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An Analysis of Raman Based Spectroscopy Bacterial Identification & Viability Assessment Platform

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Abstract

The identification of bacteria in infectious disorders is done using a range of diagnostic techniques. Irrespective of the infection type, prompt and highly precise identification of the causal organism is necessary to start the right therapy. The current techniques, while speedy, are insensitive and unable to distinguish between living and dead, and those that are, like culture, are essentially sluggish. We created a diagnostic tool based on Raman micro-spectroscopy to get over these restrictions. It can identify and analyze the viability state of a single bacteria by detecting its biochemical fingerprints. The study also demonstrates the applicability of a decontamination technique that is necessary for handling live pathogenic bacteria and does not interfere with Raman-based identification and viability testing when applied to sputum samples that include Mycobacterial strains. In addition to offering an interface for automatic classification, the minimum sample processing and multivariate analysis techniques of spectroscopic signatures also enable the capacity to forecast an unknown sample by mapping it to the available dataset.

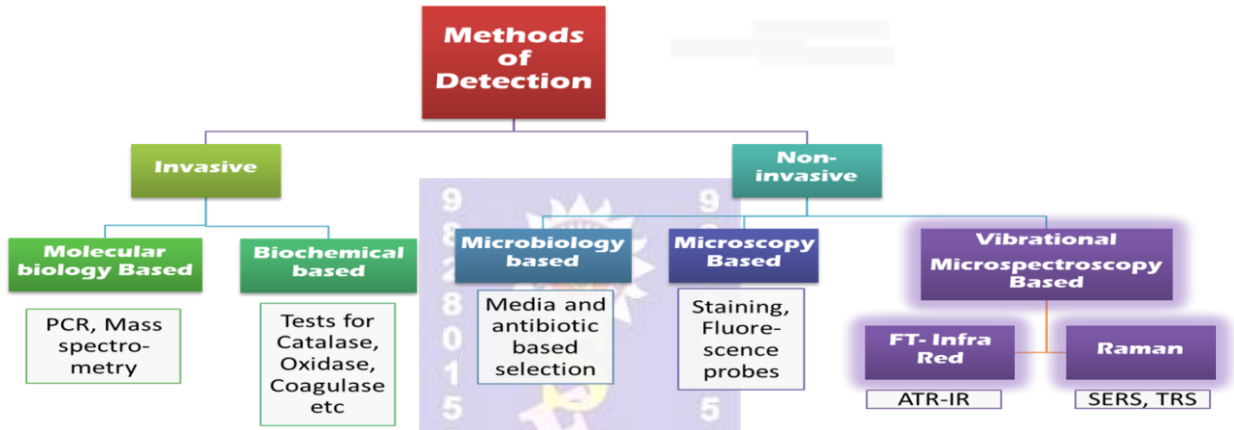
KEYBOARD: Raman Based Spectroscopy, Micro bacterial, PCR, Mass Spectrometry

INTRODUCTION

An accurate and timely diagnosis is essential to the epidemiology of infectious diseases. Finding the causal agent that can accurately identify the causative organisms and provide the viability status—which is crucial for understanding the pathogen's response to therapy and determining the best course of treatment—is one of the main challenges in the field of infectious disease epidemiology. Traditional diagnosis uses methods such as cultivating the pathogen from clinical samples or microbial staining, which are still the gold standards for identifying many infections, including M. tuberculosis, the tuberculosis (TB) causative organism. Moreover, conventional growth-based methods can only identify live culturable bacteria. Because they are metabolically viable but not culturable due to unknown growth conditions or dormancy, viable but non-culturable bacteria (VBNCs) successfully evade culturing dependent detection tests, leading to false negatives and rendering these techniques unsuitable for point-of-care diagnostics. The development of the polymerase chain reaction (PCR) and other nucleic acid-based amplification techniques, such as line amplification assay and serology-based assays, for accurate and quick prognosis and therapy without the need for pathogen cultivation has been made possible by recent developments in molecular biology. But there aren't many organisms in the sample, they have limited sensitivity, or they give false-positive findings because the sample is contaminated with bacteria from the environment. Since DNA may be extracted from both living and dead species, it is unreliable to determine if live infections are present, adding to the complexity. In addition, a major drawback of tests that depend on finding circulating antibodies against pathogen antigens is the possibility of false-positive results due to antibody cross-reactivity with antigens from closely similar bacterial species. Moreover, these tests are limited by the multistep sample processing design and require well designed controls; they do not reveal information on resistance to antimicrobial drugs unless the samples include standard mutations.

A flowchart model for the currently existing techniques for species-level bacterial identification is shown in the figure. The techniques used might be non-invasive, leaving the germs unaltered, or intrusive, treating the bacteria before detecting them. There are two methods for the invasive class: molecular and biochemical. Biomolecules are extracted and then identified via a molecular biology-based technique. In the event that DNA is the extracted biomolecule, the organism is genotyped using PCR (Polymerase Chain Reaction), which amplifies conserved areas. The size and sequence of the amplified result aid in identifying the species. Additionally, PCR methods have advanced to the point that species identification is possible with a single bacteria present in the sample. Conversely, mass spectrometry-based methods may be used to extract protein from the biomaterial. It uses

patterns in protein mass to identify species. Alternative biochemical methods entail analyzing for enzymes such as catalase, oxidase, coagulase, and so on, which aid in determining the genus because their abundance varies across different groups of bacteria. The non-invasive approach uses microscopic or microbiological analysis. The utilization of culture-based methods in microbiology capitalizes on the advantages of medium and antibiotic-specific microorganism growth, which remain the gold standard for culture typing. Microscopic inspection is a very standard procedure used in virtually all laboratories and forms the basis for Gram staining as well. It entails staining techniques combined with the examination of slides using an optical microscope to scan the distribution of dyes. For the purpose of identifying bacteria, some researchers have also looked at DNA microarrays and Fluorescence In-situ Hybridization (FISH), albeit these methods are less popular. In contrast to the other techniques discussed above, Vibrational Micro-spectroscopy (IR and Raman) provides information on the shape and biochemical makeup of bacteria, aiding in the precise identification of different strains thereof. Attenuated Total internal Reflection (ATR) mode needs less sample than conventional IR analysis, and Infra-red (IR) based techniques employ IR light as the source to examine the vibrational frequency of several biomolecules present in the microorganism.



Classification of bacterial identification methods based on sample handling

The methods that have been covered so far necessitate the growing of bacteria in order to identify unknown microorganisms since they are not detectable at the single-bacterium level. This restriction is removed by Raman micro spectroscopy, which enables strain-specific and single-species categorization of bacteria. Two new techniques to improve the technique's processing speed are Surface Enhanced Raman Spectroscopy (SERS) and Tweezer Raman Spectroscopy. To help meet the criteria, a method that can precisely examine the biochemical fingerprints of the whole bacteria can be used. Infrared and Raman spectroscopy are two vibrational micro spectroscopic methods that may be used to fingerprint an organism's whole metabolic makeup. These methods are able to record vibrations unique to bonds from the biological components of the cell. Using a microscope and spectrograph, the spatial resolution needed for a single bacterial biochemical fingerprint is attained. Using multivariate analysis with infrared and Raman micro spectroscopy, strain-specific identification of large bacterial samples has been accomplished. Achieving single bacillus analysis is difficult because of the constraints in the infra-red optics. On the other hand, high magnification and high numerical aperture (NA) objectives can be used in Raman micro spectroscopy, which primarily functions in the visible spectrum, to achieve great spatial resolution.

Materials and Procedures

Chemical and Media

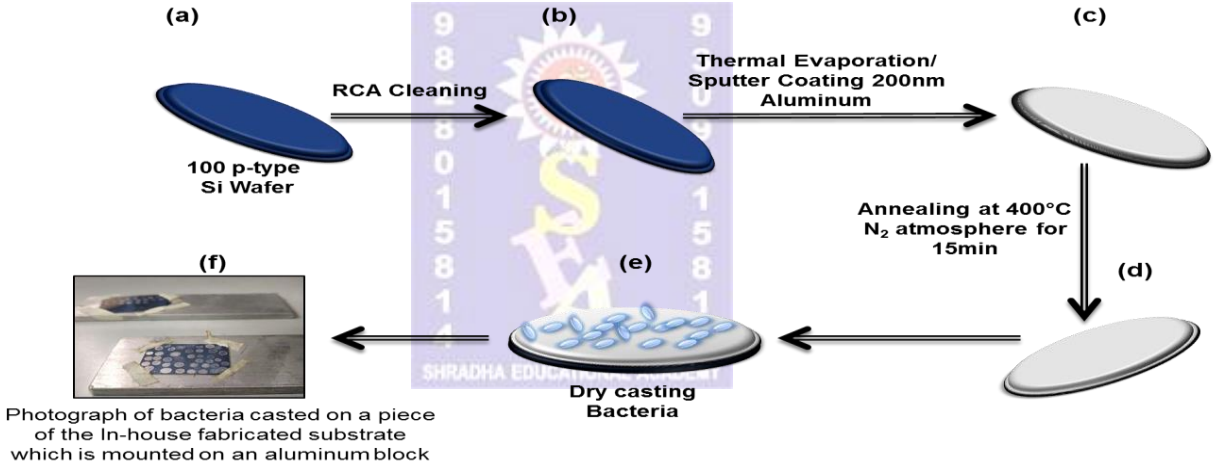
Difco provided the 7H9 medium for mycobacterial growth, whereas all Media supplied all other media components utilized in this investigation. Everything was purchased from Merck Sigma-Aldrich, including the reagents for making fake sputum. From Global Optics, quartz substrate was acquired.

Growth conditions and bacterial strains

For the purpose of creating a signature database, the study used two strains each of the Mycobacterium tuberculosis complex (MTC) and non-tuberculosis mycobacteria (NTM) clades. Escherichia coli DH5□, M. tuberculosis (H37Ra), M. tuberculosis (H37Rv), and M. bovis were from lab collection. M. smegmatis (MTCC6), M. vaccae (MTCC 954), and Klebsiella pneumoniae (MTCC 39) were obtained from the Microbial Cell Repository at Institute of Microbial Technology (IMTECH), Chandigarh, India. While other bacteria were cultivated in Luria Bertani broth, all strains of Mycobacterium were grown in 7H9 broth supplemented with 0.5% glycerol, 0.02% tween-80, and 1% albumin dextrose catalase (ADC). Every bacterial culture was kept in an incubator at 37°C until its optical density (OD) was around 0.3. Every virulent strain was managed at the Centre for Infectious Diseases, a BSL3 facility.

Substrate fabrication

A basic description of the process used to create the substrate is shown in Figure. The 100 p-type Silicon (Si) wafer was cleaned using the conventional RCA cleaning method to get rid of any oxides or ions. Next, a 200 nm layer of aluminum was deposited using sputter coating or thermal evaporation. For fifteen minutes, the aluminum deposit was annealed in N2 at 400°C. Before being used, these substrates were kept in a desiccator. The substrate, which was mounted to an aluminum holder to make attachment to the microscope stage easier, was coated with bacteria using a dry casting technique. Annealing and thermal evaporation/sputtering were carried out in the Photo Voltaics Lab (Prof. R. Dwaraknath's Lab), CeNSE, IISc.



Schematic of substrate fabrication for single bacterial Raman analysis

Analysis using Raman micro spectroscopy

The washed and/or fixed bacteria were dry-casted on the Al substrate. Raman spectroscopy was performed using the excitation wavelength of 633 nm on an upright microscope. Initial experiments were performed on Quartz substrate (Global Optics, UK) with 514 nm laser using a Renishaw InVia Raman microspectroscope. The Raman spectra of single bacteria were collected using a 100x dry objective (0.85 NA). The same objective was used to collect the backscattered light from the sample. The scattered radiation was passed through a notch filter, focussed on to a monochromator with 1200 lines/mm grating and detected using a Peltier cooled CCD camera (256x1048 pixels sensor). Following data collection, the spectra underwent pre-processing using Renishaw wire 4.2, OriginPro 8.5, and Unscrambler 10 softwares for cosmic ray removal, multipoint base line correction, Savitzky-Golay smoothing, and vector normalization. The entire dataset was used for multivariate analysis by IBM SPSS 17.0 for Canonical Discriminant Analysis (CDA) and Unscrammbler 10 for Principal Component- Linear Discriminant Analysis (PC-LDA), where predictions of unknown samples were made. The CDA uses Leave-One-Out-Cross Validation (LOOCV) method to validate the model, wherein one sample is kept out as test data and the remaining samples are used to build the training dataset. The test data is then fed to verify if it is being

Fixation of bacteria

To ensure safe sample handling, the effectiveness of three agents—2.5% glutaraldehyde, 4% para formaldehyde (PFA), and 10% sodium azide—in decontaminating and fixing samples was evaluated. Each reagent was allowed to incubate on cell pellets for 15 minutes at 37°C. Following incubation, the cells underwent the previously described washing and preparation for spectroscopic measurements. Cells were utilized for all next assays after being fixed and decontaminated for 15 minutes at 37°C using 4% PFA, or 4% paraformaldehyde prepared in 1X PBS.

CONCLUSION

Prior research has shown the use of Raman-based bacterial identification, which has also been used to the identification and typing of Mycobacterial strains in large quantities. Prior to 2003, the majority of bulk systems were used for the identification of bacteria using Raman spectroscopy. Raman imaging and single bacterial identification have been investigated since 2004 thanks to the development of confocal microscopy, high magnification objectives, and very sensitive detectors. Single-cell detection of medically and ecologically important bacteria, such as anthrax46, in urinary tract infections, water pathogens, meat-associated pathogens, and many other samples, has been reported in studies employing multivariate analytic approaches. To identify the bacterium causing tuberculosis, supervised multivariate analytic techniques such as Support Vector Machine (SVM) have been used in conjunction with Raman spectroscopy. Not only was M identified in our study. tuberculosis , we have also studied the fixation effects as well as assessed the viability status of the microorganism. Additional research has been done on the effects of antibiotics on bacteria, measuring the MIC, and examining the impact of various growth environments. In addition to standard Raman investigations, the very sensitive Surface Enhanced Raman Spectroscopy (SERS) has been used to identify both individual and bulk bacteria, including mycobacterial species and mycolates. Without requiring a lot of sample processing, the current study offers a thorough and reliable work flow for a platform based on Raman spectroscopy that is used for bacterial identification and viability evaluation at the single cell level. The work provides a design that gets beyond the sensitivity and specificity restrictions of the microbiological techniques used today to identify and type strains. The method of choice for this purpose is still culturing, which can be restrictive in a sample where bacteria are viable but not culturable (VBNCs), a major obstacle in microbiological assessment platforms worldwide. This is because it is crucial to know the viability status of the bacterial cells found in biological samples, primarily to formulate appropriate therapy.

In summary, we have accomplished the following:

(i) proven that Raman microspectroscopy is a sensitive and specific method for identifying different MTC and NTM clades of bacteria

(ii) shown that patient samples can be easily handled and processed without requiring modifications to any current microbial typing protocols; (iii) shown that this method can identify mycobacterial and other strains in artificial sputum

(iii) optimized the sample processing protocol for handling pathogenic bacteria; and

(iv) presented a simple platform for POC diagnostics.

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