Label-free identification carbapenem-resistant *Escherichia coli* based on surface-enhanced resonance Raman scattering†

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In this study, a surface-enhanced resonance Raman scattering (SERRS) method has been developed for the accurate detection and identification of carbapenem-resistant and carbapenem-sensitive *Escherichia coli*. A total of 89 human isolates of Enterobacteriaceae, comprising 41 strains of carbapenem-sensitive *E. coli* (CSEC) and 48 strains of carbapenem-resistant *E. coli* (CREC), were tested to assess the feasibility of our proposed SERRS method as a clinical tool, and the results showed almost 100% accuracy.

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这笔研究的非标准使用抗生素导致了临床抗微生物药物的耐药性，已经成为了过去几十年来最大的健康威胁。**1** 这种药物耐药性已经导致每年180万死亡。**2** 克拉维酸-β-内酰胺酶的广泛传播使**克雷伯氏肺炎克菌**（CREC）成为一种越来越严重的问题，因为它是抗生素治疗困难的病原体，导致医院出现无法治疗的感染**。** **3,4** 许多研究已经表明，克拉维酸对**克雷伯氏肺炎克菌**的高度敏感性**。** **5** 精确和准确的**CREC**识别对于选择适当的抗生素治疗和降低死亡率是非常重要的。

目前的金标准方法用于检测致病性细菌为微生物培养的测试，这主要基于表型特征。**6,7** 这些方法要求至少24 h对于细菌生长和生化特征的检测和一个24-72 h抗生素敏感性测试。作为替代，有些核酸酸基方法（聚合酶链反应和DNA测序）和蛋白质基方法（矩阵辅助激光脱附离子化-飞行时间质谱）已经用于快速细菌检测。**8,9** 然而，这些方法也涉及冗长的程序，笨重的复杂的仪器，熟练的人员，从而限制了它们的广泛应用到点-护理诊断。

表面增强拉曼散射（SERS）已经被证明是一个强有力的和有前途的工具，用于细菌的识别，因为它具有高度的敏感性，简单的准备，高速，相对低成本，和便携性。**10-13** 许多病原菌已经被SERS鉴定，如**Klebsiella pneumoniae**，**E. coli**，**Pseudomonas aeruginosa**，**Acinetobacter baumanii**，**Salmonella enterica**，**Staphylococcus aureus**，和**Lactobacillus plantarum**。**14** 质料的大量病原菌的指纹谱可以从SERS，如**CREC**，进行鉴定。当与多变量数据分析程序结合时，主要振动带的观察细菌的光谱是指向整个整个生物结构的生物，如核酸酸，蛋白质，脂质，和碳水化合物。**15** 由于抗药性和敏感性细菌在类似成分，使用SERS技术来区分抗药性细菌病原菌已经报道。

在这里，我们报告了一种表面增强拉曼散射（SERRS）方法，准确识别**CREC**和**CSEC**，基于使用表面增强
carbapenem-sensitive *Escherichia coli* (CSEC) based on the use of plasmonic silver-coated gold nanorods (Au@Ag NRs). Given that the plasmon peak of Au@Ag NRs at 785 nm matches the excitation wavelength of the laser, the sensitivity of Raman scattering spectroscopy arises from the resonance enhancement effect, leading to a high resolution and informative characteristic spectra of detection bacteria. We performed a label-free SERS test on 89 *E. coli* samples including 41 strains of CSEC and 48 CREC by mixing Au@Ag NRs with bacteria directly. High-quality SERS spectra from all bacteria samples were acquired and analyzed using the orthogonal partial least squares discriminant analysis (OPLS-DA) algorithm. This method can accurately distinguished CREC and CSEC. As far as we know, this study is the first to use plasmonic
Au@Ag NRs as SERRS substrate to achieve bacteria-sensitive detection. We believe this method indicated a new route in field applications for rapid diagnosis of drug-resistant bacterial pathogens.

All 89 strains included in this study had been prospectively collected from Affiliated Hospital of Xuzhou Medical University, and they have been identified using VITEK2 COMPACT high intelligent automatic microbial identification system (bioMérieux, La Balme-les-Grottes, France). Carbapenem resistance was defined according to the Clinical and Laboratory Standards Institute breakpoint criteria (CLSI M100, S27). Table S1† provides the bacteria sample information.

Fig. 1 shows the operating principle of the SERRS method for bacteria detection. The bacteria were mixed with Au@Ag NRs directly, and the mixture was excited by a portable Raman system with a 785 nm laser. The representative Raman fingerprinting spectra of the bacteria can be directly obtained by bringing the bacterial cell near the plasmonic Au@Ag NRs. In a typical experiment, the clinical isolation bacterial strains were cultured and grew on Columbia agar supplemented with 5% horse blood (bioMérieux, La Balme-les-Grottes, France) for 10 h. The selected colony was collected using a 1 μL sterile inoculating loop and suspended in 10 μL distilled water. Then, we mixed 5 μL of concentrated Au@Ag NRs (10-fold) with 5 μL of bacterial suspension (approximately 1 × 10⁸ cells per mL), and the mixture was pipetted on top of a clean Si substrate prior to detection. After quick drying in air, SERS signals were recorded with a portable Raman system. In measuring the Raman spectra, the accumulation time was set at 10 s, and the incident power was set at 20% of the laser power. Fig. S1a in the ESI† shows the characteristic UV-vis plasmon peak of the Au NRs is at 824 nm, as shown in Fig. 2c (black line). After Ag shell coating, the plasmon peak blue shifted to 785 nm because of the reduced aspect ratio and Au–Ag plasmon coupling.†

Subsequently, the SERS activity of Au NRs, Au@Ag NRs, Au@Ag core–shell nanoparticles (Au@Ag NPs) and commonly used Ag NPs was tested. Fig. S2 and S3 in the ESI† show the TEM images and UV-visible absorbance spectroscopy of the Ag NPs and Au@Ag NPs, respectively. These prepared nanoparticles were mixed with 5 μL E. coli sample first, and the mixtures were then dropped on the silicon chip and dried at room temperature for SERS measurement. Fig. 2d shows the recorded the SERS spectra, and the major vibrational modes of E. coli can be observed as enhanced by four kinds of nanoparticles. The typical Raman peaks at 655, 729, 958, 1244, 1325, 1371, 1461, 1585, and 1694 cm⁻¹ were observed and well confirmed by previous reports categorized in Table 1.11,29 Obviously, the SERS signal of the Au@Ag NRs (red spectrum), whose plasmon peak was adjusted to match the given laser excitation wavelength (785 nm), was approximately 4.5 times that of Au NRs (purple spectrum), and twice that of Ag NPs (black spectrum). Moreover, we found that the SERS spectrum of bacteria sample mixed with Au@Ag NRs has more Raman peaks that can provide more fingerprint information for bacterium identification. To further corroborate the relationship between the SERRS activity and particle morphology, we used Au@Ag NPs as more persuasive control samples to assess the SERS performance of Au@Ag NRs under 785 nm excitation. It can be roughly estimated that the SERS ability of the Au@Ag NRs is enhanced by at least 1.5 times compared with the spherical Au@Ag NPs from Fig. 2d. These results demonstrate that the superior SERS performance of the Au@Ag NRs is simultaneously coming from the plasmonic coupling effect and Ag shell, which is in good agreement with other reported results.31–33

Using Au@Ag NRs as SERRS substrate, we have successfully acquired high-quality SERS spectra from 89 Enterobacteriaceae species, and we also have the commonly used Au or Ag colloids, the Au@Ag NRs have two advantages: (i) the plasmon peak that matches the Raman excitation wavelength (785 nm) produced the strongest plasmonic coupling effect and (ii) the higher efficiency of Ag shells in comparison with Au NRs. We have synthesized Au@Ag NRs with longitudinal plasmon resonance peak located at 785 nm by the in situ growth of Ag shells on the surface of Au nanorods, as reported previously.35,36 Experimental section in the ESI† presents the detailed fabrication process. The transmission electron microscopy (TEM) images in Fig. 2a and b show the characteristic structures of Au NRs and Au@Ag NRs, respectively. The as-prepared Au NRs are uniform in shape and size with dimensions of approximately 70 nm × 17 nm, with an aspect ratio of 4.1. The thickness of the Ag shell can easily be controlled by varying the volumes of AgNO₃ and ascorbic acid, which makes the LSPR of Au@Ag NRs tunable for obtaining maximum SERS signals at a given excited wavelength (785 nm). After Ag shell encapsulation, the length and width of the resulting Au@Ag NRs are increased to 72 nm and 19 nm, respectively. The core–shell structure could be clearly seen in the inset of Fig. 2b, and the Ag shell thickness was approximately 2 nm. The characteristic UV-vis plasmon peak of the Au NRs is at 824 nm, as shown in Fig. 2c (black line). After Ag shell coating, the plasmon peak blue shifted to 785 nm because of the reduced aspect ratio and Au–Ag plasmon coupling.27

Fig. 1 Schematic of the SERRS measurements for bacteria detection based on Au@Ag NRs. The bacteria were mixed with Au@Ag NRs, and the mixture was excited by a portable Raman system with a 785 nm laser to obtain the SERRS signal.
isolates of human (41 strains of CSEC and 48 strains of CREC). Fig. 3 shows a comparison of the normalized mean SERS spectra obtained from CSEC and CREC samples. The red and black lines were obtained from the 41 strains of CSEC and 48 strains of CREC, respectively, and the blue line was the normalized subtracted SERS spectrum between the two groups. The positions of all the major peaks of CSEC and CREC are very similar because the two bacteria belong to a single species, but the relative intensities of the vibrational peaks showed some differences. For example, important spectral differences in the bands near 655, 729, 958, and 1325 cm$^{-1}$ can be clearly distinguished; these differences correspond to bands assigned to amino acids, nucleic acid, and proteins.$^{34,35}$ The resistance mechanism of CREC results from the combination of extended-spectrum $\beta$-lactamase production, cephalosporinase overexpression, and/or porin deficiency. Most of these are caused by plasmid-encoded carbapenemases, a form of $\beta$-lactamase that hydrolyzes the $\beta$-lactam ring, which is an essential component of $\beta$-lactam antibiotics such as cephalosporins and carbapenem.$^{36,37}$ Moreover, the resistant plasmids could be transferred through conjugation and may change the components of the cell wall such as membrane protein and lipopolysaccharide. Thus, the differences of the SERS spectra of CSEC and CREC could be ascribed to the changes of bacterial plasmid and cell wall components. This condition allows fingerprinting potential for bacteria identification and drug resistance detection.

OPLS-DA was performed to further differentiate the bacterial strains based on the SERS spectra, which can provide an accurate and intuitive view of the differences in fingerprint.$^{38}$ The OPLS-DA was performed in the entire spectral region between 550 and 1700 cm$^{-1}$ for all examined bacterial stains based on

| Raman shift/cm$^{-1}$ | Component | Band assignments |
|-----------------------|-----------|------------------|
| 655                   | Amino acids | $\delta$(COO$^-$) |
| 729                   | DNA       | Adenine, glycosidic ring mode |
| 958                   | Proteins  | $\tau$(CN) |
| 1040                  | Proteins  | CC ring breathing |
| 1128                  | Nucleic acids | $\tau$(COC), ring breathing |
| 1242                  | Proteins  | Amide III |
| 1325                  | Proteins  | $\tau$(NH$_2$) adenine, polyadenine |
| 1371                  | DNA/RNA   | Ring breathing (T/A/G) |
| 1461                  | Lipids    | $\delta$(CH$_2$) |
| 1585                  | Proteins  | Phe, amide II |
| 1694                  | Proteins  | Amide I |

* Approximate description of the modes ($\nu$, stretch; $\delta$, bend).
SIMCA 14.0 software (Umetrics, Umea, Sweden). Raman spectral data were preprocessed by removing the autofluorescence background and were smoothed and normalized with Bwspec4 (B&W Tek). Fig. 4a shows the 2D OPLS score plots of the bacteria SERS spectra for CSEC and CREC samples. The model was generated using 89 E. coli stains (41 stains for CSEC and 48 stains for CREC). We conducted five measurements for each of the samples, and all spectra were used to analyze. Data points are clustered into two completely separated groups without overlap, indicating that the SERS spectra of the CSEC and CREC samples can be clearly distinguished. The ellipse is expressed as 95% confidence interval, and the 2D scatter plot of the first two principal components with the largest explain the variance which accounts for 67.7% of the total. Moreover, the differences among the CSEC and CREC can clearly be illustrated by the loading 3D plots presented in Fig. 4b. The three principal components are the most diagnostically significant, which explains the 47.3%, 20.4%, and 15.9% of the variance in the analyzed dataset.

Furthermore, 10-fold cross-validation was applied to estimate the classification efficiency of our method. In brief, the spectrum data of all bacteria samples were divided into 10 subgroups randomly, and each subgroup (test set) was tested by a training set model built with the remaining nine subgroups. Then, the average accuracy rates of the training and test sets were obtained after 10 operations. The average accuracy rates of the training and test sets are 100% and 99.43%, respectively, thereby indicating the excellent accuracy of the OPLS-DA model (Table S2†). These results proved that the proposed SERRS methods combined with OPLS-DA analysis are a powerful tool to identify CSEC and CREC based on label-free SERS spectra.

In summary, we presented an efficient SERRS method to identify CSEC and CREC. We detected the SERS signals of 41 strains of CSEC and 48 strains of CREC using plasmonic Au@Ag NRs as SERRS substrate and analyzed the SERS spectra with OPLS-DA. Results show that the proposed method has almost 100% accuracy rate for CSEC and CREC identification. This is the first study to confirm that SERRS can potentially be used for rapid and accurate detection of drug-resistant bacterial pathogens.

Conflicts of interest
The authors report no conflicts of interest in this work.

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