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Diagnosis of bacterial pathogens in the dialysate of peritoneal dialysis patients with peritonitis using surface-enhanced Raman spectroscopy

Ni Tien, PhD [a,b], Hung-Chih Chen, MD [c,d], Shiow-Lan Gau, PhD [e], Tzu-Hsien Lin, MS [d], Hsiu-Shen Lin, MS [a,b], Bang-Jau You, PhD [f], Po-Chuan Tsai, MS [d], I-Ru Chen, MD [c,d], Ming-Fan Tsai, MS [c], I-Kuan Wang, MD, PhD [c,d], Chao-Jung Chen, PhD [g], Chiz-Tzung Chang, MD, PhD [c,d,*]

[a] Department of Laboratory Medicine, China Medical Univeristy Hospital, No. 2 Yu-Der Rd, North District, Taichung 40447, Taiwan
[b] Department of Medical Laboratory Science and Biotechnology, China Medical University, No. 2 Yu-Der Rd, North District, Taichung 40447, Taiwan
[c] College of Medicine, China Medical University, No. 2 Yu-Der Rd, North District, Taichung 40447, Taiwan
[d] Division of Nephrology, China Medical University Hospital, No. 2 Yu-Der Rd, North District, Taichung 40447, Taiwan
[e] Department of Statistics, Feng Chia University, No 100 Wenhua Rd, Seatwen, Taichung 40724, Taiwan
[f] Department of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, No. 2 Yu-Der Rd, North District, Taichung 40447, Taiwan
[g] Graduate Institute of Integrated Medicine, China Medical University, No. 2 Yu-Der Rd, North District, Taichung 40447, Taiwan

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ABSTRACT

Background: Bacterial peritonitis is the most common cause of peritoneal dialysis (PD) therapy drop-out. A quick and accurate diagnosis of the bacterial pathogen can reduce the PD drop-out rate. Surface-enhanced Raman spectroscopy (SERS) can rapidly identify bacteria using chips coated with nano-sized metal particles.
Methods: Known bacteria were loaded in the SERS-chips and illuminated with laser light to establish a reference Raman spectra library. Dialysate from PD peritonitis patients was concentrated by centrifuge and examined with the same SERS, and the resulting Raman spectra were compared with library spectra for bacteria identification. Principal component analysis was used for further confirmation. The same batches of dialysate were sent to routine culture as a reference bacteria identification method. The results of the 2 identification methods were compared.
Results: A total of 43 paired-samples were sent for study. There were 37 samples with bacteria identified but 6 were culture-negative by the reference method. 31 bacteria were identified in paired-samples by SERS, among which, 29 bacteria were exactly the same as those identified by the reference method. Bacteria not included in the reference library spectra cannot be identified.
Conclusions: SERS techniques can rapidly identify bacterial pathogens in the dialysate of PD peritonitis patients.
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1. Introduction

Peritonitis is the most common cause of patient drop-out from peritoneal dialysis (PD) therapy. Infection, especially bacterial peritonitis, is the major pathogenic cause of PD peritonitis [1]. Patients with PD peritonitis are characterized by abdominal pain and turbid dialysate. Severe PD peritonitis may be associated with fever, septic shock or patient mortality rate. Early diagnosis of bacterial pathogens and accurate antibiotic usage benefit microbial eradication and patient cure [2]. In current clinical practice, dialysate from PD peritonitis is sent for bacterial culture, and empirical antibiotics are then administered. Antibiotics chosen will be adjusted according to culture results [2]. It frequently takes 24 h or more for bacterial identification and even longer to report the antibiotic susceptibility test results [3]. A new method mandates quicker bacteria identification.

Raman spectroscopy is a spectroscopic technique that is frequently used to detect the vibrational energy of molecules [4]. To obtain weak Raman scattering, a laser light with wavelength ranging from ultraviolet to near infra-red region is employed. The emitted scatterings from molecules after excited by the laser are collected by a lens and passed through a monochromator. The elastic radiation, or radiation at the corresponding wavelength of laser light, is filtered out by a filter and the remaining of collected light or inelastic radiation is dispersed on a detector and turned into a specific Raman spectrum [5]. The Raman shifts are defined as the wavelength difference between incident and scattered light in the Raman spectrum. Molecules can be identified through their specific fingerprint spectra. However, the signal of spontaneous Raman scattering is very weak and not easily discerned. The Raman signal can be augmented by absorbing molecules on the surface

Abbreviations: MRSAq, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*; PD, peritoneal dialysis; SERS, surface-enhanced Raman spectroscopy.

* Corresponding author at: No. 49, Hsueh-Shih Rd, North District, Taichung 40402, Taiwan.
E-mail address: d19863@mail.cmuh.org.tw (C.-T. Chang).

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of Raman substrates, frequently nanosized metal such as gold or silver [6]. Surface enhanced Raman spectroscopy (SERS) can increase Raman scattering intensity by a factor of 10^{10} to 10^{11} [7]. SERS techniques can be applied in bacterial identification [8–10]. It is a culture-free detection method for the quick diagnosis of bacterial infection in body fluids [11]. To detect bacteria in a fluid sample, we frequently need to evaporate water from the aqueous sample to allow the contact of bacteria with the SERS substrate. The presence of water in the sample can cause uncertain spreading of aqueous samples on the SERS substrate and result in problems of reproducibility [12]. Recently, a sensitive cylindrical SERS substrate array was developed. The cylindrical SERS chip allows spontaneous contact of the specimen with SERS substrate and increases the sensitivity and reproducibility of detection [13].

In the current study we used cylindrical SERS substrate array to identify bacteria in the dialysate of PD peritonitis. The cylindrical SERS was fabricated by decorating silver nanoparticles on the tip of 2-mm diameter polymethylmethacrylate (PMMA) rod. The minimum sample volume for one analysis is small and can be <5 μl. Also SERS spectra can be acquired without drying the samples [13].

2. Methods

2.1. Patients

PD patients with abdominal pain and turbid dialysate in China Medical University Hospital from June 2014 to May 2015 were recruited for study. We retrieved 40 ml of dialysate from patients with PD peritonitis. 10 ml of the dialysate was sent to dialysate routine examinations, including white blood cell (WBC) count, red blood cell count, and differential count. These examinations were performed manually by hospital bacterial laboratory technicians. We included only dialysate with WBC over 100/μl and neutrophil count over 50% of the WBC count according to PD peritonitis criteria [14]. Peritonitis patients who used antibiotics before dialysate sampling were excluded. Dialysate retrieval from patients was performed after getting patient informed consent. The study followed the regulation of the institutional review board.

2.2. Reference bacterial culture

Ten milliliters of dialysate was inoculated into aerobic (BD BACTEC Plus Aerobic/F) and another 10 ml into anaerobic (BD BACTEC Plus anaerobic/F) blood culture vials (BD) separately as reference culture [15]. These vials were sent to a hospital laboratory for bacterial identification. These vials were incubated in Phoenix Automated Microbiology System (BD) for microbial identification following the manufacturer`s manual [16]. Reference cultures were used as positive control of the study.

2.3. Establishment of standard Raman bacteria spectra library

Bacteria commonly seen in PD peritonitis were purchased from ATCC (American Type Culture Collection) such as methicillin-sensitive *Staphylococcus aureus* (MSSA, ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), Group A Streptococcus (GAS, ATCC 19615), *Escherichia coli* (*E. coli*; ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Klebsiella pneumoniae* (ATCC 70063), *Pseudomonas aeruginosa* (ATCC 27853) and *Acinetobacter baumannii* (ATCC 10591). Other bacteria were isolated from hospital patients such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus salivarius*, *Streptococcus oralis*, *Corynebacterium striatum*, and *Enterococcus faecium*. These samples were used as standard bacteria to establish a reference Raman spectra library. The bacteria were grown in culture plates. A sterile culture loop was used to fetch the bacteria and dissolve them in 10 ml distilled water. To avoid contaminating the bacterial wall with culture material, the bacteria solution was centrifuged at 700 rpm for 10 min separate bacteria from the WBCs, immune-cells and cellular debris. The precipitated bacteria was then washed with 10 ml distilled water and centrifuged again. The resulting bacterial precipitate was dissolved in distilled water and adjusted to a concentration of 5 McFarland (MCF) (3×10^9 CFU/ml) [17], which was then further diluted by distilled water to different concentrations. 3 μl of dialysate was loaded into a cylindrical Raman SERS-chip (Labguide Co.) [13]. The SERS-chip was illuminated with laser light and detected by a Raman spectrometer (QE Pro, Ocean). The laser wavelength was 785 nm with a laser power of 20 mW. The integration time was 5 s. The resulting spectra were analyzed and served as the standard reference spectra library.

2.4. Bacteria identification using SERS-chips

Ten milliliters of dialysate from peritonitis patients was centrifuged at 700 rpm for 10 min to separate bacteria from the WBCs, immune-cells, and cellular debris. The supernatant, containing target pathogens, was then mixed with 10 ml distilled water and centrifuged at 13,000 rpm for another 10 min to concentrate the bacterial samples. The precipitate was subsequently diluted with distilled water to an optical density of 5 MacFarland (MCF) [17]. Concentrated dialysate (3 μl) was loaded onto a Raman SERS-chip. The SERS-chip was illuminated with a Raman spectrometer. The resulting spectrum was then compared to spectra from the reference spectra library using RM.View software (Ocean) to identify possible bacteria in the sample.

2.5. Principal component analysis

The Raman spectra (400-2000 cm^{-1}) of reference bacteria and dialysate bacteria after RM.View software analysis were further analyzed by principal component analysis (PCA) using SPSS ver 22 [18]. Dots of PCA from dialysate bacteria that co-localized with dots of reference bacteria were deemed dots from the same type of bacteria.

2.6. Antibiotic susceptibility test

To determine the antibiotic susceptibility of bacteria, we added antibiotics to bacteria to observe any bacteria-specific Raman shift changes. Oxacillin and vancomycin were added to MSSA and MRSA and incubated for 6 to 24 h. Bacteria after antibiotic treatment were loaded onto the SERS chips and the resulting Raman spectra were compared to that of bacteria without antibiotic treatment. Other bacteria were also tested by different kinds of antibiotics. The concentrations of antibiotics used were higher than or equal to the minimal inhibitory concentration (MIC) [19].

2.7. Statistical analysis

Positive and negative predictive values were calculated using the culture results from hospital laboratory as reference.

3. Results

3.1. The reference Raman spectra from known bacteria

The bacteria from ATCC or hospital patients were washed with distilled water and prepared as MCF = 5.0 (1×) or 1.5×10^9 CFU/ml. Solutions were then diluted with distilled water at 10-fold dilution (10×), 100-fold dilution (100×), 1000-fold dilution (1000×) or 10,000-fold dilution (10,000×). A representative Raman shift of *E. coli* without dilution (1×) was shown in Fig. 1A (left). Raman shift spectra from serial dilutions of *E. coli* were merged and shown in Fig. 1A (right). Raman shifts of different *E. coli* concentrations revealed the same fingerprints. The representative spectra of MSSA (1×) and serial dilutions were shown in Fig. 1B. Similarly, Raman spectra of MRSA without and with

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