Rapid Antimicrobial Susceptibility Testing Directly from Blood culture Broth by Surface-enhanced Raman Scattering of Spectral Biomarkers

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Abstract

Background

As effective management of sepsis requires timely administration of appropriate antibiotics, a reliable and rapid antimicrobial susceptibility testing (AST) is crucial. To meet clinical needs, we developed a novel AST, referred to as SERS-AST, based on the surface-enhanced Raman Scattering (SERS) technology. SERS-AST determines antibiotic susceptibility of bacteria based on variations in bacterial SERS signals derived from secreted purines and their derivatives.

Methods

SERS-AST was applied to blood culture samples of patients with bacteremia. Eight common causative organisms, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Enterococcus faecium, Escherichia coli*, *Enterobacter cloacae, Klebsiella pneumoniae*, and *Acinetobacter baumannii* and seven most prescribed antibiotics, including oxacillin, levofloxacin, vancomycin, ampicillin, cefotaxime, ceftazidime, levofloxacin, and imipenem were tested. Receiver operating characteristic analysis was performed to obtain the optimal cutoff SERS signal value to determine the susceptibility of tested bacteria. The results of SERS-AST were compared with those of VITEK 2.

Results

A total of 164 bacterial isolates from blood samples were examined, and the agreement rates between SERS-AST and VITEK 2 results were 96% for Gram-positive bacteria and 97% for Gram-negative bacteria.

Conclusions

By directly assaying positive blood cultures without additional subcultures, SERS-AST can be completed within 4 hours with high accuracy. It can be an alternative AST method to provide critical information to clinicians for timely administration of appropriate antibiotics to treat patients with blood stream infections.

Background

Sepsis is a medical emergency with a high mortality rate, and the prevalence of sepsis and costs related to its management continue to rise (1). It is recommended that obtaining blood cultures and lactate levels before antibiotic treatment and administration of broad-spectrum antibiotics and 30 ml/kg of crystalloid fluid for hypotension be done within 3 hours of presumptive sepsis diagnosis (2). Timely appropriate antibiotic administration is crucial for the outcomes of patients with sepsis (3), but it relies on fast pathogen identification and antimicrobial susceptibility testing (AST). However, conventional bacterial

culture and AST take 3–5 days to complete even with the aid of modern automated microbiological analysis systems (4). As empirical antibiotic usage may not be effective and may contribute to the development of antibiotic-resistant bacterial strains (5), there is an urgent need to develop rapid and reliable AST methods to precisely guide early antibiotic treatment for sepsis.

During the past two decades, tremendous progress has been made in the development of rapid microbiological diagnostics, such as matrix-assisted laser desorption ionization-time of flight mass spectroscopy (6), next-generation sequencing (7), nucleic acid amplification technologies (8), and single-molecule detection. These techniques can provide rapid identification and determine antibiotic susceptibility of causal organisms even for those that are non-culturable or in low concentrations (9). However, the accuracy of these diagnostics may be impaired by unsatisfactory specimen preparations, interference of co-existing human DNA fragments or proteins, or the inability to distinguish dead from live bacteria. Furthermore, most genome- or proteome-based methods are not capable of detecting emerging antibiotic-resistant microorganisms and determining the minimum inhibitory concentration (MIC) of the antibiotics to a certain pathogen.

Surface-enhanced Raman scattering (SERS) is an optical technique for rapid microbiological testing with minimal sample preparation (10). Although successful identification of bacteria by SERS has been reported (11–13), its clinical application remains challenging due to problems such as low reproducibility of SERS signals (14), low concentration of targeted bacteria in clinical samples, and inhibitory substances in samples (15). We have improved the manufacturing process of the SERS device and its sensitivity (16) and have used it to identify various *Mycobacterium* species (17), determine antibiotic susceptibility of certain bacteria (18, 19), and monitor environmental pollution (20) and food safety (21).

The SERS spectra of bacteria determined in our studies (18, 19, 22) are similar to those obtained by other investigators (23–25). Bacterial SERS signals are derived from secreted purines and their derivatives (e.g., adenine, hypoxanthine, xanthine, guanine, uric acid, and adenosine monophosphate) (26, 27). SERS signal patterns, such as the 730-cm^{-1} peak for *Staphylococcus aureus* and the 724-cm^{-1} peak for *Escherichia coli*, are used to determine changes in the concentration of live bacteria after antibiotic treatment (18). We have developed a SERS-based AST, referred as SERS-AST, and performed a proof-of-principle study on 32 *S. aureus* and 43 *E. coli* isolates from blood cultures. The consistency of our SERS-AST results of *S. aureus*-oxacillin (OXA) and *E. coli*-cefotaxime (CTX) combinations was 93% compared with those of conventional AST methods (19).

To apply our SERS-AST to bacteria causing sepsis, more tests on various bacterium-antibiotic combinations are required. As SERS-AST is based on variations in bacterial metabolism in response to antibiotic treatment (28, 29), protocol modifications are needed to test different bacteria and antibiotics. In this study, we developed SERS-AST protocols to determine the susceptibility of 8 common bacterial pathogen of sepsis to 7 frequently used antibiotics.

Methods

Aim and Study Design

This study was aimed to improve our original SERS-AST method (19) for determination of antibiotic susceptibility of bacteria causing bloodstream infections. Eight most frequently isolated bacteria from the blood cultures of patients (30, 31) were tested, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Enterococcus faecium*, *E. coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii.* Seven antibiotics with different mechanisms of action that are commonly used for bloodstream infections (32) were tested, including oxacillin (OXA), levofloxacin (LVX), vancomycin (VAN), ampicillin (AMP), cefotaxime (CTX), ceftazidime (CAZ), levofloxacin (LVX), and imipenem (IMP). SERS-AST results were verified by comparing them with those determined by the VITEK 2 automated microbial diagnostic system (bioMérieux, Hazelwood, Missouri, USA).

Ethical Approval Statement

This study was conducted according to the guidelines of the Declaration of Helsinki-based Ethical Principles for Medical Research and was approved by the Ethics Committee of National Taiwan University Hospital (NTUH) (approval number: 201107031RC). Written informed consent was obtained from each patient before the study was initiated, and patient confidentiality was strictly protected.

Preparation of blood samples

SERS-AST was performed as described previously (19). The entire test included blood sample preparation, antibiotic treatment, SERS measurement, and receiver operating characteristic (ROC) analysis. Five ml of the blood culture broth that grew a single species of bacteria was obtained for each SERS-AST after bacterial identification and AST had been completed by VITEK-2. To prevent blood components, such as hemoglobin (33), from interfering with SERS measurements, red blood cells were selectively lysed with Ammonium-Chloride-Potassium (ACK) buffer (34) followed by ultrasonication to facilitate sonoporation (35). The bacteria in the ACK-treated sample were pelleted and resuspended in Mueller-Hinton broth (MHB) to 2.5×10^7 CFU/ml based on OD_{600} value, which was previously correlated with results of plate count. They were then treated with or without a selected antibiotic at various concentrations and incubated at 37°C for 2 to 3 hours. The bacteria in the untreated control sample were then pelleted and resuspended in deionized water to $3.0 \cdot 10^9$ CFU/ml, and those in each antibiotic-treated sample were resuspended with the same amount of deionized water as that for the control sample. For *A. baumannii*, the cell suspension in deionized water was further incubated in a shaking water bath at 37°C for 30 minutes to ensure sufficient amounts of secreted purine derivatives for SERS-AST.

Antibiotic treatment

Various antibiotic concentrations were tested as follows: 0.0625, 0.125, 0.25, and 0.5 μg/ml for the *S. epidermidis*-OXA combination; 0.5, 1, 2, and 4 μg/ml for *S. aureus*-OXA, *S. aureus*-LVX, *S. epidermidis*-LVX, *E. coli*-CTX, and *E.* cloacae-CTX combinations; 1, 2, 4, and 8 μg/ml for *E. faecalis*-LVX, *E. faecium*-LVX, *E.*

coli-LVX, *K. pneumoniae*-CTX, *K. pneumoniae*-IPM, *A. baumannii*-LVX, *A. baumannii*-IPM combinations; 2, 4, 8, and 16 μg/ml for *E. faecium*-VAN, *E. coli*-CAZ, *E. cloacae*-CAZ, *K. pneumoniae*-CAZ, *K. pneumoniae*-LVX combinations; and 4, 8, 16, and 32 μg/ml for *E. faecium*-AMP and *A. baumannii*-CAZ combinations.

SERS assay and spectral analysis

The SERS device was a glass slide coated with anodic aluminum oxide with embedded Ag nanoparticles (AgNP/AAO) (16). For the SERS assay, the AgNP/AAO slide was placed inside an aluminum trough, which holds the slide in place. A 1-mm thick aluminum sheet with 2 rows of 4 holes of 1.5-mm in diameter separated from each other by 2.5 mm was hung on the edges of the trough covering the slide 7 mm above. One microliter of each bacterial suspension was then placed onto the slide with a pipet to deliver it through the holes on the aluminum sheet with the 4 antibiotic-treated samples forming 4 spots in the top row on the slide and the 4 non-treated control samples forming another 4 spots in the bottom row at precise locations. The slide was then removed from the trough and placed on a hot plate to dry the samples at 55°C for 15 minutes before SERS measurements.

The SERS instrument was composed of a He-Ne laser emitting a 632.8 nm light, an upright optical microscope (BX61WI, Olympus), a Raman probe (Superhead, Horiba), a spectrometer (HE 633, Horiba), and a thermoelectric-cooled charge-coupled (CCD) camera. The laser beam was delivered through an optical fiber into a 20× objective lens by the Raman probe. The scattered light that bounced back from the sample on the AgNP/AAO slide was collected and delivered by the same objective lens through another optical fiber to the spectrometer and CCD for spectral recording and analyses.

The height of the dominant peak in a SERS spectrum was used to determine antibiotic susceptibility. For Gram-positive bacteria, a dominant spectral peak at 730 cm^{-1} was observed due to secreted adenine. For Gram-negative bacteria, a dominant spectral peak at 724 cm^{-1} was observed because of secreted hypoxanthine. For *A. baumannii*, which secrets primarily xanthine (26), a dominant spectral peak at 654 cm^{-1} was observed. The SERS-AST signal ratio was calculated by dividing the peak signal height value of the antibiotic-treated sample by that of the adjacent non-treated control. For each bacterial-antibiotic test, four SERS-AST signal ratios corresponding to four different antibiotic concentrations were obtained and used to draw receiver operating characteristic (ROC) curves.

Receiver operating characteristic curve

As the ROC curve is commonly used to illustrate the diagnostic ability of a binary classifier, ROC analysis was performed to determine the optimal cutoff signal ratio for antibiotic susceptibility of the bacterium in each bacterium-antibiotic combination. The optimal cutoff signal ratio (r_{op}) was the ratio that maximized the Youden index (J = sensitivity + specificity − 1) and accounted for all antibiotic-resistant samples. As ROC is a binary classification system (i.e., resistant or susceptible), a bacterium determined to be intermediate susceptible to an antibiotic was classified as resistant. For a bacterium-antibiotic test, the antibiotic concentration that yielded a signal ratio smaller than r_{op} indicated that the bacteria in the sample were inhibited at that concentration. The lowest antibiotic concentration at which the inhibition

occurred was defined as the MIC of the tested bacterium. The final antibiotic susceptibility of the tested bacterium was determined by comparing the MIC to that of the AST standards of the Clinical and Laboratory Standards Institute (CLSI) (36).

Comparative evaluation of SERS-AST results with those of clinical standards.

As the VITEK 2 system is the method routinely used in our lab for microbial identification and antibiotic susceptibility testing, the results of SERS-AST were compared with those of VITEK and were classified as agreement and disagreement. Since the effectiveness of VITEK 2 in AST has been repeatedly compared to other reference methods such as broth microdilution (37–39), agar dilution (40), double-disk diffusion (41), and PCR for *mecA* (42), such comparison was not performed in this study.

Results

From March 2016 to June 2019, a total of 164 bacterial isolates from blood samples were analyzed, including *S. aureus* (n = 20), *S. epidermidis* (n = 21), *E. faecalis* (n = 20), *E. faecium* (n = 21), *E. coli* (n = 20), *E. cloacae* (n = 20), *K. pneumoniae* (n = 21), and *A. baumannii* (n = 21). Three samples failed to generate analyzable SERS signals, including one *S. epidermidis* and one *E. faecium* sample with insufficient growth and one *A. baumannii* sample that generated unrecognizable SERS signals. Results of three *K. pneumoniae*-IPM tests were also excluded because of improper antibiotic preparation. There were 141 bacterium-antibiotic tests excluded from ROC analyses due to insufficient numbers (< 3) of antibiotic-resistant samples, including *S. aureus*-VAN, *S. epidermidis*-VAN, *E. faecalis*-AMP, *E. faecalis*-VAN, *E. coli*-IPM, *E. cloacae*-LVX, and *E. cloacae*-IPM tests. A total of 401 datasets were analyzed by ROC.

SERS-AST results of Gram-positive Bacteria.

The SERS-AST signal ratio (signal value of treated sample divided by that of untreated) of spectral peaks at 730 cm^{-1} (r_{730}) was used to analyze the antibiotic response of each Gram-positive bacterium (Fig. 1). For *S. aureus* treated with OXA or LVX, the patterns of r_{730} value variation were similar for susceptible isolates with a low r_{730} value at the susceptible breakpoint and higher concentrations, while the r_{730} values of resistant isolates remained high for all 4 antibiotic concentrations. The areas under the ROC curve (AUC) were 0.99 for OXA and 0.95 for LVX (Fig. 2). There was one disagreement in each of *S. aureus*-OXA and *S. aureus*-LVX tests, in which SERS-AST determined the isolate as resistant, but VITEK 2 determined it as susceptible. The agreement rate between the results of SERS-AST and VITEK 2 was 95% for both tests (Table 1). Good performance of SERS-AST was also observed in *S. epidermidis* treated with OXA or LVX and *E. faecium* treated with AMP or VAN. Only one disagreement was seen in *S. epidermidis*-LVX tests, in which SERS-AST determined the isolate as resistant, whereas VITEK 2 determined it as susceptible. The agreement rate between the results of SERS-AST and VITEK 2 was 100% for *S. epidermidis*-OXA, *E. faecium*-AMP, and *E. faecium*-VAN tests, and 95% for *S. epidermidis*-LVX tests. The

SERS-AST results of *E. faecalis* and *E. faecium* treated with LVX were less satisfactory as low AUC values (0.90 for *E. faecalis*-LVX tests and 0.67 for *E. faecium*-LVX tests) and low agreement rates with the results of VITEK 2 (85% for *E. faecalis*-LVX tests and 95% for *E. faecium*-LVX tests) were observed. There were 3 disagreements for *E. faecalis*-LVX tests and one disagreement for *E. faecium*-LVX tests, in which SERS-AST determined the isolates as resistant, while VITEK 2 determined them as susceptible. The overall agreement rate between SERS-AST and VITEK-2 results of the four Gram-positive bacteria was 96%.

For Gram-positive bacteria, the decrease in SERS-AST signal ratio as a result of LVX treatment was generally smaller than those of other antibiotic treatments (Fig. 1). Therefore, a higher r_{op} for AUC was obtained (0.83 vs. 0.36 in *S. aureus*-LVX and *S. aureus*-OXA tests; 0.43 vs. 0.25 in *S. epidermidis*-LVX and *S. epidermidis*-OXA tests; 0.68 in *E. faecium*-LVX, 0.61 vs. 0.33 and 0.44 in *E. faecium*-LVX, *E. faecium*-VAN, and *E. faecium*-AMP tests) (Table 1).

Table 1
SERS-AST results of Gram-positive bacteria.

Bacterial species	Antibiotic	Number of S and R isolates determined by VITEK 2		r_{op}	AUC value	Disagreement (No.)	Agreement rate (%)
		S	R				
S. aureus	OXA	12	8	0.36	0.99	1	95
	LVX	16	4	0.83	0.95	1	95
S. epidermidis	OXA	6	14	0.25	1	0	100
	LVX	12	8	0.43	0.99	1	95
E. faecalis	LVX	17	3	0.68	0.90	3	85
E. faecium	LVX	3	17	0.61	0.67	1	95
	VAN	4	16	0.33	1	0	100
	AMP	9	11	0.44	1	0	100

S and R stand for susceptible and resistant, respectively. r_{op} is the optimal cut-off SERS-AST signal ratio. The bacteria treated with a certain antibiotic concentration that yielded a SERS-AST signal ratio lower than r_{op} were considered as being inhibited at that concentration. AUC is the area under the receiver operating characteristic (ROC) curve. Numbers of isolates with disagreement results and agreement rate (%) were determined by comparing the results of SERS-AST with those of VITEK 2.

SERS-AST results of Gram-negative Bacteria.

The SERS-AST signal ratio (signal value of treated sample divided by that of untreated) of a spectral peak at 654 cm^{-1} (r_{654}) was used to determine the antibiotic response of *A. baumannii* and that at 724

cm^{-1} (r_{724}) was used to analyze other Gram-negative bacteria (Fig. 3). For *E. coli* treated with CTX, CAZ, or LVX, the patterns of r_{724} value variation were similar for susceptible isolates with a low r_{724} value at the susceptible breakpoint and higher concentrations, while the r_{724} values of resistant isolates remained high for all 4 antibiotic concentrations. The AUC values of the three antibiotic tests (CTX, CAZ, and LVX) were all 1, indicating a 100% agreement between the results of SERS-AST and VITEK 2 (Table 2). The SERS-AST was also successfully applied to other Gram-negative bacteria with an AUC value of 1 for all tests, including *K. pneumoniae*-IPM, *A. baumannii*-CAZ, *A. baumannii*-LVX, *and A. baumannii*-IPM tests. The AUC values of *E. cloacae*-CTX, *E. cloacae*-CAZ, *K. pneumoniae*-CTX, and *K. pneumoniae*-LVX tests were 0.96, 0.98, 0.98, and 0.96, respectively. There was only one disagreement in each of the 4 tests, in which SERS-AST determined the isolates as resistant, but VITEK 2 determined them as susceptible. For *K. pneumoniae*-CAZ tests, the AUC was 0.91, and there were 3 disagreements, in which SERS-AST determined the isolates as resistant, while VITEK 2 determined them as susceptible. The overall agreement rate of the four Gram-negative bacteria between SERS-AST and VITEK 2 results was 97%.

Table 2
SERS-AST results of Gram-negative bacteria.

Bacterial species	Antibiotic	Number of S and R isolates determined by VITEK 2		r_{op}	AUC value	Disagreement (No.)	Agreement rate (%)
		S	R				
E. coli	CTX	14	6	0.65	1	0	100
	CAZ	14	6	0.53	1	0	100
	LVX	14	6	0.72	1	0	100
E. cloacae	CTX	12	8	0.68	0.96	1	95
	CAZ	12	8	0.69	0.98	1	95
K. pneumoniae	CTX	12	9	0.18	0.98	1	95
	CAZ	12	9	0.13	0.91	3	86
	LVX	16	5	0.68	0.96	1	95
	IPM	15	3	0.37	1	0	100
A. baumannii	CAZ	10	10	0.56	1	0	100
	LVX	10	10	0.52	1	0	100
	IPM	10	10	0.49	1	0	100

S and R stand for susceptible and resistant, respectively. r_{op} is the optimal cut-off SERS-AST signal ratio. The bacteria treated with a certain antibiotic concentration that yielded a SERS-AST signal ratio lower than r_{op} were considered as being inhibited at that concentration. AUC is the area under the receiver operating characteristic (ROC) curve. Numbers of isolates with disagreement results and agreement rate (%) were determined by comparing the results of SERS-AST with those of VITEK 2.

Discussion

The whole SERS-AST process could be completed within 4 hours. By directly assaying positive blood cultures without additional subcultures, the time needed for a SERS-AST is significantly shorter than that for VITEK 2, which took 9 ± 1.3 hours to complete an AST directly from blood cultures in the study of Hogan et al. (43) and is comparable to that for the EUCAST rapid disc diffusion method, which takes 4–8 hours (44).

In SERS-AST, bacterial response to antibiotics is determined by changes in SERS peak height, while conventional ASTs are mainly based on changes in bacterial number, which may be determined by OD_{600} readings. In this study, we found that approximately 30% of the results determined by OD_{600} values of antibiotic-treated bacterial cultures did not agree with those of SERS-AST or VITEK 2. Most of the

disagreements were of Gram-negative bacteria treated with β-lactam antibiotics, such as *E. cloacae*-CTX tests (Fig. 4). It has been shown that the initial response of *E. cloacae* to treatment with a β-lactam antibiotic is cell elongation, instead of decreased number of cells (45). Therefore, the OD_{600} value of the treated culture may not change within 2 hours of treatment. Such morphological change is postulated to be a repair process for survival (46). In contrast, SERS-AST measures the amounts of secreted purines and their derivatives in response to antibiotic treatment. Such measurements are less affected by changes in cell morphology as in the case of our *E. cloacae*-CTX tests.

Several modifications of the SERS-AST protocol were done to obtain reliable results from certain bacteria, e.g., *A. baumannii*, which failed to generate recognizable SERS signals even when the incubation time of antibiotic treatments was extended to 3 hours. This problem may be due to the low permeability (approximately 1/100 that of *E. coli*) of its outer membrane to small molecules, thus hindering the secretion of xanthine (47, 48). To determine the optimal condition for *A. baumannii* SERS-AST, the antibiotic-treated cell suspension in water was incubated in a shaking water bath at 25, 37, or 50°C for 30, 60, or 90 minutes. As the result showed that an additional 30-minute incubation in a shaking water bath at 37°C rendered a 2.2-fold increase in SERS signal, this condition was used for all subsequent SERS-AST tests for *A. baumannii.* We postulate that this signal improvement is mainly due to the impact of placing bacteria in a nutrient-deficient environment as this starvation process has been shown to stimulate more secretion of purines and their derivatives (49–51).

In this study, 14 SERS-AST tests gave results that did not agree with those of VITEK 2, including 3 each of *E. faecalis*-LVX and *K. pneumoniae*-CAZ tests, and one each of *S. aureus*-OXA, *S. aureus*-LVX, *S. epidermidis*-LVX, *E. faecium*-LVX, *E. cloacae*-CTX, *E. cloacae*-CAZ, *K. pneumoniae*-CTX, and *K. pneumoniae*-LVX tests (Table 1, 2). Seven (50%) of these 14 cases were LVX tests with mostly Gram-positive bacteria (Table 1). Among the seven antibiotics tested, LVX is the only one not acting on cell wall synthesis. It is a quinolone antibiotic that inhibits gyrase and topoisomerase IV leading to impaired DNA replication, repair, and recombination (52). It has been shown that gyrase is the primary target of quinolones in Gram-negative bacteria, while topoisomerase IV is the main target of quinolones in Gram-positive bacteria (52). In DNA replication, inhibition occurs within minutes when the antibiotic acts on gyrase (53) but takes place later if it targets topoisomerase IV (54). However, bacterial responses to quinolones have been shown to vary on a species-by-species and drug-by-drug basis (55). It is likely that a longer time is required for LVX to inhibit the growth of slow-growing Gram-positive bacteria, such as *E. faecalis*. In an attempt to optimize the condition for *E. faecium*-LVX tests, we obtained more clear-cut SERS-AST results when the antibiotic treatment time was extended to 3 or 4 hours. Based on these results, we recommend that the antibiotic treatment time be extended to 3 hours for all LVX tests in future studies.

Five (36%) of the aforementioned 14 cases were of *K. pneumoniae* tested with CAZ, CTX, or LVX (Table 2). *K. pneumoniae* is known to produce a pronounced polysaccharide capsule covering the entire bacterial surface resulting in a mucoid phenotype (56) with reduced ability to secrete purines and their derivatives, thus yielding weak SERS signals. *K. pneumoniae* is also known to produce the CTX M β-

lactamase, which is an extended-spectrum β-lactamase (ESBL) and can degrade third-generation cephalosporin antibiotics at different rates (57). Compared to CTX, which is the preferred target of CTX M β-lactamase, CAZ is relatively resistant to that enzyme and may require more than 2 hours to be degraded. Therefore, some CAZ-resistant bacteria may be determined by laboratory testing as susceptible, which is inconsistent with clinical manifestations. The Advanced Expert System (AES) of VITEK 2 can identify bacteria with ESBL by special software and modify the primary laboratory results accordingly (58). In this study, there were five AES-revised *K. pneumoniae*-CAZ results. Three of them that were determined as susceptible by SERS-AST after the 2-hour antibiotic treatment were interpreted by AES as resistant.

The SERS technology has also been used by Tien et al. (59) to detect antibiotic-resistant bacteria in urine samples with the aid of principal component analysis (PCA). By using magnetic separation and SERS technology with AgNP colloid, Li et al. (60) accurately identified antibiotic-resistant strains of *S. aureus, A. baumannii*, and *P. aeruginosa* from 77 clinical blood samples. In the study of Novelli-Rousseau et al. (61), Raman spectrometry coupled with PCA and support vector machine (SVM) algorism was used to determine the MIC of gentamicin, ciprofloxacin, and amoxicillin against *E. coli* strains. Ho et al. (62) have generated an extensive dataset of bacterial Raman spectra and employed deep learning with convolutional neural network (CNN) algorism to train the computer to identify 30 common bacterial pathogens. With this approach, they achieved 99.0 ± 1.9% accuracy in the identification of 25 clinical isolates from 50 patients and 89.1 ± 0.1% accuracy in the differentiation between methicillin-resistant and methicillin-susceptible *S. aureus.* Our results are consistent with these observations.

This study has several strengths. With optimized specimen processing protocols and effective SERS device, reproducible bacterial SERS spectra were acquired for AST. This study also successfully developed methods, such as starvation, to perform SERS-AST on bacteria (i.e., *A. baumannii*) that were previously not assayable. The finding that extending the LVX treatment time to 3 hours, especially for Gram-positive bacteria such as *E. faecalis* that grows slowly, can generate satisfactory SERS-AST results is very significant.

To move the SERS-AST forward for clinical applications, further improvements are needed. As different bacteria emit different SERS signals, methods for discriminating mixed SERS spectra from samples of patients with polymicrobial infections remain to be developed. The impact of patient treatment on SERS-AST also remains to be investigated. Although *P. aeruginosa* is a major causative organism of sepsis, we have not been able to perform SERS-AST on it because of interference by its fluorescent pigments. As SERS-AST is based on changes in bacterial metabolism due to antibiotic treatment, it is possible to modulate the metabolic activity of bacteria by altering their growth environments with substances such as culture media and cations (e.g., Mg^{2+}, Ca^{2+}, and Na^{+}) (63) or with pH adjustment (46). A limitation in the performance of SERS-AST is that the instrument is homemade and is currently not commercially available. Since the SERS-AST instrument that we have used is a prototype, there is room for improvements to increase its sensitivity and specificity.

Conclusions

In this study, we performed SERS-based rapid AST on 20 bacterium-antibiotic combinations of eight most common pathogens causing bacteremia and seven commonly used antibiotics. Distinct from culture-based and genome- or proteome-based ASTs, SERS-AST analyzes changes in bacterial metabolism due to antibiotic treatment by measuring the amounts of secreted purines and their derivative. By directly assaying positive blood cultures without additional subcultures, the whole process can be completed within 4 hours and the agreement rates between SERS-AST results and VITEK 2 results were 96% for Gram-positive bacteria and 97% for Gram-negative bacteria. SERS-AST can be an alternative AST method to provide critical information to clinicians for timely administration of appropriate antibiotics to treat patients with blood stream infections.

Declarations

Ethics approval and consent to participate:

This study was conducted according to the guidelines of the Declaration of Helsinki-based Ethical Principles for Medical Research and was approved by the Ethics Committee of National Taiwan University Hospital (NTUH) (approval number: 201107031RC). Written informed consent was obtained from each patient before the study was initiated, and patient confidentiality was strictly protected.

Consent for publication:

Not applicable

Availability of data and materials:

The datasets used and analyzed in the current study are available from the corresponding author upon reasonable request.

Competing interests:

The authors declare that they have no competing interests.

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Author contributions:

Y-Y. H., J-K. W., and Y-L.W. designed and conducted the study. W-C. C., K-L. C., and Y. C. performed experiments. Y-Y. H. and J-K. W. interpreted the results and wrote the manuscript. L-J.T. and C-T. W.

participated in the analysis of the results. Y-L.W. reviewed the results and edited the manuscript.

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Figures

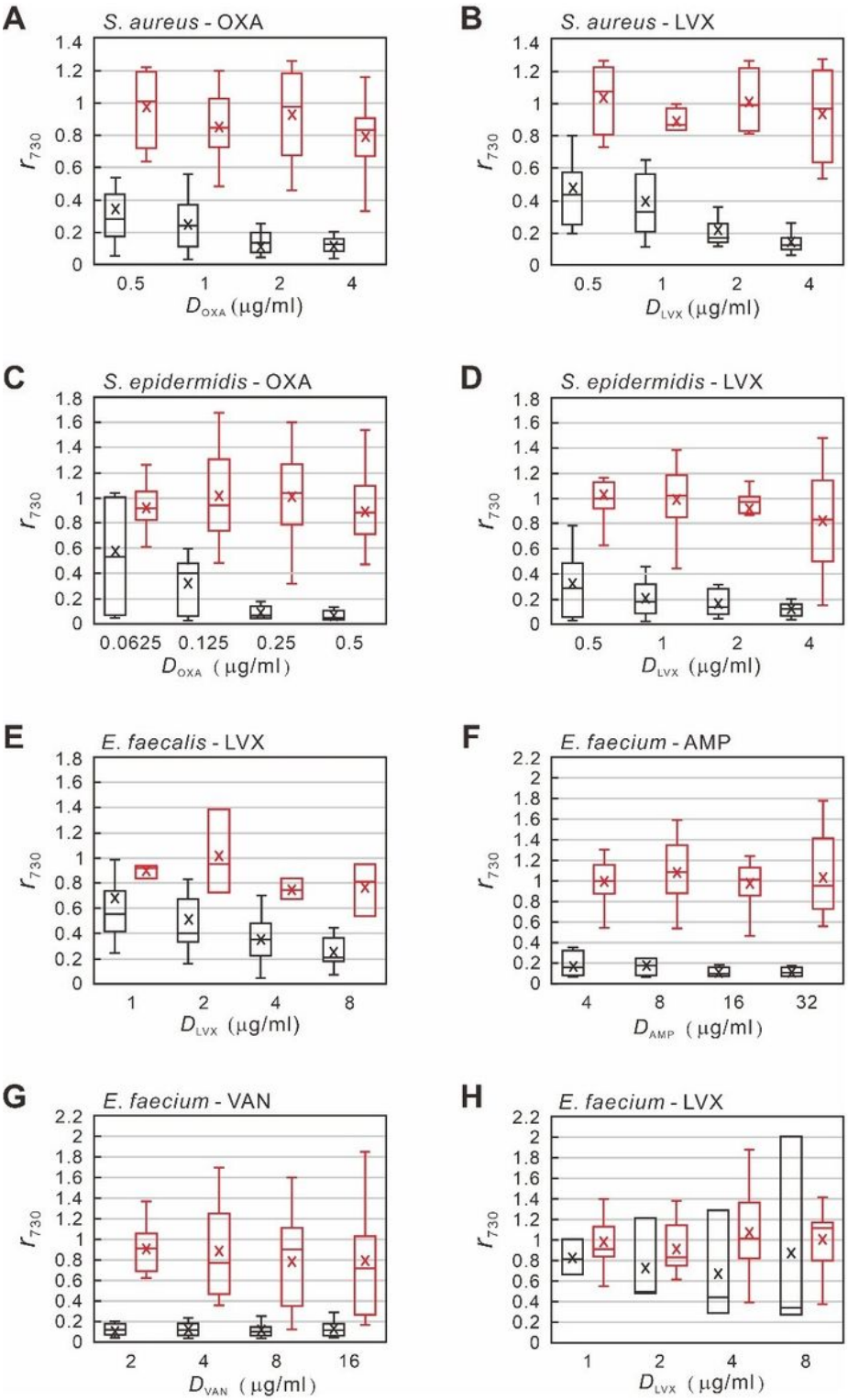

Figure 1

Box-and-whisker plots of SERS-AST results of Gram-positive bacteria treated with various concentration of antibiotics. A. *S. aureus* with OXA, B. *S. aureus* with LVX, C. *S. epidermidis* with OXA, D. *S. epidermidis* with LVX, E. *E. faecalis* with LVX, F. *E. faecium* with AMP, G. *E. faecium* with VAN, and H. *E. faecium* with LVX. Values on the Y-axis are SERS-AST signal ratios (r_{730}) calculated by dividing the peak height value at 730 cm^{-1} of the antibiotic-treated sample by that of the non-treated control sample. Values on the X-

axis are antibiotic concentrations. The boxes represent 25th to 75th percentiles of samples, with the 50th percentile indicated with a small line in the boxes. The 10th and 90th percentiles of samples are indicated with whiskers. The x letter inside each box represents the mean r_{730} value. Red and black boxes represent the data of resistant and susceptible samples, respectively. VAN, vancomycin; OXA, oxacillin; AMP, ampicillin; LVX, levofloxacin.

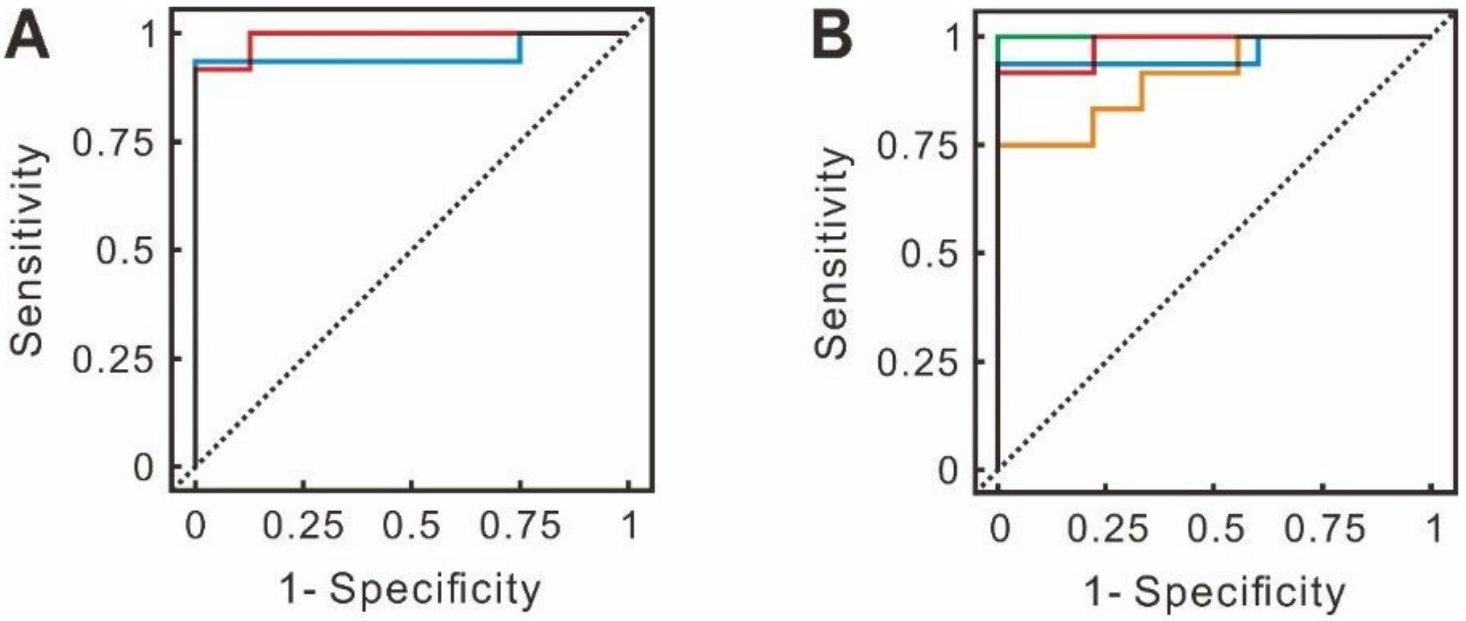

Figure 2

Representative ROC curves of SERS-AST results. A. Results of *S. aureus* treated with OXA or LVX. Red and blue curves represent results of OXA and LVX treatments, respectively. The black segments indicate overlaps between the two results. B. Results of *K. pneumoniae* treated with CTX, LVX, IPM, or CAZ. Red, blue, green, and orange curves represent results of CTX, LVX, IPM, and CAZ treatments, respectively. The black segments indicate overlaps among the results.

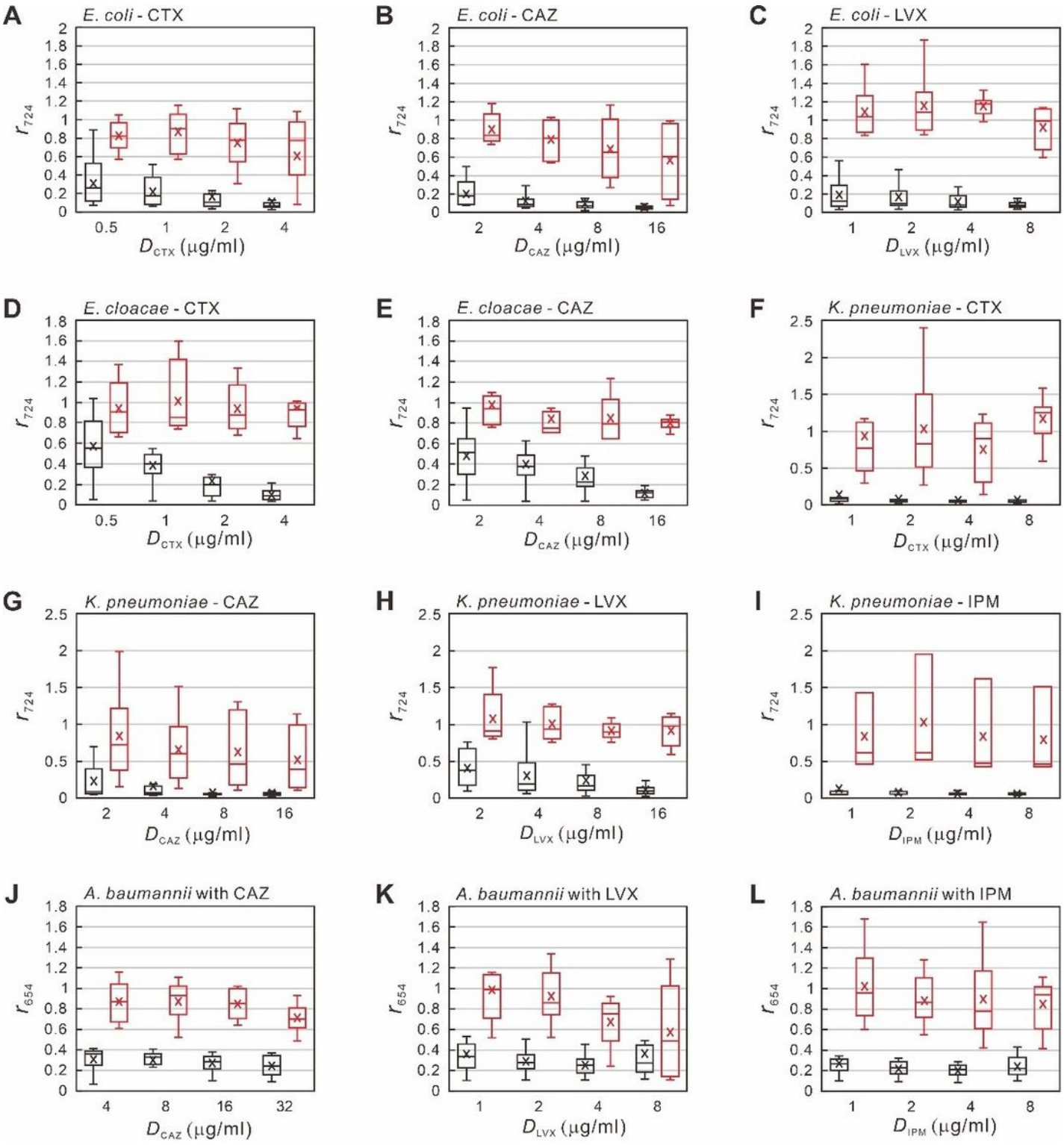

Figure 3

Box-and-whisker plots of SERS signals of Gram-negative bacteria treated with various concentrations of antibiotics. A. *E. coli* with CTX, B. *E. coli* with CAZ, C. *E. coli* with LVX, D. *E.* cloacae with CTX, E. *E. cloacae* with CAZ, F. *K. pneumoniae* with CTX, G. *K. pneumoniae* with CAZ, H. *K. pneumoniae* with LVX, I. *K. pneumoniae* with IPM, J. *A. baumannii* with CAZ, K. *A. baumannii* with LVX, L. *A. baumannii* with IPM. For A-I, values on the Y-axis are SERS-AST signal ratios (r_{724}) calculated by dividing the peak height value at

724 cm^{-1} of the antibiotic-treated sample by that of the non-treated control sample. For J-L, values on the Y-axis are SERS-AST signal ratios (r_{654}) calculated by dividing the peak height value at 654 cm^{-1} of the antibiotic-treated sample by that of the non-treated control sample. Values on the X-axis are antibiotic concentrations. The boxes represent 25th to 75th percentiles of samples, with the 50th percentile indicated with a small line in the boxes. The 10th and 90th percentiles of samples are indicated with whiskers. The x letter inside each box represents the mean r_{724} or r_{654} value. Red and black boxes represent the data of resistant and susceptible samples, respectively. LVX, levofloxacin, CTX, cefotaxime, CAZ, ceftazidime, and IPM, imipenem.

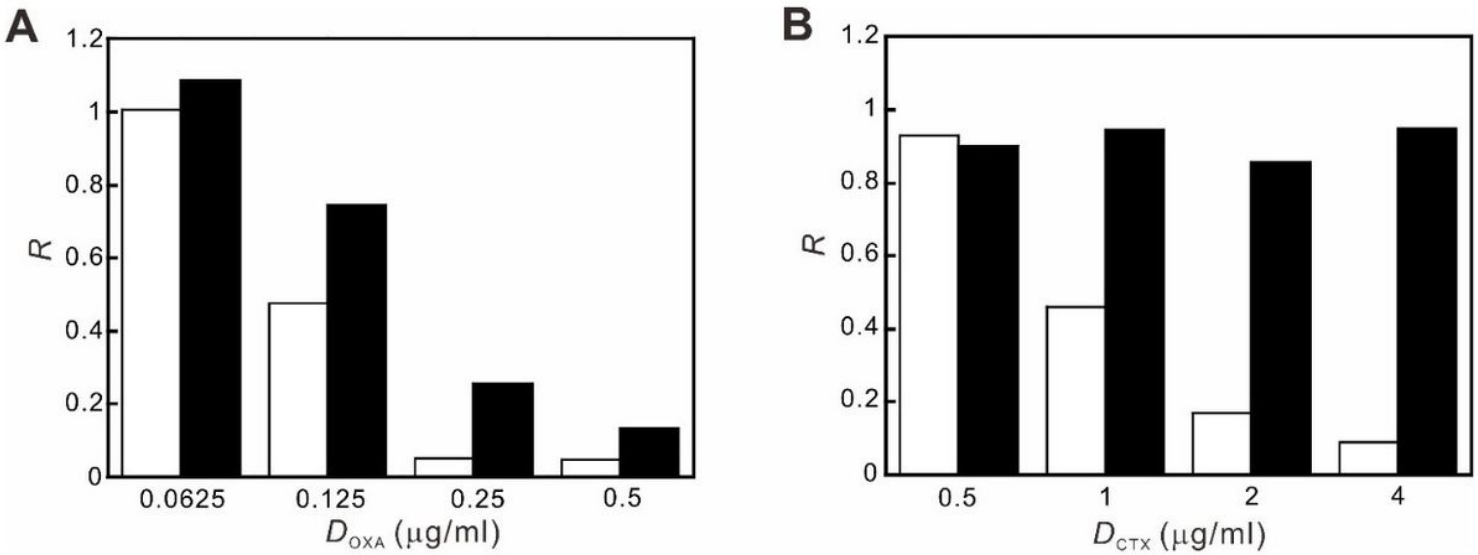

Figure 4

SERS-AST signals versus optical density of antibiotic-treated cultures. A. Variations in SERS-AST signal ratios and OD_{600} ratios of OXA-susceptible *S. epidermidis* cultures. B. Variations in SERS signal ratios and OD_{600} ratios CTX-susceptible *E. cloacae* cultures. Values on the Y-axis are ratios of OD_{600} or SERS peak height values calculated by dividing the value of the antibiotic-treated sample by that of the non-treated control sample. Values on the X-axis are antibiotic concentrations.