 37 °C in a shaker at 250 rpms. The optical density (OD) of the cells was measured by absorbance spectrometry at 600 nm wavelength. Cultured cells were initially normalized to 1 OD by centrifugation and resuspension in an appropriate volume to bring the concentration to 1 OD/mL. Washing was performed by centrifugation at 14,000 rpms for 5 min, discarding the supernatant and resuspension with ddH₂O. The washing process was repeated three times to remove all broth components followed by a final resuspension of the pellet in ddH₂O back to 1 OD. Cell count was determined by plating the 10⁴, 10³, 10² and 10¹ cell dilutions on TSB Nutrient Agar media. Colony forming units (CFU) were counted the following day to confirm bacterial species concentrations of fresh cultures at 1 OD suspended in ddH₂O. Fresh pure cultured bacteria suspended in ddH₂O will be referred to as PRELOADED bacteria.

**Lysis filtration and recovery of bacteria**

Lysis filtration is a simple and well established method by which bacteria can be separated from biological matrices such as serum or even whole blood. A lysis filtration method for separation of bacteria from whole blood was adapted from Fothergill et al. [22]. PRELOADED bacteria suspended ddH₂O at 1 OD were first concentrated by an order of magnitude via centrifugation and resuspension in ddH₂O. Concentrated bacteria were spiked into pooled human serum, 100 μl bacteria into 900 μl serum, and briefly vortexed to generate a bacterial concentration of 1 OD in serum. For purification of bacteria, 500 μl of lysis buffer was added to the serum-bacteria mixture and incubated at room temperature for 2 min. The lysate was then passed through a 0.45 μm filter with vacuum. The filter was removed from the vacuum apparatus and placed in a micro-centrifuge tube with 1 mL of ddH₂O. The micro-centrifuge tube was vortexed to remove bacteria from the filter for 5 min. The filter was discarded and the micro-centrifuge tube was centrifuged for 5 min at 14,000 rpm. Supernatant was discarded and pellets were washed/centrifuged three times in wash buffer and three times with ddH₂O. The resulting pellet was re-suspended in 1 mL of ddH₂O, back to the original volume prior to filtration. Serial dilutions were prepared from the recovered ddH₂O suspended bacteria and plated on TSB Nutrient agar media. Colony forming units were counted the following day to confirm bacterial concentrations of the recovered bacterial lysates. Bacteria that underwent lysis filtration and resuspension will be referred to as RECOVERED bacteria.
Evaluating bacterial viability

The percentage of viable cells was determined for RECOVERED bacteria. LIVE/DEAD® BacLight™ Bacterial Viability Kits were acquired from Life Technologies (Carlsbad, CA). A kill curve was prepared for each bacterial strain at $10^8$ CFU/mL in ddH$_2$O. Cell count percentages of 0, 25, 50, 75, and 100% of 1 mL cultures were separated and exposed to 100% isopropanol for 1 h. Cells killed by isopropanol were washed with ultrapure ddH$_2$O and recombined with the remaining untreated bacteria to create the varying ratios of live to dead bacteria. SYTO Green and Propidium Iodide were mixed in a 1:1 ratio and 3 µL of this mixture was added to 1 mL of RECOVERED bacteria and kill curve samples. Samples were incubated for 15 min at room temperature in the dark. Fluorescence readings were taken at an excitation of 485 nm and emission readings at 530 nm for SYTO Green and 630 nm for Propidium Iodide. The ratio of the SYTO Green/Propidium Iodide was calculated to give the ratio of live to dead bacteria.

SERS measurements

All bacterial samples were pipetted into substrate wells and allowed to dry for 3 h at 60 °C. Two different cultures of each bacterial species were examined for both PRELOADED and RECOVERED bacteria. Each recessed well holds 10 µl microliters of solution. A 10 µl sessile drop of water requires approximately 1 h to dry at room temperature [23]. Spectra of deionized water evaporated at room temperature for 3 h contributed significantly to background signal. Evaporation of water at 60 °C in a convection oven decreased background signal resulting in responses similar to fresh, unaltered AgNR substrates. Spectral measurements were taken using a handheld Raman spectrometer (FirstDefender RM, Thermo Fisher Scientific Inc.). A laser wavelength of 785 nm, spot size of 100 µm diameter at the focal point, power of 75 mW, and measurement time of 500 ms were utilized for all spectral measurements. The total time of analysis that included substrate loading, drying, and measurement was approximately 4-5 h. In addition to comparing the various species of bacteria, the ability to distinguish between different strains of a single bacterial species was determined using four strains of *S. aureus*: SA-17 (IQ 0070), SA-18 (XEN-40), SA-19 (ATCC 33591) and SA-22 (TCH 1516). A 1:1 mixed culture of *A. baumannii* and *S. aureus* was evaluated to determine the possibility of identifying multiple bacteria.
Chemometrics and data analysis

Data were processed using MATLAB R2013b (The MathWorks Inc., Natick, MA) and OriginPro 9.1 (OriginLab Corporation, Northampton, MA). Chemometric analysis was performed using PLS_Toolbox software (Version 7.5, Eigenvector Research Inc., Wenatchee, WA). Two tailed t-tests using a significance of 0.05 were performed to determine statistical differences between datasets. The limit of detection was the lowest concentration of bacteria that the system is capable of measuring. Limit of detection was calculated by taking the average absolute peak intensity at 735 cm$^{-1}$ for *E. coli* samples at varying concentrations and comparing to background solvent. A statistical difference in peak intensity at 735 cm$^{-1}$ was considered positive for microbial presence. Variability in Raman signal across SERS spectra was determined by averaging the coefficient of variation (%CV) at every frequency.

Principal component analysis (PCA) and partial least squares-discriminant analysis (PLSDA) were performed on SERS spectra using PLS_Toolbox. The statistical tests were used to reduce spectral dimensionality and observe grouping of bacteria for subsequent classification and identification. Preprocessing of spectra for PCA and PLSDA involved taking the first derivative followed by normalization and mean centering. In order to evaluate effectiveness of pure culture bacteria as a SERS reference library, calibration models for PCA and PLSDA were developed using spectra collected for PRELOADED bacteria. Spectra for RECOVERED bacteria, considered the validation set, were projected into the PCA and PLSDA space of the calibration model for classification and comparison. Models of four principal components (PC) for PCA or latent variables for PLSDA were built using a leave-one-out cross validation. Score plots using the first two PCs were generated for calibration and projected validation data set visualization. Similarly, a complex matrix reference library was evaluated by validating RECOVERED bacteria using a model calibration data set of serum RECOVERED bacteria. Strict class predictions were used to provide the safest class assignment. Samples found belonging to multiple classes or no classes were considered unclassified.
RESULTS

Bacterial recovery from serum

Plate counts of PRELOADED and RECOVERED bacteria prepared at a concentration of 1 OD are summarized in Table 1 and Figure 1. PRELOADED bacterial concentrations were not statistically different from one another (p ≥ 0.26). Similarly, RECOVERED bacteria concentrations were not statistically different (p ≥ 0.13). Viable RECOVERED cell concentrations were 1-2 logarithms lower in concentration compared to PRELOADED bacterial concentrations. Changes in concentration were statistically different for E. coli (p = 0.017) and P. aeruginosa (p = 0.046) but not for A. baumannii (p = 0.22), K. pneumoniae (p = 0.28), and S. aureus (p = 0.35). Recovery yields were typically 1-5% of the initial PRELOADED concentrations. The lowest and highest observed recovery yields were for P. aeruginosa and S. aureus respectively, Figure 1. Both A. baumannii and P. aeruginosa experienced cell death due to lysis filtration as shown in Figure 2. The percentage of living RECOVERED bacteria was 2.5% for P. aeruginosa, 68% for A. baumannii, and 100% for all other species.

Table 1. Bacterial concentrations for PRELOADED and RECOVERED bacteria were determined by plate count. Parentheses show standard deviation for n = 2 cultures with 2 measurements per culture for each species.

<table>
<thead>
<tr>
<th>Species</th>
<th>PRELOAD Concentration (CFU/mL)</th>
<th>RELOAD Concentration (CFU/mL)</th>
<th>Percent Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. baumannii</td>
<td>2.6x10^{10} (2.4x10^{10})</td>
<td>2.3x10^{8} (1.1x10^{8})</td>
<td>2.6 (3.4)</td>
</tr>
<tr>
<td>E. coli</td>
<td>1.5x10^{10} (8.8x10^{9})</td>
<td>1.6x10^{8} (9.3x10^{7})</td>
<td>1.6 (1.2)</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>1.7x10^{11} (2.9x10^{11})</td>
<td>5.0x10^{8} (3.9x10^{8})</td>
<td>1.1 (0.9)</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>3.1x10^{10} (2.4x10^{10})</td>
<td>2.0x10^{8} (1.8x10^{7})</td>
<td>0.5 (0.2)</td>
</tr>
<tr>
<td>S. aureus</td>
<td>7.6x10^{11} (1.5x10^{12})</td>
<td>2.1x10^{9} (2.2x10^{9})</td>
<td>10.7 (19.6)</td>
</tr>
</tbody>
</table>
Figure 1. The average cell concentrations of PRELOADED and RECOVERED bacteria for each species at initial concentration of 1 OD as determined by plate count. RECOVERED concentrations were 1-2 logarithms lower than PRELOADED concentrations. Error bars show standard deviation for \( n = 2 \) cultures with 2 measurements per culture for each species (wide error bars are due to logarithmic differences between some data points). * Indicates statistical difference between PRELOADED and RECOVERED concentrations where \( p < 0.05 \).

Figure 2. The average percentage of living RECOVERED bacteria as determined by Live/Dead assay. All species of bacteria except for \( P. \) aeruginosa showed viability after lysis filtration. Error bars show standard deviation for \( n = 2 \) cultures with 1 measurement per culture for each species.

**Bioanalytical sensitivity of handheld SERS**

\( E. \) coli bacteria were used as a model species to determine limits of detection for the handheld SERS system. A limit of detection of approximately \( 10^9 \) CFU/mL was observed for
*E. coli* suspended in water. The SERS intensity band at 735 cm$^{-1}$ was significantly higher ($p = 0.002$) at $10^9$ CFU/mL compared to background spectra of wells containing only deionized water. **Figure 3.** Prominent peaks at 735 cm$^{-1}$ and 1330 cm$^{-1}$ are indicative of the presence of bacteria. Bacterial SERS spectra were not distinguishable from background at concentrations $\leq 10^8$ CFU/mL.

**Figure 3.** The SERS spectra of *E. coli* as a function of bacterial concentration in CFU/mL. A limit of detection of $10^9$ CFU/mL was observed for the system. Each spectrum represents an average measurement from five wells.

**Reproducibility of bacterial SERS**

The reproducibility of absolute SERS signal intensity and normalized SERS signal is summarized in **Table 2**. Calculating the %CV for SERS spectra is a common means of assessing signal reproducibility [24, 25]. Unprocessed SERS signal intensity had greater variability prior to normalization with %CV range of 20-48% for PRELOADED bacteria and 27-47% for RECOVERED bacteria. The change in variability following normalization for both PRELOADED and RECOVERED bacteria is shown in **Figure 4**. Normalizing spectra for each species revealed greater consistency in peak intensity and distribution with a %CV range of 3-13% for PRELOADED bacteria and 8-15% for RECOVERED bacteria. Signal variability increased for most bacterial species after lysis filtration was performed. Spectral reproducibility for *A. baumannii* remained the most consistent between PRELOADED and RECOVERED bacteria.
Table 2. The average %CV for unprocessed and normalized SERS spectra across 779 frequencies between 400-1800 cm\(^{-1}\) for all bacterial species before and after recovery from serum. Parentheses show standard deviation for \(n = 16\) bacterial samples for each average and \(n = 2\) cultures per species.

<table>
<thead>
<tr>
<th>Species</th>
<th>PRELOADED</th>
<th></th>
<th>RECOVERED</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average %CV of</td>
<td>Average %CV of</td>
<td>Average %CV of</td>
<td>Average %CV of</td>
</tr>
<tr>
<td></td>
<td>unprocessed spectra</td>
<td>normalized spectra</td>
<td>unprocessed spectra</td>
<td>normalized spectra</td>
</tr>
<tr>
<td>A. Baumannii</td>
<td>48.1 (3.3)</td>
<td>13.1 (4.5)</td>
<td>47.3 (5.9)</td>
<td>11.6 (6.9)</td>
</tr>
<tr>
<td>E. Coli</td>
<td>22.7 (0.8)</td>
<td>10.0 (1.2)</td>
<td>44.6 (3.1)</td>
<td>14.5 (2.3)</td>
</tr>
<tr>
<td>K. Pneumoniae</td>
<td>25.9 (2.0)</td>
<td>3.6 (1.3)</td>
<td>26.9 (1.9)</td>
<td>9.6 (2.0)</td>
</tr>
<tr>
<td>P. Aeruginosa</td>
<td>20.0 (0.9)</td>
<td>3.3 (2.0)</td>
<td>27.1 (4.2)</td>
<td>7.6 (4.6)</td>
</tr>
<tr>
<td>S. aureus</td>
<td>8.1 (1.6)</td>
<td>5.6 (1.3)</td>
<td>30.3 (0.8)</td>
<td>10.5 (1.6)</td>
</tr>
</tbody>
</table>

Figure 4. Spectra of (A) absolute intensity and (B) normalized intensity of PRELOADED E.coli compared to (C) absolute intensity and (D) normalized intensity of RECOVERED E.coli. Normalization reduced the amount of variation between spectral measurements. A total of \(n = 16\) bacterial samples from \(n = 2\) cultures per species were measured.
Raman spectra of pure culture bacteria and bacteria recovered from pooled human sera

The handheld analyzer generated unique SERS spectra for each bacterial species. Normalized SERS spectra of all five bacterial species are summarized in Figure 5. The total PRELOADED and RECOVERED SERS spectra for all samples can be referenced from supplemental information, Figures S1-S5. All species except for P. aeruginosa displayed characteristic bacterial SERS peaks at 735 cm\(^{-1}\) and 1325 cm\(^{-1}\). Differences among species are marked by differences in relative peak intensities. Noticeable changes in peak intensity occurred for E. coli and K. pneumoniae after recovery from serum. Peak intensity for spectra of K. pneumoniae increased at 654 cm\(^{-1}\) and 794 cm\(^{-1}\) and decreased at 1031 cm\(^{-1}\). Peak intensity for spectra of E. coli increased at 798 cm\(^{-1}\). Both A. baumannii and S. aureus maintained consistent relative peak intensity and distribution after recovery, with a slight increase at 655 cm\(^{-1}\) for S. aureus.

![Normalized intensity vs Raman shift](image)

**Figure 5.** The average SERS spectra of (A) PRELOADED bacteria and (B) RECOVERED bacteria. Shifts in the relative peak intensities are most evident between 600 and 800 cm\(^{-1}\). Each spectra represents an average of \(n = 16\) samples from \(n = 2\) separate cultures.

**Discrimination between bacterial species and strains**

A series of PCA scores plots using the first two PCs were generated for comparison and classification of bacterial species. The first model generated using PRELOADED bacteria showed the first four PCs accounting for 97% of the variance, Figure 6-A, B. All bacteria coming from a pure culture environment were unique and distinguishable from water. RECOVERED bacteria were also unique compared to water except for several samples of
P. aeruginosa that had low recovery yields from lysis filtration. The second model generated using PRELOADED bacteria excluded the spectra for water and had the first four PCs accounting for 93% of the variance, Figure 6-C, D. Spectral reproducibility was greater for bacteria from pure culture compared to RECOVERED bacteria. Distinct clusters were observed for each of the five species of PRELOADED bacteria. RECOVERED bacteria projected onto the second model grouped based on species but with notable overlap amongst some samples.

The third model was generated using a sample set of serum RECOVERED bacteria and had the first four PCs accounting for 86% of the variance, Figure 6-E, F. A second data set of RECOVERED spectra from a separate culture was projected onto the third model. Species distributed similarly in both the model and validation data sets. Insufficient recovery yields of P. aeruginosa prevented its inclusion in the validation data set. More overlap in bacterial species was observed for the third model indicating less specificity.

The SERS spectra of the various S. aureus strains are summarized in Figure 7. Similarities amongst the four strains are evident by the similarities in distribution and intensity of SERS peaks, Figure 7-A, B. Relative peak intensities changed after lysis filtration as was the case for other species. Principal component analysis showed close grouping between the strains. These results indicate that while species level discrimination is possible, differentiation between strains is still a challenge. A mixed culture of A. baumannii and S. aureus (1:1) yielded spectra that were different from pure cultures of either species, Figure 8-A. The PCA score plot confirms this with distinct grouping of individual species and mixed culture data points, Figure 8-B.
Figure 6. Score plots for (A) water and PRELOADED bacteria as a model data set with (B) water and RECOVERED bacteria as an evaluation data set; (C) PRELOADED bacteria as a model data set with (D) RECOVERED bacteria as an evaluation data set; (E) RECOVERED bacteria as a model data set with (F) a second different culture of RECOVERED bacteria as an evaluation set. Each data point represents one sample spectra.
Figure 7. SERS spectra of (A) PRELOADED and (B) RECOVERED *S. aureus* strains with associated PCA score plots for (C) PRELOADED and (D) RECOVERED samples. Data points showed similarities and overlap amongst the various strains indicating that strain level detection is not possible.
Figure 8. (A) Spectra and (B) scores plot for pure culture \textit{S. aureus} (SA), \textit{A. baumannii} (AB) and their mixed culture (AB+SA). Mixed culture AB+SA samples at a 1:1 ratio generated unique spectra compared to either pure culture species demonstrating the potential to identify the presence of polymicrobial infections.

The sensitivity and specificity of bacterial class prediction at the species level for models generated from PRELOADED and RECOVERED bacteria are summarized and compared in Table 3. PRELOADED bacteria as a reference library successfully predicted RECOVERED \textit{A. baumannii} with 94% sensitivity and 88% specificity. RECOVERED \textit{S. aureus} was also predictable but with less sensitivity of 69% and higher specificity of 96%. The four successfully RECOVERED samples of \textit{P. aeruginosa} were also correctly classified. The model based on PRELOADED bacteria was not capable of independently classifying RECOVERED \textit{E. coli} or \textit{K. pneumoniae}. Generation of a library using RECOVERED bacteria as the model enabled the prediction of \textit{E. coli} and \textit{K. pneumoniae}. The RECOVERED model also improved the sensitivity to \textit{A. baumannii} and \textit{S. aureus} and all species could be identified and differentiated with 100% sensitivity and $\geq88\%$ specificity.
Table 3. Sensitivity and specificity of PLSDA models. Utilizing RECOVERED bacteria as a model improved identification sensitivity for all species. \( ^a \) Model generated with four latent variables accounting for 91% of variance; \( ^b \) Model generated with four latent variables accounting for 80% of variance (--- no viable test samples of \( P. \) aeruginosa were available).

<table>
<thead>
<tr>
<th>Species</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( A. ) baumannii</td>
<td>94</td>
<td>88</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>( E. ) coli</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>88</td>
</tr>
<tr>
<td>( K. ) pneumoniae</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>( P. ) aeruginosa</td>
<td>100</td>
<td>100</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>( S. ) aureus</td>
<td>69</td>
<td>96</td>
<td>100</td>
<td>96</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The purpose of the present study was to determine if a SERS diagnostic system could accurately detect and identify pathogenic bacteria from human serum. Two major components of the diagnostic system include sample preparation by lysis filtration and sample measurement by SERS. The process of lysis filtration was characterized first by examining the number of viable bacteria recovered for each bacterial species. Lysis filtration decreased the initial bacteria concentration for all species with statistically significant changes observed for \( E. \) coli and \( P. \) aeruginosa. This finding in conjunction with the 100% viability of species like \( E. \) coli reveals that cells are likely being trapped or retained in filters rather than being killed. This was not the case for \( P. \) aeruginosa as nearly 100% of recovered cells were killed. These results demonstrate the unique effects of the recovery process on each bacterial species. Serum appeared to have an important role in protecting bacteria during the lysis filtration process. Lysis filtering Bacillus subtilis, \( E. \) coli, and \( K. \) pneumoniae suspended in deionized water rather than serum yielded no viable bacteria in almost all cases as determined by plate count (data not shown). This finding indicates that serum components appear to provide some protection to the bacteria from the
detergent used in the lysis buffer. Recovery of viable bacteria may not be necessary for whole cell SERS characterization but it will be an essential consideration for a biosensor system that concomitantly characterizes antimicrobial resistance of detected bacteria. The effects of modifying lysis buffer types and concentration on bacterial viability will need to be further examined. Milder detergents, for instance, may better protect the more susceptible species such as *P. aeruginosa*.

The limit of detection for an infection diagnostic system determines the lowest discernable concentration of bacteria in a test sample that can be successfully identified. Low limits of detection are important for early identification of bacteria. The bacterial limit of detection is a function of substrate design, Raman instrumentation, and sample processing. The observed limit of detection of 10⁹ CFU/mL for the SERS system used in this study was high. Severe septicemia can result in bacterial loads of 10³ CFU/mL circulating in blood [26], which is considerably less than the detection limit of the AgNR handheld SERS biosensor. Although this high detection limit is currently a constraint, clinically relevant low limits of detection can be attained. Silver microwells of 1.2 µm diameter have been reported to have limits of detection that approach 10⁵ CFU/mL for *E. coli* [24], while coating AgNR substrates with vancomycin or surfactants such as Poly(oxyethylene) C18-alkyl ether has been shown to increase bacterial binding, protect substrates from environmental contamination and improve limits of detection down to 10³ CFU/mL [16, 27, 28]. Handheld SERS systems have limitations in their instrumentation which can also adversely impact limits of detection. A tabletop Raman spectrometer would likely improve detection limits greatly in exchange for portability. Applying technological advancements to the nanoparticle substrates and handheld spectrometer hardware will enable the application of the SERS biosensor as a clinical diagnostic.

Reliability of measured spectra in SERS characterization of biomolecules has been a longstanding challenge for the technology and is paramount for accurate diagnosis in the future. Variation in unprocessed spectra reflects Raman signal reproducibility of a single 4x10 substrate. Differences were observed in background intensity and overall Raman signal amplitude. The importance of signal processing was noted by the reduction in %CV range of 8.1-48.1% for all unprocessed spectra down to 3.3-14.5% after processing, Table 2. The observed signal reproducibility is consistent with other silver nanoparticle-based SERS systems measuring live
bacteria [20, 24]. Variability ranging between 24-45% was reported for the first generation of multi-well AgNR substrates during measurement of Raman probe molecule 1,2-Bis(2-pyridyl)ethylene [21]. The notable improvement in consistency of these substrates is likely due to improved manufacturing. Gold nanoparticle coated SiO$_2$ substrates have shown absolute scattering intensity variation of approximately 15% for bacterial samples from a single culture [29]. Close contact between bacteria and substrate helps with reproducibility and signal strength and will depend greatly on the hydrophobicity/hydrophilicity of the bacteria [27]. Accounting for cell hydrophobicity can aid in producing higher quality SERS spectra. This undertaking can be challenging because some bacteria such as $S.\ aureus$ can modify cellular hydrophobicity, which is normally for the purpose of protection from bactericidal activity of host defenses [30].

The observed shifts in bacterial grouping in the PCA scores plots are attributed to the changes in relative peak intensities as observed in the normalized SERS spectra. The handheld SERS system with AgNR substrates measures the extracellular, whole cell spectra of bacteria. If no changes occur to the bacteria, the SERS spectra are a characterization of the cell envelope with intensity correlating to the contents of the bacterial cell wall and plasma membrane. The SERS spectra of PRELOADED samples are representative of the intact, living cell. The relative SERS peak intensity changed for all bacteria to varying extents after separation from serum. All RECOVERED samples of $E.\ coli$ and $K.\ pneumoniae$ were unclassifiable by PLSDA when utilizing a pure culture reference library, which was due to the additional peaks of the RECOVERED samples that were not accounted for by the model data set, Table 3. The change in position of bacterial groupings observed using PCA, Figure 6, reflects the changes to the bacterial cell surface and biomolecular composition due to lysis filtration.

The extent of change in bacterial cell surface composition by lysis filtration can be due to physical and biomolecular disparities between species. Hydrophilic microbes include $A.\ baumannii$ [31] and $S.\ aureus$ [32] whereas $P.\ aeruginosa$ [33], $K.\ pneumoniae$ [34], and $E.\ coli$ [35] are hydrophobic. $A.\ baumannii$ followed by $S.\ aureus$ appear to be the least susceptible to lysis filtration compared to all other bacteria based on the consistency of the scores plots for PRELOADED and RECOVERED samples. RECOVERED $E.\ coli$, while maintaining full viability, underwent biochemical changes such that the SERS peak distribution rendered it
distinct from PRELOADED E. coli. The abstraction of cell membrane proteins, rather than death of bacteria, is likely the cause of change in bacterial SERS spectra as RECOVERED bacteria were viable for nearly all species, Figure 2. Mild non-ionic detergents are effective in solubilizing integral membrane proteins in E. coli with high specificity and having an extraction efficiency closely correlated to detergent critical micelle concentration [36]. Protein extraction would alter Raman peak distribution for the whole cell SERS spectrum of E. coli and potentially other hydrophobic species. Research has shown that changes in the biomolecular composition of cell surface structures and cell metabolic activity are responsible for relative changes in SERS peak intensity [37]. These peak intensity changes must be accounted for when generating reference libraries in the future for use in species identification.

The pattern of major SERS intensity bands observed between 650-800 cm\(^{-1}\) are highly similar to SERS spectra of E. coli from mung bean sprout samples with high intensity peaks at 654 cm\(^{-1}\), 730 cm\(^{-1}\), and 795 cm\(^{-1}\) [16]. Bacterial cell wall constituents normally characterized by SERS include peptidoglycans, phospholipids, glycoproteins, and lipopolysaccharides [38, 39]. Nucleic acid bases generate strong intensity SERS peaks. Origins of common SERS peak intensities at 650 cm\(^{-1}\) and 1248 cm\(^{-1}\) have been assigned to guanine [40], and peaks at 731 cm\(^{-1}\) and 1325 cm\(^{-1}\) are assigned to adenine and adenine containing molecules [41]. The prominent peaks observed at 735 cm\(^{-1}\) and 1330 cm\(^{-1}\), Figure 5, are highly characteristic of bacterial cells [42-45]. Relevant biomolecules containing adenine include DNA, RNA, riboflavin, flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD), adenosine diphosphate (ADP), and adenosine triphosphate (ATP). Peaks at ~730 cm\(^{-1}\) also include contributions from a vibrational mode of the glycosidic ring of polysaccharides such as N-acetyl-D-glucosamine and N-acetylmuramyl acid [46]. Peaks at 790 cm\(^{-1}\) have been assigned out of plane bending of C-H [47]. Vibrational bands between 930-1130 cm\(^{-1}\) have been attributed to membrane phospholipids [48]. Raman peaks at 1030 cm\(^{-1}\) have been assigned to the in-plane C-H bending mode of phenylalanine. The strain of P. aeruginosa examined, XEN41, is a bioengineered strain derived from PAO1 that is constitutively bioluminescent. The bioluminescence may be the cause for the lack of the typical prominent SERS bands expected for P. aeruginosa [49-53].

The lysis filtration technique can also cause bacterial inactivation. Lysis buffers using detergent and high pH have been shown to leave bacteria such as S. aureus physically intact but
with significantly inhibited growth [54]. Based on the results, it is unlikely that bacterial inactivation causes dramatic changes to SERS spectra, but this has not yet been confirmed. Furthermore, increased adenine and guanine molecular signature may be due to increased permeability of DNA, FAD, NAD, ADP, and ATP molecules through the cell membrane caused by compromised cell walls. The influence on SERSs peak distribution of bacterial inactivation and cell membrane integrity will be determined in future studies. It may also be possible to account for, or predict changes to, SERS signal intensity by understanding detergent extraction efficiency of, and specificity toward, membrane proteins and the resultant influence on cell membrane integrity.

Separation techniques can be accounted for by generating a reference library or model using microbes that have undergone separation in addition to those from pure culture. Drawbacks to this approach include the lack of general applicability of the model data set and the time required to generate new reference libraries. For example, iterations of sample preparation may require a new reference data set to be prepared. Research into novel separation technologies and methodologies is currently underway. Nano-sieves based on capillary action can be applicable for protein removal and concentration of microbes without need for vacuum filtration [55]. The use of hydrogels and porous poly(ethylene glycol) microgels have been shown to be effective at trapping bacteria with yields averaging 70% even in the presence of albumin [56, 57].

Dielectrophoresis is a promising method of continuously separating bacteria from biological samples with substrates having sorting efficiencies of 80% and 99% for S. aureus and red blood cells respectively and working limits of $10^6$ CFU/mL [58]. Exclusion of separation and processing steps of biological samples altogether would be ideal. Bimetallic substrates modified with antibiotics have shown potential for SERS compatibility with whole blood without the need for any separation techniques [59]. Generating an easy to use, field-ready platform for separating bacteria from biological samples will be a necessary step in the development of the current generation of SERS technology.

The handheld SERS biosensor successfully identified bacteria at the species level from pure culture and serum samples. Strain level detection was not observed, likely due to the high limits of detection requiring $10^9$ CFU/mL bacteria for measurements. Discrimination between bacterial strains is advantageous because many strains of a single species have differences with
respect to properties such as pathogenesis, antibiotic resistance, biofilm production, and generation of toxins. Many SERS substrates are capable of generating sufficiently large enhancement factors to discern between bacterial strains necessitating spectrometer instrumentation to be on an equal level with respect to measurement sensitivity. Microfluidic SERS systems have achieved strain level resolution with support vector machine accuracy of 92% [60]. Hardware improvements to the handheld SERS biosensor are necessary to reach strain level resolution. Polymicrobial detection is another major concern as more than one species will likely be present in a chronic infection. The separate PCA score grouping of the combined pure culture sample of A. baumannii and S. aureus, Figure 8, demonstrates the potential to identify the presence of a mixed species of Gram positive and Gram negative bacteria. Additional studies will need to be pursued to determine SERS spectra of mixed cultures as a function of more species and varying cell concentration. The effects of serum processing on SERS spectra of polymicrobial populations also need to be addressed.

**CONCLUSIONS**

The effective recovery of live bacteria is important for consistent whole cell SERS characterization. The SERS biosensor utilized in this study was capable of detection, identification, and classification of a majority of the bacteria of military interest sampled from human serum. Bacteria were identifiable at the species level, and the potential for detecting polymicrobial cultures by the unique spectra that they generate was demonstrated. Raman signal for RECOVERED bacteria is likely generated from a combination of inactive cells, active viable cells, and altered cell membranes, but it should be noted that SERS measurement is sensitive to even the smallest changes in biomolecular composition. Lysis filtration successfully purified hydrophilic bacteria without significantly affecting Raman spectra. However, shifts in relative peak intensities of SERS spectra for hydrophobic bacteria due to lysis filtration were seen and must be overcome. Generation of a reference library using pure culture samples can be effective for identifying hydrophilic bacteria such as A. baumannii and S. aureus. Bacteria sensitive to lysis filtration will require a more complex reference library for SERS identification or milder conditions for separation.
MILITARY SIGNIFICANCE

While bacterial and fungal infections are not normally common in individuals with a properly functioning immune system, severe trauma can cause immunosuppression, paving the way for sepsis and invasive fungal infections. This is particularly true for warfighters who suffer from severe trauma on the battlefield. A field deployable detection platform is needed for timely and accurate determination of bacteria associated with infection. Accuracy of diagnosis down to the species level can aid in decision support for antibiotic administration. Our results demonstrate that a label-free SERS technique can be utilized to identify bacterial pathogens of military interest in complex biological matrices such as serum. Future experiments of polymicrobial detection and field-ready separation techniques will continue to make SERS biosensors a viable technology for in the field diagnosis of infection.

References


**Figure S1.** Absolute (A) and normalized (B) SERS spectra of **PRELOADED** *A. baumannii*; Absolute (C) and normalized (D) SERS spectra of *A. baumannii RECOVERED* from human serum by lysis filtration (n = 16 bacterial samples for each average, n = 2 cultures per species).
Figure S2. Absolute (A) and normalized (B) SERS spectra of PRELOADED *E. coli*; Absolute (C) and normalized (D) SERS spectra of *E. coli* RECOVERED from human serum by lysis filtration (n = 16 bacterial samples for each average, n = 2 cultures per species).
Figure S3. Absolute (A) and normalized (B) SERS spectra of PRELOADED *K. pneumoniae*; Absolute (C) and normalized (D) SERS spectra of *K. pneumoniae* RECOVERED from human serum by lysis filtration (n = 16 bacterial samples for each average, n = 2 cultures per species).
Figure S4. Absolute (A) and normalized (B) SERS spectra of PRELOADED *P. aeruginosa*; Absolute (C) and normalized (D) SERS spectra of *P. aeruginosa* RECOVERED from human serum by lysis filtration (n = 16 bacterial samples for each average, n = 2 cultures per species).
Figure S5. Absolute (A) and normalized (B) SERS spectra of PRELOADED *S. aureus*; Absolute (C) and normalized (D) SERS spectra of *S. aureus* RECOVERED from human serum by lysis filtration (n = 16 bacterial samples for each average, n = 2 cultures per species).
# Surface Enhanced Raman Spectroscopy for the Rapid Detection and Identification of Microbial Pathogens in Human Serum

**Authors:** Christian N. Kotanen, PhD; Luis Martinez, PhD; Rene Alvarez, PhD

**Performing Organization:** Naval Medical Research Unit San Antonio
3650 Chambers Pass, BHT-2, Bldg 3610
JBSA, Fort Sam Houston, TX 78234-6315

**Performing Organization Report Number:** Report No. 2015-15

**Sponsoring/Monitoring Agency:** BUMED Core Program / Advanced Medical Development
7700 Arlington Blvd. Suite 5113
Falls Church, VA 22042-5113

**Security Classification:** UNCL

**DISTRIBUTION/AVAILABILITY STATEMENT:** Approved for public release; distribution is unlimited.

**Abstract:**
A rapid and sensitive means to diagnose bacterial agents would decrease the time to diagnosis, allow providers to administer the most effective antimicrobial earlier during the course of treatment and reduce mortality and morbidity due to infection. Biosensors based on Surface Enhanced Raman Spectroscopy (SERS) hold great promise for the rapid and sensitive detection of bacterial pathogens of military interest. The objective of this study was to evaluate a hand-held SERS-based diagnostic system to identify bacteria in pooled human sera using lysis filtration as a means of separating bacteria. Furthermore, the usefulness of pure culture bacteria in generating a reference library for identification of bacterial species recovered from serum was examined. Measurement of SERS spectra was carried out for bacteria from pure culture and bacteria recovered from pooled human sera using lysis filtration. *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were evaluated for generation of SERS-based “molecular fingerprints” followed by Principal Component Analysis and Partial Least Squares Differential Analysis to determine uniqueness and commonalities of measured spectra. The successful detection, identification and classification of bacteria from human serum using a hand-held Raman spectrometer were demonstrated. Pure culture bacteria were readily identifiable and distinguishable by their SERS-based “molecular fingerprints” at the species level, but greater similarities existed in SERS spectra amongst different species recovered from serum. Lysis filtration successfully recovered hydrophilic bacteria without significantly affecting SERS spectra. However, shifts in relative peak intensities of SERS spectra were observed primarily for hydrophobic bacteria after recovery from serum. Hydrophilic bacteria, such as A. baumannii and S. aureus, recovered from serum using lysis filtration can be readily identified by SERS. However, bacteria sensitive to lysis filtration will require a more complex reference library for SERS identification and milder conditions for separation.

**Subject Terms:**
Raman spectroscopy, SERS, bacteria, lysis filtration, rapid diagnostics, silver nanorods

**Security Classification of:**
15a. REPORT UNCL
15b. ABSTRACT UNCL
15c. THIS PAGE UNCL

**Number of Pages:** 37