Original Article

Rapid Identification of *Legionella* Pathogenicity by Surface-Enhanced Raman Spectroscopy*

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Abstract

Objective To establish Surface-enhanced Raman Spectroscopy (SERS) can be used as a rapid and reliable method to distinguish virulent strain and mild strain of *L. pneumophila*.

Methods We isolated and characterized of bacterial strains from ATCC and water samples strains, while we analyzed data from SERS technology using gold nanoparticles as a base and cell infections were employed to rapidly detect *L. pneumophila* strains. Origin 8.0 was used to collect Raman spectra, smooth and homogenize data, and to contrast spectra. Principal component analysis (PCA) was conducted to discriminate differences between groups using the multivariate analysis package PyChem 3.0.5.

Results Our results indicated that the peaks of high virulence strains reached ≥4000. This criterion was verified by subsequent cell experiments. In addition, we also conducted SERS rapid identification on the virulence of several collected clinical strains and obtained accurate results.

Conclusion The present study indicates that the established SERS protocol can be used as a rapid and reliable method to distinguish virulent and mildly virulent strains of *L. pneumophila*, which can be further used in clinical samples.

Key words: *Legionella pneumophila*; Surface-enhanced Raman Spectroscopy; Pathogenicity; Gold nanoparticles

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INTRODUCTION

Legionella was successfully isolated in 1977[1] and named because of its first massive outbreak resulting in the death of 34 people[2] during the Philadelphia veterans annual conference in 1976. *Legionella* is a Gram-negative Bacillus that spreads through the air in the form of aerosols. *Legionella* pneumophila is the most pathogenic of the >50 known *Legionella* species[3]. *Legionella* infection mainly results in Pontiac fever in humans and *Legionellosis*, whose main clinical symptoms includes acute fever with a short incubation period and pneumonia symptoms with a long incubation period[4].

Rapid and efficient detection of *Legionella* is of great significance to safeguard public health and reduce production losses. At present, widely used pathogen detection methods include PCR[5], enzyme linked immunosorbent assays (ELISAs)[6], real time-PCR[7-9], and traditional bacterial culturing. However, all of these procedures require the pretreatment of samples and are time consuming. Therefore, there is an urgent need to develop a more rapid, accurate, and convenient detection method for pathogenic bacteria.

The Raman spectrum was first discovered by the Indian scientist Raman and used to detect material structures in 1928[10]. Several decades later, SERS technology, which is characterized by its high speed, high efficiency, and accuracy, was invented by Fleischman in 1974[11]. The application of SERS has improved the sensitivity of pathogen detection to a large extent and drastically shortened the time required for detection[12-15]. Indeed, researchers are able to obtain accurate detection results within a few minutes when not using sample labels[16]. Compared to traditional Raman techniques, SERS improves the signal-to-noise ratio (SNR) of maps and detection sensitivity by taking advantage of metals, like gold and silver, with rough surfaces to absorb the molecules being tested. Noble metal nanoparticles are widely employed in SERS detection due to their large surface-to-volume ratio and superior sensitivity[17-18]. When a photon undergoes an inelastic scattering, Raman spectroscopy is based on the physical phenomenon that the frequency changes, precisely matching the difference in vibrational energy levels. Therefore, the Raman spectra can provide rich information about the analyte molecules. However, the weak signal intensity of Raman spectra hinder their further application[19]. SERS occurs at/near the rough surface of metal nanoparticles when analyte molecules are absorbed onto them, enhancing the Raman signal of the absorbed molecules 105-1014. The Raman spectrum intensity is directly affected by the dipole moment as a product of the molecular polarizability and the applied electric field. The excellent surface plasmon resonance properties of gold nanoparticles generate strong electromagnetic fields, which increase the induced dipole moment, leading to enhanced Raman scattering and resulting in enhanced Raman signal intensity[20].

Recently, SERS has been extensively used in a series of research fields, such as the food, chemistry, and pharmaceutical industries. Concerning pathogenic microorganisms, SERS is usually used for rapid detection, isolation, and identification of mixed strains, but the applicability of this rapid analysis technology to determine a pathogen's virulence has not been extensively studied.

METHODS

Isolation and Characterization of Bacterial Strains

The *Legionella* strains used in this study are listed in Table 1. Five reference strains of four species were purchased from the American Type Culture Collection (ATCC). Three tested *L. pneumophila* strains were isolated from water samples in Beijing, China.

Table 1. List of Reference and Tested Strains

Strain	Species	Sources	Virulence
ATCC33152	*L. pneumophila*	ATCC	strong
ATCC33156	*L. pneumophila*	ATCC	strong
ATCC43878	*L. brunensis*	ATCC	weak
ATCC35249	*L. spiritensis*	ATCC	weak
ATCC35252	*L. cherrii*	ATCC	weak
Strain1	*L. pneumophila*	Water sample, Beijing	strong
Strain 2	*L. pneumophila*	Water sample, Beijing	strong
Strain 3	*L. pneumophila*	Water sample, Beijing	weak

Note. ATCC, American Type Culture Collection.

All of the strains were streaked onto buffered charcoal yeast extract (BCYE) agar plates and incubated at 35 °C in 2.5% CO_2 for 48 h. Colonies were picked and identified by morphology, biochemical reaction (oxidase, nitrate reduction, urease, liquefaction of gelation, and hydrolysis of Hippuric acid), and serum agglutination tests. The colonies with characteristics matching *Legionella* species were used for further research.

Culturing and Single-colony Purification of Legionella

The protocol for the collection and pre-treatment of environmental water samples was according to ISO 11731 (1998). Water samples (500 mL) were collected from a reservoir or condensation pan in sterile screw-capped containers. As in the preparation of the mock sample, chlorine in the environmental water samples was inactivated by the addition of 0.05% sodium thiosulfate. Two hundred milliliters of water sample was centrifuged at 6000×g for 10 min in a 300-mL centrifuge bottle. Pellets were resuspended in 3 mL distilled water for further analysis. Resuspended pellets were serially diluted 10-fold with sterile water. Diluted and undiluted samples (100 μL each) were plated onto GVPC agar (Oxoid, England), and plates were incubated at 37 ˚C for 10 d. Colonies were identified as *Legionella* using the L-cysteine requirement test, Gram-staining, and slide agglutination using polyclonal antisera (Tokyo, Japan).

Synthesis of 100-nm Au-tiopronin Nanoparticles

One hundred-nanometer Au-tiopronin nanoparticles (NPs) were synthesized as reported[21]. Fifteen-nanometer Au-citrate NPs were prepared by the standard citrate reduction method. Briefly, 1.5 mL 1% (w/v) sodium citrate was quickly added to 50 mL 0.01% boiling $HAuCl_4 \cdot 3H_2O$ with vigorous stirring to obtain 15-nm gold seeds. One hundred-nanometer citrate-functionalized gold NPs were synthesized using the seed growth method. Briefly, 150 mL mixture solution contained 0.24 mL of 15-nm gold seed solution and 2.49 mL 10 mmol/L $HAuCl_4 \cdot 3H_2O$ solution and was gently stirred. Then, 100 mL 0.4 mmol/L ascorbic acid was added at a rate of 10 mL/min to obtain 100-nm Au-citrate NPs. Finally, a surface molecule exchange reaction was adopted to obtain 100-nm Au-tiopronin NPs using the 100-nm Au-citrate NPs and the addition of an aqueous solution containing a large excess of tiopronin. When the exchange reaction finished, the Au-tiopronin NPs solution was centrifuged three times at 10,000 rpm for 30 min to remove any residual salt and unbound tiopronin.

Stability of SERS

The six standard ATCC strains were streaked on plates and incubated at 35 °C in 2.5% CO_2 for 48-72 h. Colonies were picked onto a clean microslide, 5 μL 100-nm Au-tiopronin NPs was added, the cells and NPs were incubated for 5-10 s, and then SERS was performed. Trials were repeated five times to collect data.

SERS Analysis of Standard and Clinical Strains

Strains were cultured as above. Single colonies were picked into 1.5-mL centrifuge tubes containing distilled water and shaken at 6500 rpm twice for 5 min. Strains were mixed with 100 μL distilled water, and 10 μL was absorbed onto clean microslides. The 100-nm Au-tiopronin NPs were added as above prior to SERS analysis with a microscopic confocal laser Raman spectrometer (Via-Reflex, Renishaw, UK). A 532-nm laser was employed with a laser power of 100% and 10 exposures/s. Samples were focused under a 50×objective, and the spectra were collected from the 100-2000 cm^{-1} Raman shift range[22].

Cell Infections

Legionella strains were incubated on BCYE plates until they reached early stationary phase. Then, they were resuspended and diluted (1:10) in RPMI 1640 tissue culture medium. Bacterial suspensions (1 mL) were added to J774 cells in 24-well dishes (MOI=6.72), incubated at 35 °C in 2.5% CO_2 for 1.5 h, washed three times with PBS, and then incubated at 35 °C in 2.5% CO_2. The supernatant was discarded at 0 and 72 h, and 1 mL/well distilled water was added to the suspended cells and transferred to 1.5-mL centrifuge tubes. Inoculation on BCYE plates occurred after gradient dilution. The virulence of a *Legionella* strain is high if the amount of bacteria incubated after 72 h exceeded that incubated for 0 h.

Data Analysis

Origin 8.0 was used to collect Raman spectra, smooth and homogenize data, and to contrast spectra. Principal component analysis (PCA) was conducted to discriminate differences between groups using the multivariate analysis package PyChem 3.0.5. To highlight the fingerprint region for further multivariate analyses, the acquired spectral ranges of all samples were reduced to 400-1800 cm^{-1}.

RESULTS

Characterization of 100-nm Au-citrate NPs

To characterize the Au-tiopronin NPs, a Tecnai G2 20 S-TWIN Transmission Electron Microscope (TEM; Philips, Netherlands) with 200 kV acceleration voltages and a Nano ZS Zetasizer (Malvern, England) were used in this work. The morphology of the as-prepared nanoparticles is shown in Figure 1A. The TEM image reveals that the Au-tiopronin nanoparticles were spherical with a uniform shape. The sizes of the as-prepared nanoparticles were measured, statistically analyzed, and found to be approximately 100 nm. In addition, the hydrodynamic diameter of the Au-tiopronin NPs was measured by dynamic light scattering (DLS), which is shown in Figure 1B. The hydrodynamic diameter of as-prepared nanoparticles was 103.3 nm, with a narrow size distribution. It is important to note that SERS was only possible for the rest of the experiments with the aid of AuNPs.

Stability of SERS

Three *Legionella* strains from the ATCC (ATCC33156, ATCC43878, and ATCC35252) were tested to evaluate the stability of SERS. Five replicate samples of each strain were analyzed (Figure 2). The spectral region of ATCC33156 was concentrated on 750 cm^{-1}, 1300 cm^{-1}, and 1535 cm^{-1} (Figure 2A), the peaks of ATCC43878 were focused on 1270 cm^{-1}, 1400 cm^{-1}, and 1650 cm^{-1} (Figure 2B), and the peaks of ATCC35252 were focused on 750 cm^{-1}, 1200 cm^{-1}, 1400 cm^{-1}, 1600 cm^{-1}, and 1700 cm^{-1} (Figure 2C). Therefore, the overlaid spectra of each individual *Legionella* strain demonstrate a high degree of spot-to-spot and substrate-to-substrate reproducibility.

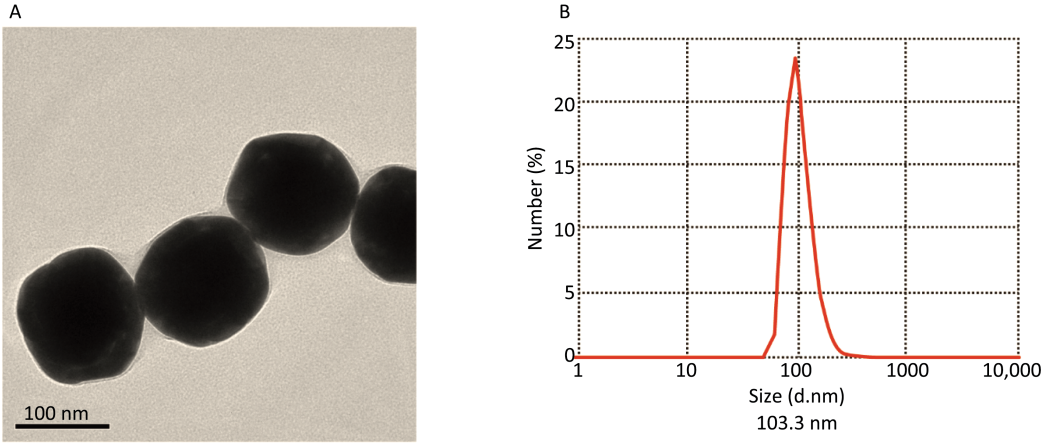

Figure 1. Electron micrograph of the Au-tiopronin NPs. (A) The 100-nm Au-tiopronin NPs were round and equally distributed in size with good dispersion. (B) The hydrodynamic diameter of the Au-tiopronin NPs was measured by dynamic light scattering. The hydrodynamic diameter of as-prepared nanoparticles was 103.3 nm, with a narrow size distribution.

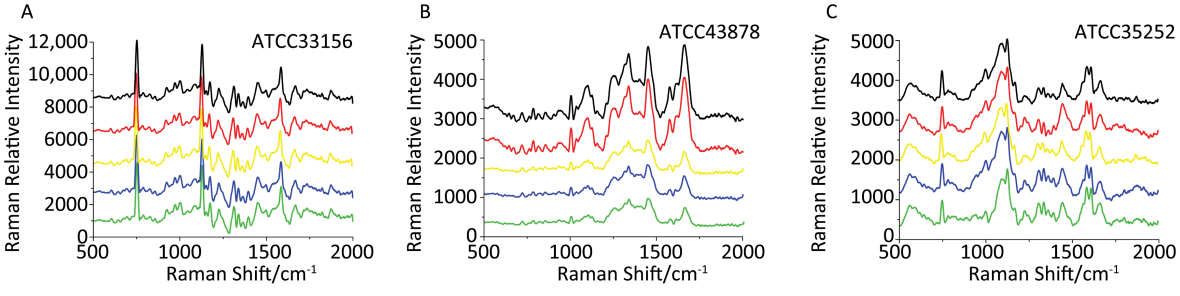

Figure 2. Three *Legionella* strains, ATCC33156 (A), ATCC43878 (B), and ATCC35252 (C), were analyzed using SERS. The SERS spectra were obtained by scanning five replicate samples of each strain.

SERS Test of Five ATCC Legionella Strains with Differential Virulence Characteristics

Five *Legionella* strains (ATCC33152, ATCC33156, ATCC35252, ATCC43878, and ATCC35249) were used to evaluate the capacity of SERS to distinguish pathogenic strains with differential virulence. Among them, ATCC33156 and ATCC33152 had high intracellular growth ability, demonstrating that they were highly virulent and that the other three were low-virulence strains (Figure 3A). In the SERS patterns (Figure 3B), the highest difference of peak height for ATCC33156 and ATCC33152 was >4000. In contrast, the greatest difference in peak height for ATCC35252, ATCC43878, and ATCC35249 was <2000. The SERS results were consistent with that of intracellular growth tests, suggesting that SERS can distinguish the virulence levels of pathogenic *Legionella* strains.

SERS Determination of Three Clinical Legionella Strains with Different Virulence Characteristics

Three clinical *Legionella* strains (strain 1, strain 2, and strain 3) with different intracellular growth abilities were tested to evaluate the clinical applicability of SERS to distinguish *Legionella* strains with different virulence levels. Among these strains, strain 1 and strain 2 displayed high intracellular growth, while strain 3 did not (Figure 4A). In the SERS patterns, the highest difference of peak height for strain 1 and strain 2 was >4000, but the highest difference of peak height for strain 3 was <2000 (Figure 4B). The consistency between SERS and intracellular growth tests demonstrated that strain 1 and strain 2 were highly virulent, and strain 3 was lowly virulent.

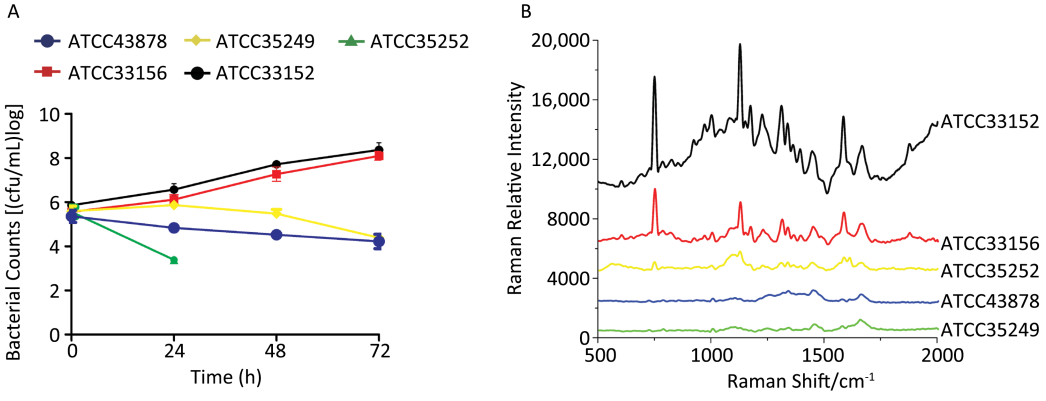

Figure 3. SERS and intracellular growth ability tests of five *Legionella* strains from the ATCC. The test strains were ATCC33152, ATCC33156, ATCC35252, ATCC43878, and ATCC35249. (A) Intracellular growth of the five *Legionella* strains. (B) SERS patterns of the five tested strains. The abscissa is the Raman shift, and the ordinate is the Raman relative intensity.

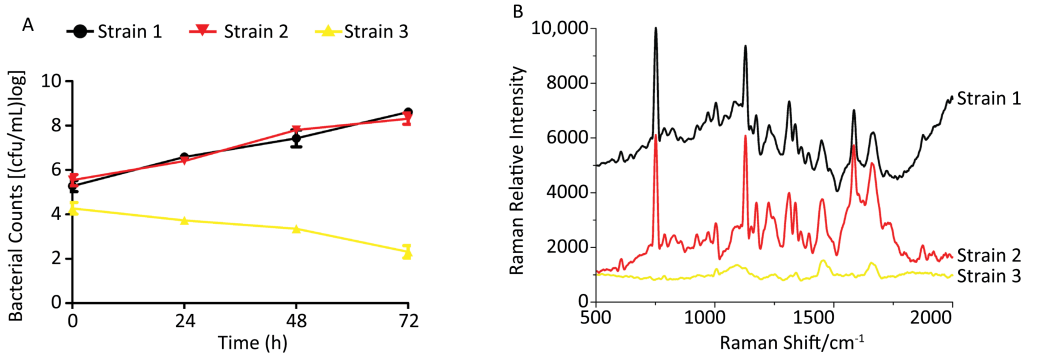

Figure 4. SERS and intracellular growth ability tests of two clinical *Legionella* strains and one environmental strain. The test strains were designated strain 1, strain 2, and strain 3. (A) Intracellular growth of five *Legionella* strains. (B) SERS patterns of the five tested strains. The abscissa is the Raman shift, and the ordinate is the Raman relative intensity.

PCA Analysis

PCA was used to further determine whether individual strains could be differentiated using SERS spectra. Figure 5 shows the PCA score plots of the two principal components (Group 1 and Group 2) for the data. Four strains (ATCC33152, ATCC33156, strain 1, and strain 2) with strong virulence were found to constitute group 2, while group 1 included the weakly virulent strains ATCC35252, ATCC35249, ATCC43878, and strain 3. The PCA results are consistent with the SERS analysis and cell infection results, suggesting that our SERS assay is sensitive, reproducible, and can be used for *Legionella* pathogenicity detection.

DISCUSSION

Legionella bacteria are a group of common pathogenic strains that are mainly found in fresh water, mud, and other aquatic environments[23]. In addition to aerosol dissemination[22], intake of potable water containing *Legionella* can also result in Legionnaires' disease[24]. After internalization by alveolar macrophages, *L. pneumophila*-containing phagosomes do not acidify[25] or fuse with lysosomes[26]. Instead, the mitochondria, smooth vesicles, and rough endoplasmic reticulum (RER) near these *L. pneumophila*-containing vacuoles are recruited, and *L. pneumophila* begins to multiply in this unique niche[27]. This altered endocytic pathway is considered to be controlled by the Dot/Icm type IV protein secretion system[28-30]. The dot/m genes are essential for the intracellular growth of *L. pneumophila*[31-32].

It is well known that the enhanced Raman field is within 10 nm away from the surface of nanoparticle. We have reviewed the literature concerning unlabeled AuNPs interacting with *Legionella*. Interestingly, Stojak et al. report that after exposure to AuNPs, the AuNPs are absorbed onto the *Legionella* surface and eventually absorbed into the cells, where they aggregat[33]. There are also reports on other bacteria exposed to unlabeled AuNPs in which the unlabeled AuNPs attach to the bacteria surface[34]. Therefore, it is possible that AuNPs bind to bacteria surfaces via nonspecific absorption.

Additionally, it has been reported that virulent *Legionella* strains contain unique a Dot/Icm complex that is regarded as the key to host-infection because this complex is responsible for translocation pore formation between the host-pathogen membranes[31]. Therefore it is presumably located on/near the bacteria membranes. AuNPs clustered near such a complex would greatly enhance the unique Raman signal responding to the complex, and thus, the virulent *Legionella* strains could be distinguished from mildly virulent strains via SERS.

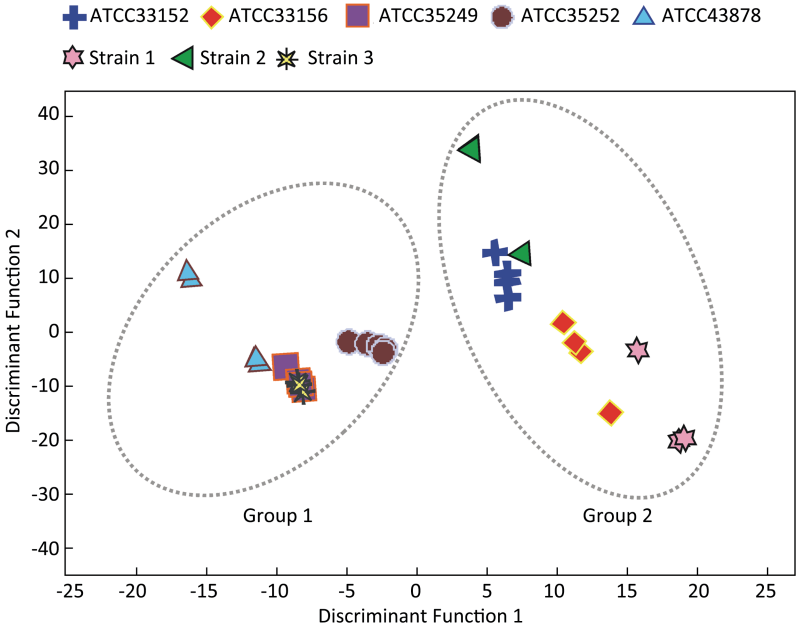

Figure 5. PCA of the relationships among the eight different strains. Group 1 includes ATCC35252, ATCC35249, ATCC43878, and Strain 3. Group 2 includes ATCC33152, ATCC33156, Strain 1, and Strain 2.

Rapid detection of the virulence levels of pathogens has important implications for environmental monitoring and clinical diagnosis. Because the *Legionella* strains selected in this study included various types, the results indicate that our method and analysis criteria are applicable for the joint detection of various *Legionella* species. Currently, the amount of research on rapid detection of pathogen virulence strength is relatively small[35]. The traditional methods of isolated culture and serological detection include PCR, ELISAs, and real time-PCR, which require lengthy testing times. The presence of the Dot/Icm loci in several species of *Legionella* was shown by Southern or PCR analysis[36]. However, the contributions of these loci to the pathogenesis of other species have yet to be investigated.

PCA is based on the sign of the second derivative of the SERS spectrum obtained on in situ-grown Au cluster-covered SiO_2 substrates, resulting in improved reproducibility and enhanced specificity for bacterial diagnostics. The barcode-generated clustering results are systematically compared to those obtained from corresponding spectral intensities, first derivatives, and second derivatives for the SERS spectra of closely related cereus group Bacillus strains[37]. PCA plots and corresponding hierarchical cluster analysis (HCA) dendrograms illustrate the improved bacterial identification resulting from the barcode spectral data reduction. Supervised DFA plots result in slightly improved group separation but show more susceptibility to false positive classifications than the corresponding PCA contours. In addition, this PCA treatment was used to highlight the enhanced bacterial species specificity observed for SERS as compared to normal bulk (non-SERS) Raman spectra. The identification algorithm described here is critical for the development of SERS microscopy as a rapid, reagentless, portable diagnostic tool for bacterial pathogen detection. The testing time was inclusive from sample treatment to data analysis, which was cut down from >1 h to 5 min.

However, these researchers did not apply SERS to identify the virulence strength of pathogens. Because showers, drainage facilities, and other pieces of factory equipment are increasingly exposed, which significantly increases the chances for *Legionella* infection, early sample collection and rapid detection of *Legionella* virulence is very important.

In this study, the results of five repeated measurements for three ATCC type strains of *Legionella* indicated the strong stability of SERS technology, while the analysis of six strains with SERS was consistent with that of cell infection experiments (Figures 3 and 4). Therefore, the virulence of *Legionella* strains can be confirmed as follows in accordance with the Raman spectrograms of test strains: within the abscissa range of 500-2000 (Raman shift/cm^{-1}): if ≥1 peaks with ordinate (counts) strength >4000 is observed, the strain is highly virulent; otherwise, its virulence is low.

In this study, we found that the significant peaks consistently found in virulent strains correlated to common virulence factors found in these strains by SERS, and the criterion was verified by subsequent cell experiments. The strains were thus divided to high and low virulence strains. The result explored the use of SERS to detect the virulence strength of *Legionella* strains with the hope that certain guiding effects would be achieved for the rapid identification of virulence in clinical settings. In future research, we will determine the key substance or element corresponding to the peaks.

CONCLUSION

The present study indicates that the established SERS protocol can be used as a rapid and reliable method to distinguish virulent and mildly virulent strains of *L. pneumophila*, which can be further used in clinical samples.

1) Surface enhanced Raman spectroscopy technology (SERS), using gold nanoparticles as a base, was developed for rapid detection of *L. pneumophila* strains.

2) Established SERS can be used as a rapid and reliable method to distinguish virulent strain and mild strain of *L. pneumophila*.

3) SERS rapid identification method used in clinical strains also obtained the accurate results.

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