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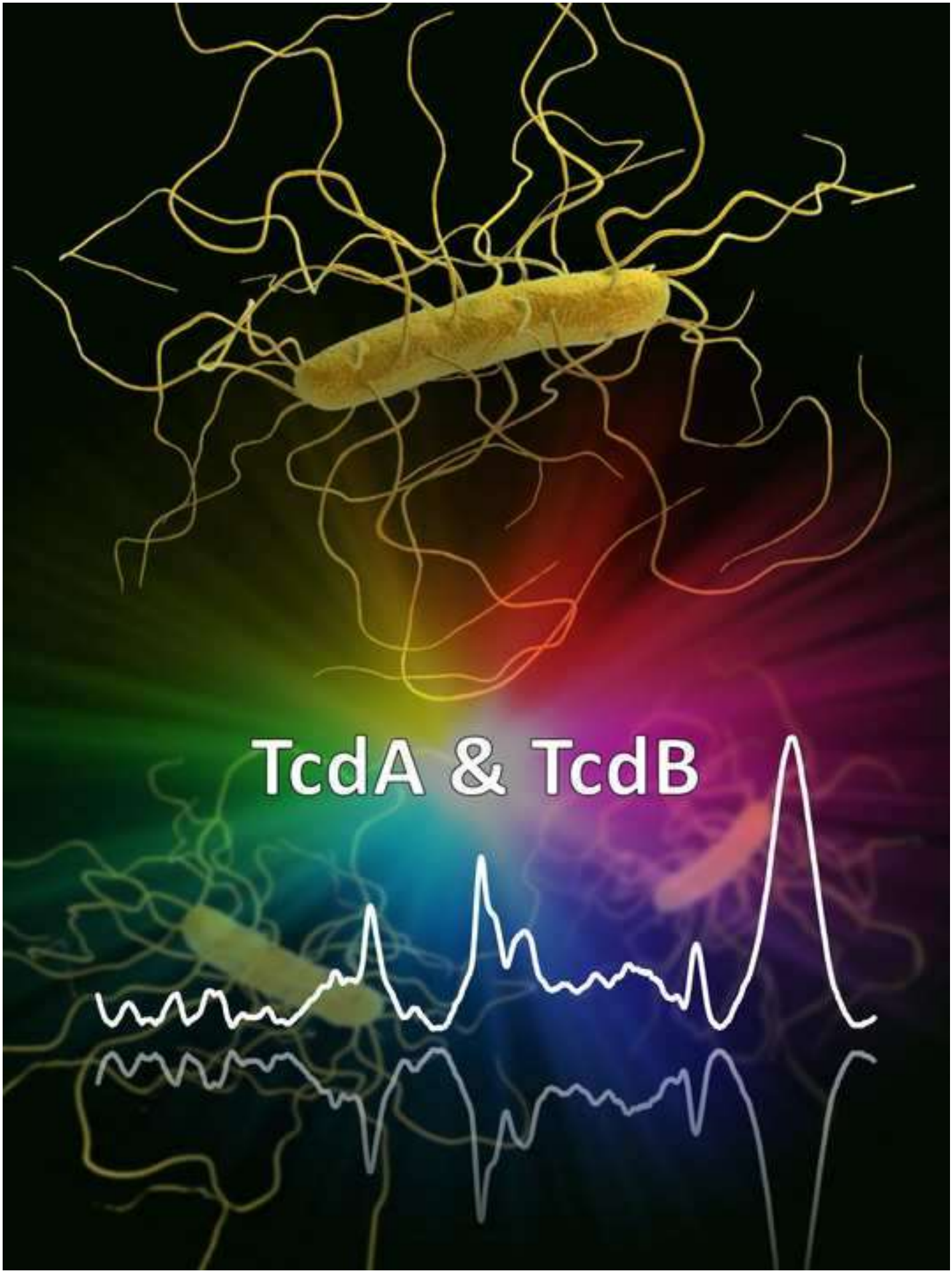

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Manuscript Title

Rapid Detection of *Clostridium difficile* Toxins in Stool by Raman Spectroscopy.

Short title

Rapid detection of TcdA & TcdB in stool

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Author's contributions: SKK was involved in study design, protocol development, toxin-spiked stool preparation, statistical and computational analysis, interpretation of the data, and manuscript preparation. SY was involved in acquisition of Raman spectra from stool samples, study design, Raman protocol development and manuscript approval. CH was involved in preprocessing of Raman spectra and manuscript approval. MB created the cover image. MB, GWA and LND were involved in all aspects of this study including study design, analysis, manuscript preparation and final approval. GWA and LND are co-principle investigators.

Abstract

Background: Clinical practice guidelines define *Clostridium difficile* infections (CDI) as diarrhea (≥3 unformed stools in 24 hrs.) with either a positive *C. difficile* stool test or detection of pseudomembranous colitis. Diagnostic modalities such as toxigenic culture (TC) and nucleic acid amplification testing (NAAT) can identify presence of toxigenic *C.*

difficile in stools. But these tests are confounded by the presence of asymptomatic colonization of toxigenic *C. difficile* and lead to overdiagnosis of CDI. The presence of two large toxins, toxin A & B (TcdA and TcdB) is necessary for pathogenicity. Detection of toxins using toxin enzyme immunoassay (Toxin-EIA) is difficult as it has low sensitivity and moderate specificity. Raman spectroscopy (RS) is a novel technology that is used to detect bacteria and their toxins. RS does not require any reagents for detection such as antibodies, enzymes, primers or stains. We hypothesize that RS is a sensitive method to detect *C. difficile* toxins in stool and will solve the problem of overdiagnosis of CDI.

Materials and Methods: CDI negative stool samples were spiked with concentrations (1 ng/ml, 100 pg/ml, 1 pg/ml and 0.1 pg/ml) of TcdA and TcdB. RS was performed on air-dried smeared samples of stool supernatant on a mirror polished stainless-steel slide. Since RS of feces is difficult due to confounding background material and autofluorescence, samples were photo-bleached prior to spectral acquisition to reduce autofluorescence. Raman spectra were obtained, background corrected, and vector normalized. The data was split into training (70%) and test (30%) data sets. The machine learning methods used on the training data set were Support Vector Machine (SVM) with Linear and Radial Kernels, Random Forest (RF), Stochastic Gradient Boosting Machine (GBM) and Principle Component Analysis - Linear Discriminant Analysis (PCA-LDA). Results were validated using a test data set. The best model was chosen, and its accuracy, sensitivity and specificity were determined.

Results: In our preliminary results, at all concentrations (1 ng/ml, 100 pg/ml, 1 pg/ml and 0.1 pg/ml) TcdA or TcdB spiked stool was distinguished from un-spiked stool by all models with accuracies ranging from 64% to 77%. GBM, PCA-LDA and SVM Linear Kernel performed best with sensitivities ranging from 69% to 90% and specificities ranging from 43% to 78%.

Conclusions: Using RS, we successfully detected TcdA and TcdB in stool samples albeit with moderate to high sensitivity and low to moderate specificity. Sensitivity and specificity could be further increased with the implementation of deep learning methods, which require large sample sizes. In terms of sensitivity, RS performs better than Toxin-EIA and has the potential to rapidly detect *C. difficile* toxins in stool at clinically relevant concentrations and thereby help mitigate overdiagnosis of CDI.

Key words:

Clostridium difficile Infection, CDI, TcdA, TcdB, CDI diagnostic test, Raman spectroscopy, rapid detection, diagnostic test.

Introduction:

Clostridium difficile infections (CDI) are the major cause of antibiotic-associated diarrhea (1/3rd of all cases), antibiotic-associated colitis (3/4th of all cases) and antibiotic-associated pseudomembranous colitis (90-100% of all cases) (1, 2). In the USA alone, CDI accounts annually for half a million new cases resulting in nearly 30,000 gastroenteritis-associated deaths within 30 days of diagnosis (3). It was estimated that the annual health care burden of CDI in USA is around $6 billion (3). Pathogenicity of CDI is due to toxins, toxin A (TcdA) and toxin B (TcdB) (4). Accurate diagnosis of CDI is challenging due to poor sensitivity, specificity and the long turnover time of the available diagnostic modalities. Current approaches to diagnosing CDI prioritize either detection of the organism itself, or detection of toxins produced by the organism. Each of these approaches has its own limitations (Table 1). Diagnosis of CDI with modalities that detect the presence of toxigenic *C. difficile,* such as toxigenic culture (TC) and nucleic acid amplification testing (NAAT), are confounded by the presence of asymptomatic colonization of toxigenic *C. difficile (5-7).* Around 3-26% of acute care hospital patients, 5-7% of long-term care facility inhabitants and less than 2% of individuals that never had any exposure to a health care facility, are asymptomatic carries of *C. difficile (8).* Thus, reliance on NAAT alone leads to overdiagnosis of CDI (6, 9-12). While the presence of toxin is necessary for disease, detection of toxins in stool is difficult. Cell Culture Cytotoxicity Neutralization Assay (CCNA) is a gold standard test for the detection of TcdA and TcdB in stool with 94-100% sensitivity and 99% specificity. However, CCNA is a labor-intensive test requiring a cell culture facility and has a 24 to 48 hours turnaround time. Therefore, CCNA is not routinely performed (13). Detection of toxins using TcdA and TcdB enzyme linked immuno-assays (Toxin-EIA) has low sensitivity and moderate specificity making the diagnosis complex (14). For these reasons, clinical practice guidelines for CDI in adults and children (2017) gave weak recommendations based on low quality evidence for diagnosis of CDI (8). According to these guidelines, if clinicians and lab technicians at the institutional level set guidelines to only test samples from patients likely to have CDI, then NAAT alone is enough for diagnosis of CDI. In the absence of such guidelines, it is recommended to perform a multiple step algorithm in which, initial screening of the samples is done by a poorly specific Glutamate Dehydrogenase Enzyme Immunoassay (GDH), followed by a confirmatory Toxin-EIA or Toxin-EIA arbitrated by NAAT or NAAT and Toxin-EIA. However, even with the multiple step algorithm approach, problem still exist resulting in either under or over diagnosis of CDI (8).

There is a great need for a single confirmatory diagnostic test that is rapid and can detect TcdA and TcdB in stool with high sensitivity and specificity. We propose a Raman Spectroscopy (RS) based diagnostic test for rapid detection of TcdA and TcdB in stool which has great potential as a standalone point of care testing unit. RS has been used as a diagnostic modality for infectious diseases and toxin detection (15, 16). RS is a reagent-less optical technique that provides a unique spectroscopic fingerprint

of a pathogen or toxin that is being detected (17). 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 stool. Using RS, we identified TcdA and TcdB in toxin-spiked serum at biologically relevant concentrations (1 ng/ml to 0.1 ng/ml) with high sensitivity and specificity with the purpose of diagnosing severe CDI (17). This work has been extended to a modality to detect TcdA and TcdB in stool to diagnose mild and moderate CDI. The RS based diagnostic modality is cost effective (doesn't require any reagents such as antibodies, enzymes, primers or stains), rapid (< 30 min turnaround time requiring minimal sample preparation) and has potential to be a standalone point of care test for diagnosis of CDI. This test will need verification in clinical trials and comparison with CCNA, which is a gold standard method for detection of TcdA and TcdB in stool.

Material and methods:

Ethics Statement: All the experiments were done in biosafety level II laboratory. No human or animal testing was done in this study. Stool samples used in this study were obtained from OpenBiome (Boston, MA).

Toxin-spiked stool 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 (PBS). *C. difficile* negative stool samples were obtained from OpenBiome (Boston, MA). Stool samples (4 biological replicates) were thawed as per OpenBiome specifications, spun down on tabletop centrifuge to remove particulate matter. Toxins (TcdA or TcdB) were spiked into stool supernatant at the levels of 1 ng/ml, 100 pg/ml, 1 pg/ml and 0.1 pg/ml. Un-spiked stool supernatant was used as negative control. In total 36 stool samples (4 unspiked and 32 toxin-spiked) were used for the analysis. Due to the toxicity of TcdA and TcdB, the preparation, dilutions and RS were done in biosafety level II lab.

Acquisition of Raman spectra: For Raman acquisition, a pipetted drop (10 µl) of un-spiked or toxin-spiked stool supernatant was placed on a sterile mirror polished stainless-steel substrate (alloy 304, Stainless Supply, Monroe, NC) and was airdried by gently blowing sterile air for 5 min. Since the RS of stool samples have excessive autofluorescence, each sample was photobleached by exposing it to laser light of 514 nm using 50X objective for a time period of 10 min. Photobleaching reduced autofluorescence significantly (data not shown). Immediately after photobleaching, the Raman spectra of air-dried stool samples were recorded with an inVia Raman microscope (Renishaw, Gloucestershire, UK) equipped with a 514 nm excitation laser, 1200 l/mm grating, 576 x 400-pixel thermoelectric cooled charge coupled device, and WiRE 3.4 software (Renishaw Inc., Hoffman Estates, IL). The laser light was focused onto the sample through a 50x N-plan Leica microscope (Leica Inc., Allendale, NJ)

objective with numeric aperture of 0.75 and working distance of 0.75 mm was used for measurements. Spectra were acquired using 10% laser power (corresponding to 4 mW at the sample) over a spectral range of 100-3200 cm^{-1} with 5 accumulations at an integration time of 10 seconds. From the 36 samples, a total of 422 Raman spectra were obtained. Among them, 62 spectra were eliminated due to excess residual autofluorescence (supp table 1).

Pre-processing of Raman spectra: After acquisition, raw spectra were preprocessed by applying an in-house developed LabVIEW procedure for background subtraction and normalization. Modified Morphology method was used for background subtraction (18). Cosmic ray spikes were removed by eliminating peaks with large second derivative values. All the spectra were normalized by unit vector normalization.

Multivariate Analysis: Background subtracted, vector normalized Raman spectra were split into 70% training and 30% test data sets. Machine learning methods such as Principle Component Analysis - Linear Discriminant Analysis (PCA-LDA), Support Vector Machine - Linear and Radial kernels (SVM), Random Forest (RF) and Stochastic Gradient Boosting Machine (GBM) were used on the training data set and their results were validated on the test data set (19, 20). The best performing model for each analysis was chosen and confusion matrix, accuracy, sensitivity and specificity were calculated. Machine learning was performed using CARET Package (C_lassification _A_nd _RE_gression _T_raining) in R programming environment (21, 22).

Learning curves: For each multivariate analysis (un-spiked vs. toxin-spiked stool at 1 ng/ml, 100 pg/ml, 1 pg/ml and 0.1 pg/ml concentrations) learning curves were plotted using Random Forest method. A learning curve is a diagnostic tool for machine learning algorithms that learn from a training dataset incrementally. At each increment, performance of the training data set model is validated using the test data set (20% of the total data), and the measured performance for both are plotted as a learning curve. When the gap between training and testing performance curves is narrow, the model accurately represents the general population. When the gap is wide, the model is overfit to the training data set and is not representative of the general population. Receiver Operating Characteristic (ROC) was used as a measure of model performance. ROC is a ratio of true positive rate over false positive rate at various threshold settings. Learning curves were calculated and plotted using CARET Package (C_lassification _A_nd _RE_gression _T_raining) in R programming environment (21, 22).

Results:

Using the 360 spectra acquired from 36 samples (supp table 1), the feasibility of RS to detect *C. difficile* toxins; TcdA and TcdB in stool was demonstrated at concentrations of 1 ng/ml, 100 pg/ml, 1 pg/ml and 0.1 pg/ml. Figure 1 shows the mean Raman spectra of

the un-spiked, TcdA spiked and TcdB spiked stool at all concentrations (1 ng/ml, 100 pg/ml, 1 pg/ml and 0.1 pg/ml). For multivariate analysis, each toxin-spiked stool (TcdA or TcdB) sample was compared with un-spiked stool separately at each concentration. Multivariate analysis revealed biomarker signatures from several Raman spectral regions with spectroscopic differences enough to distinguish toxin-spiked stool from un-spiked stool. However, the accuracy of preliminary testing models was moderate, ranging from 64% to 77% with Kappa statistic (compares observed accuracy with expected accuracy) ranging from 0.26 to 0.52 (fair to moderate). The sensitivity ranged from 71% to 91% and specificity ranged from 43% to 78% (Table 2). The GBM model performed better for higher spiked concentrations, while the PCA-LDA and SVM linear kernel models performed better for lower concentrations.

Discussion:

Due to the high autofluorescence of stool, samples preparations steps were required. Therefore, samples were spun down to remove particulate matter and thoroughly airdried on a stainless-steel slide. To enhance the signal, several changes to our initial Raman spectroscopy protocol were made including: 1) a change in laser excitation wavelength from 785 nm (red) to 514 nm (green), 2) a reduction in laser power from 100% to 10%, 3) a reduced spectral acquisition time from 20 sec to 10 sec and 4) photobleaching the samples for 10 min prior to Raman spectral acquisition. For spectral preprocessing, a modified Morphology method was used to remove the background from each Raman spectra and to elucidate the spectral features (18). All of these steps significantly reduced autofluorescence (data not shown). Despite these efforts, around 14% of spectra (62 out of 422) that were acquired from 36 samples had excessive autofluorescence and were eliminated from the analysis (outliers).

The diet of a person brings great variation in the contents of the stool sample which results in great variation among Raman spectra- as RS is highly sensitive. For this reason, it is necessary to use a very conservative approach for data analysis. A modified multivariate analysis approach was adapted in which the data was split into 70% training and 30% test data sets for each group of Raman spectra. For each machine learning method, training data was used to build the model and the results were validated on the test data. This was done to avoid overfitting the model on the existing data and any biases associated with it. We chose the best performing model for each concentration based-on the accuracy and Kappa statistic and calculated sensitivity and specificity.

The sensitivity of RS to detect TcdA (90.9% to 76.5%) is higher than TcdB (76.5% to 68.8%) and the overall sensitivity is moderate to high. Specificity of RS to detect TcdA (42.9% to 77.8%) and TcdB (54.5% to 72.7%) is somewhat similar with overall specificity being low to moderate for the limited data set. We attempted to improve

model performance of the limited data set by detecting both TcdA and TcdB together in stool. However, this approach did not improve overall sensitivity (data not shown).

The sensitivity of the RS test is moderate to high and specificity is low to moderate. This could be due to the variation in Raman spectra corresponding to the variation in stool samples in our initial data set (36 samples and 360 spectra). In general, as the training sample number increases, predictive power of the model increases, resulting in higher sensitivity and specificity. We tested this hypothesis by plotting learning curves. Learning curves suggested that, at all concentrations (1 ng/ml, 100 pg/ml, 1 pg/ml and 0.1 pg/ml) tested, as the training data sample number is increased, the sensitivity and specificity of the testing data is increased, resulting in ROC close to 1 (Figure 2). Further the gap between training and testing curves narrowed suggesting that the model is representative of general population.

Although few challenges exist, preliminary results demonstrate the feasibility of RS as a diagnostic modality for rapid detection of TcdA and TcdB in stool at clinically relevant concentrations. Compared to the Toxin-EIA test, that has low sensitivity and moderate specificity, RS can detect free toxins with moderate to high sensitivity and low to moderate specificity. Future work will include, using large number of patient samples accompanied by robust multivariate analysis methods such as deep learning to improve sensitivity and specificity and comparing results to CCNA which is a gold standard test for detection of TcdA and TcdB in stool.

Conclusion: RS is a rapid (< 30 min turnaround time), cost effective (no reagents required) diagnostic test for detection of TcdA and TcdB in stool. Due to these advantages, RS has great potential to be a standalone point of care diagnostic modality to detect TcdA and TcdB in stool at clinically relevant concentrations and help mitigate overdiagnosis of CDI.

Disclosure
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Figure Captions

Figure 1: Mean Raman Spectra: Mean Raman spectra for un-spiked stool (solid black line), TcdA-spiked stool (solid red line) and TcdB-spiked stool (solid blue line) with respective standard deviation shaded in their respective colors for 1 ng/ml (top), 100 pg/ml (2^{nd} from top), 1 pg/ml (3^{rd} from top) and 0.1 pg/ml (bottom) were plotted on X-axis for Raman shift 300-3200 cm^{-1} and their intensities in arbitrary units on y-axis.

Figure 2: Learning Curves: were plotted for multivariate analysis of all concentrations (1 ng/ml, 100 pg/ml, 1 pg/ml and 0.1 pg/ml). Training (blue) and testing (red) curves with standard deviation (grey) were plotted using ROC as a measure of performance on Y-axis and training data size on X-axis.

Table 1: Available diagnostic tests for CDI and their limitations. Currently available diagnostic tests for CDI were listed in decreasing order of sensitivity. Limitation of each test is given (adapted from table 3 in (8) and (23)).

Table 2: Machine Learning Model Performance Metrics. For each concentration, accuracy of the machine learning algorithm, Cohen's kappa statistic which compares an observed accuracy with an expected accuracy (random chance), sensitivity, specificity and the best performing machine learning model were given.

Supplemental table 1: Sample number. For each stool sample, number of Raman spectra that were used in analysis were given.

Table 1

Diagnostic Modality	Substance Detected	Cost	Sensitivity	Specificity	Limitations
Toxigenic Culture (TC)	Vegetative cells or spores	Low	High	Low	• Labor intensive • Long turnaround time (days) • Asymptomatic carriage.
Nucleic Acid Amplification Test (NAAT)	Toxin genes	High	High	Low/ Moderate	• Asymptomatic carriage • Expensive
Glutamate Dehydrogenase Enzyme Immunoassay (GDH)	Common antigen	Low	High	Low	• Doesn't differentiate toxigenic & non-toxigenic strains • Must be combined with a toxin test for confirmation
Cell Culture Cytotoxicity Neutralization Assay (CCNA)	Free toxins	High	High	High	• Labor intensive • Long turnaround time (48 hours) • Requires cell culture facility • Not routinely performed in hospitals.
Toxin A and B Enzyme Immunoassays (Toxin-EIA)	Free toxins	Low	Low	Moderate	• Variable accuracy

Table 2

Un-spiked vs. Toxin Spiked Stool	*Accuracy*	*Kappa*	*Sensitivity*	*Specificity*	*ML Method*
TcdA 1ng/ml	64%	0.32	90.90%	42.90%	GBM
TcdB 1ng/ml	66.70%	0.32	68.80%	63.60%	GBM
TcdA 100pg/ml	72%	0.41	80%	60%	PCA-LDA
TcdB 100pg/ml	64%	0.26	71.40%	54.50%	PCA-LDA
TcdA 1pg/ml	68%	0.36	75%	61.50%	PCA-LDA
TcdB 1pg/ml	72%	0.42	85.70%	54.50%	SVM Linear Kernel
TcdA 0.1pg/ml	76.90%	0.52	76.50%	77.80%	PCA-LDA
TcdB 0.1pg/ml	72%	0.44	71.40%	72.70%	PCA-LDA

Figure 1

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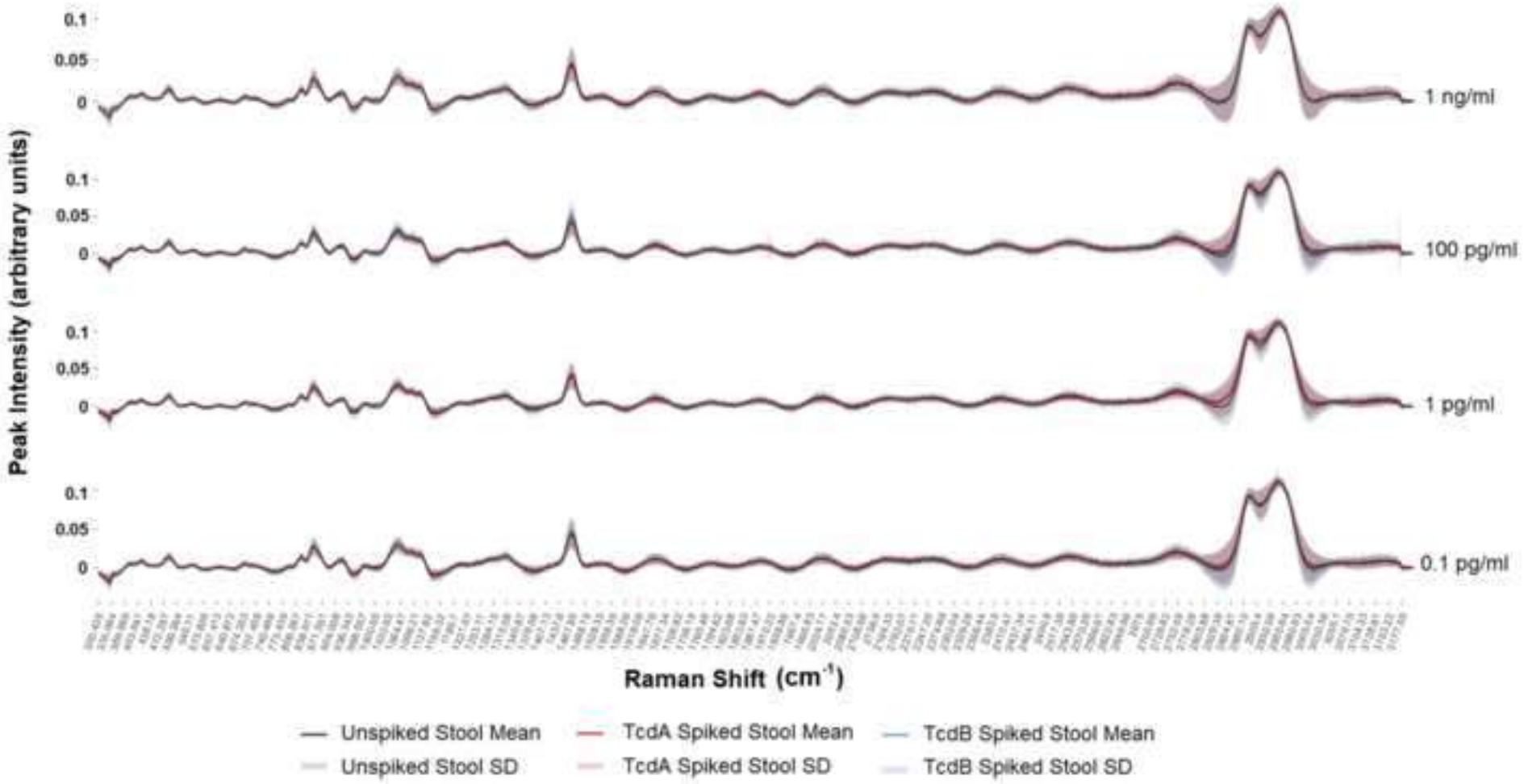

Figure 2

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