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Rapid detection of *Clostridium difficile* toxins in serum by Raman spectroscopy

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ABSTRACT

Background: *Clostridium difficile* infection (CDI) is due to the effects of toxins, toxin A and toxin B on the host. Severe CDI is associated with systemic signs of infection. Animal models of CDI demonstrate a strong correlation between systemic toxemia and the occurrence of severe disease. However, current technologies have low sensitivity to detect *C difficile* toxemia in human subjects. Raman spectroscopy (RS) is an upcoming technology that is used to detect bacteria and their toxins. We speculate that RS may be a sensitive method to detect clinically relevant concentrations of *C difficile* toxins in serum.

Materials and methods: Serum samples were spiked with varying concentrations of toxin A, toxin B, and both. RS was performed on an air-dried serum drop that was placed on a mirror-polished stainless steel slide. Raman spectra were obtained, background corrected, vector normalized, and analyzed by Partial Least Square Linear Discriminant Analysis and Support Vector Machine for Classification. Model accuracy was measured by cross-validation and bootstrap methods.

Results: Toxin-spiked sera of various concentrations (1 ng/mL, 1 pg/mL, and 0.1 pg/mL) were distinguished from control serum 100% with cross-validation error rate ranging from 0% to 18% and bootstrap error rate ranging from 0% to 12% for various concentrations. The sensitivity ranged from 87% to 100% and specificity ranged from 77% to 100% for various concentrations of toxin-spiked serum.

Conclusions: We conclude that RS may be a sensitive method to detect clinically relevant concentrations of *C difficile* toxins in serum and thus to help diagnose severe CDI in patients in real-time at the point of care.

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Introduction

Clostridium difficile is the most common health care pathogen, with approximately a half million new cases with more than 29,000 deaths attributed to *Clostridium difficile* infection (CDI) each year.[1] Signs of severe CDI include hypotension and the need for pressors, profound leukocytosis, mental status changes, and worsening organ failure.[2] These systemic manifestations of CDI have been attributed to *Clostridium* pathogenic factors, principally toxin A (TcdA) and toxin B (TcdB), as well as the immunoinflammatory host response to infection.[3-5]

Animal studies suggest that *C difficile* systemic toxemia alone indicates severe disease.[6,7] Passive immunotherapy with intravenous administration of immunoglobulin resulted in remission of refractory *C difficile* colitis suggesting that toxemia may occur in fulminant CDI in humans.[8] This gives us the impetus to measure toxin levels in serum in severe CDI patients. Reports of toxemia in humans are rare and this is due to low levels of circulating toxins that are below the detection limit of assays.[8,9]

Cell cytotoxicity assay (CCAT) is the gold standard test for laboratory diagnosis of toxemia in serum, due its high sensitivity and specificity. However, this test is not performed by many laboratories due to slow turnaround time (24-72 h), requirement of cell culture facility, and technical complexity.[10] This method was improved drastically by ultrasensitive rapid immunocytotoxicity assay (ICT) for detection of TcdA at doses as low as 1-10 pg/mL. This was achieved by enhancing toxicity of TcdA on Fc gamma receptor I-expressing cells by adding anti-TcdA monoclonal antibody.[11] Turnaround time for ICT was brought down to approximately 3 h by using cryopreserved cells and measuring electrical impedance as a measure of cytotoxicity using real-time cell electronic sensing.[11] However, this test is not sensitive to TcdB at low concentrations (~10 pg/mL limit) and presence of toxin-specific neutralizing antibodies in sera further increased level of detection for both toxins.[9] A simple, rapid, and relatively cheap diagnostic assay for detection of TcdA and TcdB with high sensitivity and specificity is still lacking and is desirable.[9,12]

We propose a rapid, ultrasensitive Raman spectroscopy (RS)-based diagnostic modality for detection of TcdA and TcdB in serum. RS has been used as a diagnostic modality for infectious disease and toxin detection.[13,14] RS is a noncontact, nondestructive, reagent-less optical technique, which provides a unique spectroscopic fingerprint of a pathogen or toxin that is being detected. RS is very sensitive to slight changes in concentrations of toxins even at very low concentrations making it an ideal platform for detection of *C difficile* toxins in serum.

The RS-based diagnostic modality is cost-effective, rapid (<30 min turnaround time), and ultrasensitive (as low as 0.1 pg/mL) and may be employed as a point-of-care testing modality for continuous monitoring of patients and critically ill patients. This test will need verification in clinical trials and comparison with other modalities such as CCAT and ICT.

Materials and methods

Ethics statement

This study was approved by Institutional Review Boards of Wayne State University and Detroit Medical Center. Serum that was used in this study was obtained from healthy volunteer who provided written informed consent.

Toxin-spiked serum preparation

Recombinant TcdA and TcdB were purchased from R&D Systems (Cat# 8619GT020) and diluted to 2 μg/mL stock solutions in phosphate-buffered saline. Blood was collected using venipuncture into BD vacutainer redtop tubes from a healthy volunteer who provided a written consent. Blood was allowed to clot by leaving it undisturbed at room temperature. The clot was removed by centrifuging at 2000 × *g* for 10 min and was aliquoted into 0.5 mL tubes and stored at −80°C until RS. Toxins (TcdA alone, TcdB alone, or both TcdA and TcdB) were spiked into serum at the levels of 1 ng/mL, 1 pg/mL, and 0.1 pg/mL. Serum was used as negative control. Because of the toxicity of TcdA and TcdB, the preparation, dilutions, and RS were done in biosafety level II lab. Toxin-spiked serum samples were stored at −80°C until RS measurements were made.

Raman spectroscopy

RS probes molecular vibrations or rotations associated with chemical bonds in a sample to obtain information on molecular structure, composition, and intermolecular interactions. With this technique, a sample is illuminated with monochromatic light of a certain wavelength, typically from a laser. Although light can interact with the sample via the process of absorption, reflection, or scattering, it is the scattering event that is exploited in RS. Light can be scattered from a sample at the frequency of the incident light, termed Rayleigh (or elastic) scattering.[15] This occurs without a net energy transfer between the incident light and the sample. Light can also be scattered at higher (anti-Stokes shift) or lower (Stokes shift) frequencies than the incident light via an inelastic process and involves a net energy transfer between the incident photons and sample. An inelastic process, termed the Raman Effect, occurs in approximately 1 in 10^7 photon interactions with matter and depends on a change in the polarizability of a molecule as it vibrates or rotates.[16-18] By monitoring the intensity profile of inelastically scattered light as a function of frequency, the unique spectroscopic fingerprint of a sample (pathogen or toxin) is obtained. By convention, the frequency of scattered light is converted to Raman shifts, which is the difference in frequency between the incident and scattered light (usually in units of wave number). The wave numbers at which Raman bands occur is characteristic of vibrational modes of specific bond types in a molecule, with the intensity directly proportional to the concentration of species that give rise to the bands.[19] Since each sample has a unique composition, the spectroscopic profile arising from Raman-active