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Detection and Classification of Bacteria using Raman Spectroscopy Combined with Multivariate Analysis

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ABSTRACT

Vibrational spectroscopic techniques have advantages over conventional microbiological approaches towards identification & detection of pathogens. Since unique spectral fingerprint is obtained, one can identify very closely related bacteria using such methods. In this study Raman microspectroscopy in combination with chemometric method has been used to classify four strains of *E. coli* (two pathogenic & two non-pathogenic). Different multivariate approaches such as hierarchical cluster analysis, principal component analysis & linear discriminant analysis were explored to obtain efficient classification of the Raman signals obtained from the four strains of *E.coli*. It was observed that multivariate analysis was able to classify the bacteria at strain level. Linear discrimination analysis using PC scores (PC-LDA) was found to give very good result with as high as 100 per cent accuracy. This hybrid technique (Raman spectroscopy & multivariate analysis) has tremendous potential to be developed as a tool for bacterial identification.

Keywords Raman microspectroscopy; Pathogen; Multivarate analysis; Hierarchical cluster analysis; Principal component analysis; Linear discriminant analysis; PC-LDA

1. INTRODUCTION

Rapid and specific methods for the detection and identification of pathogens are becoming very important in the field of medicine and defence[1-6]. Conventional methods employed by microbiologists include phenotypic and genotypic methods. Growth temperature, salt concentration and oxygen requirement are useful physiological parameters for classification and identification of micro-organisms. Standard biochemical methods include enzymatic activity, gas production and compound metabolism for identification of bacteria. Genotypic techniques such as analysis 16s rRNA and other PCR based methods are also used frequently among microbiologists. Additionally, mass spectrometry based methods have also been used[7,8]. All the methods mentioned above are time consuming and require extensive sample preparation[9,10].

Molecular spectroscopic technique such as Raman spectroscopy is a well established tool that employs molecular vibrations to decipher molecular structure and chemical composition of samples. Raman spectroscopy is based upon the principle of inelastic scattering of photons incident on the sample. It has found applications in the area of materials science as well as in biology[11-15]. In the realm of biolchemical analysis it is essential that a technique is non-invasive, preferably label-free and non-destructive. All these unique features are provided by Raman spectroscopy based approaches. However, biological samples are very complex as even a single cell or a bacterium is composed of a number of biomolecules such as proteins, lipids, carbohydrates and nucleic acids. Different types of analysis techniques are used to extract information from biological samples[12,16].

Since the molecular structure of the biomolecules are unique, distinctive spectral fingerprints can be obtained through Raman spectroscopic analysis. Therefore, these spectral markers can be used for tracking diseases, studying effectiveness of drugs in cells and tissues, identification of pathogens and many other biological processes[17-20]. Every molecule will have unique Raman spectra. Moreover, aqueous samples can be easily studied as water is a weak Raman scatterer and enables monitoring of biological systems in live condition. Furthermore, the level of specificity can be enhanced by combining this technique with chemometric methods[21,22] as even the most subtle changes are expected to be picked up by this technique.

Raman spectroscopy based methods have gained popularity in the last few decades due to rapid development in instrumentation that has led to enhanced sensitivity and resolution. In the area of pathogen detection and identification, several studies have been reported in the last few years[22-28]. However, there is not a single method available so far to

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differentiate and detect pathogenic strains from non-pathogens of the same species. This work aims at the detection of human pathogenic EHEC *E.coli* strains and differentiating it from its non-pathogenic variants using Raman spectroscopy coupled with chemometrics (multivariate analysis). *E. coli* comes under the family of Enterobacteriaceae which is a gram-negative, non-spore forming, facultatively anaerobic rod shaped bacteria. It is predominantly found in the mucous layer of mammalian colon[29]. Most of the strains of *E.coli* are non-pathogenic except a few which cause diseases such as UTI, diarrhoea, sepsis and meningitis[30]. The objective of this study is to explore the effectiveness of Raman spectroscopy coupled with multivariate analysis towards discrimination of *E. coli* at strain level.

2. MATERIALS & METHODS

2.1 Sample Preparation

2.1.1 Strains Used and Sample Preparation

The *E. coli* ATCC 10536, *E. coli* MTCC 1687, *E. coli* DFRL 302 and *E.coli* DFRL 303 strains used in the study were procured from DFRL, Mysore culture collection. The latter two strains are of O157:H7 serotype and pathogenic to humans. Formalin inactivation of bacterial cells was carried out according to Stockel *et al*. with slight modifications[31]. Briefly, single colonies of *E. coli* pure culture from LB agar plate were inoculated into 5 mL LB broth followed by its cultivation overnight at 37 °C in shaker incubator. After overnight incubation, the cells were harvested by centrifuging at 12,000 rpm for 4 min and re-suspended in sterile physiological saline to final concentration of 10^{10} CFU/mL. The samples were mixed with formaldehyde to achieve final concentration of 10-20 per cent and continuously shaken for 1-3 h in shaker incubator. Then the treatment was stopped by centrifuging the suspension at 12,000 rpm for 4 min. The supernatant was discarded and the pellet was re-suspended in sterile physiological saline. To determine a successful inactivation, 10 µl of the bacterial suspension was inoculated into LB broth followed by incubation at 37 °C for 3 days. Only samples without visible growth were used for Raman spectroscopic measurements.

2.1.2 Raman Instrumentation

2.5 µl of the sample was drop-cast on a Raman grade MgF_2 coverslip. Raman spectra were obtained from the dried drop using Renishaw in-Via upright microscope, Renishaw Inc., UK. The spectra were acquired from different regions of the drop. The excitation wavelength selected for the study was a 785 nm diode laser. The excitation light was focused onto the sample using a 100 x microscope objective with a spot size of ~2 µm. The laser power at the sample was less than 50 mW. The scattered Raman signals pass through the monochromator where they are diffracted using a 1200 -grooves/mm grating and collected with a thermoelectrically cooled charge-coupled device (CCD) camera with a spectral resolution of ~5 cm^{-1}. The Raman spectra were acquired for 6 s with 4 accumulations. The Raman spectra were acquired at different positions within the spots and the number of spectra ranged from 30-100. Wavenumber calibration was performed using Si standard peak at 520.5 cm^{-1} before acquiring the spectra. The collected spectra were truncated to the finger print region of 600 to 1700 cm^{-1} and cosmic rays, if any, were removed by using Wire 4.2 software.

2.2 Data Analysis

The analysis of the Raman spectra was carried out in two steps: (i) Univariate analysis, and (ii) Multivariate analysis (MV), which was preceded by pre-processing.

Pre-processing was performed using Wire 4.2, Origin 9 and Unscrambler-X software. Each spectrum was subjected to the same pre-processing steps in order to reduce the contribution from noise and also the spectral variations due to the data collection procedure[32]. Firstly, the background and the cosmic spikes of the spectra were removed. Importance was given to background correction to minimise the effects of various backgrounds, caused by fluorescence of the sample or thermal fluctuations on the CCD. Baseline subtraction was performed with the help of MATLAB program, using fixed points for all the samples. Before multivariate analysis, all these spectra were further subjected to baseline subtraction by linear offset method followed by unit vector normalisation using Unscrambler X to ensure comparability between the spectra. In addition, noise was minimised by a 7 point Savitzky Golay smoothing filter.

Univariate as well as multivariate data analysis method were employed, after the data pre-processing. Since all the four strains of bacteria are genetically very similar, it was challenging to search for subtle differences using univariate approach. However, it was anticipated that multivariate analysis method could be useful in this regard.

Two unsupervised approaches were attempted under multivariate analysis, hierarchical cluster analysis (HCA) and principal component analysis (PCA) to reduce the dimensionality of the problem. Additionally, linear discriminant analysis (LDA), which is a supervised technique, was also performed to further analyse the scores obtained from PCA[21]. The number of the chosen PCs was the best compromise between a minimal number of PCs and classification accuracy.

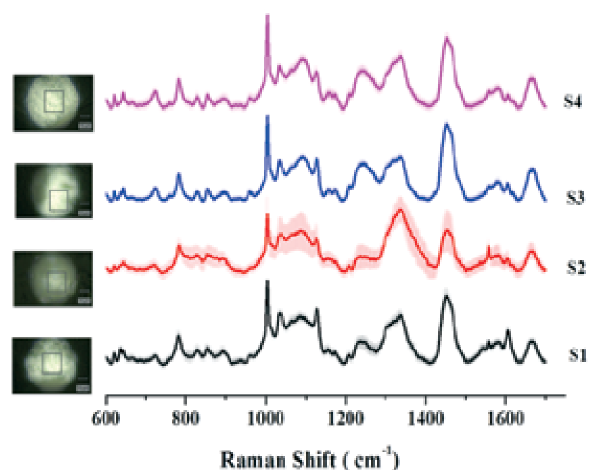

Figure 1: Averaged Raman spectroscopic signal from four strains of Escherichia *coli.* (S1-S4), where S1 is *E.coli* ATCC 10536, S2 is E. coli MTCC1687, S3 is *E. coli* O157:H7 DFRL 302 & S4 is *E. coli* O157:H7 DFRL 303. The left panel depicts the white light image of the spot from where Raman spectra were acquired.

Table 1. Tentative Raman band assignments for the four strains of *Escherichia coli*. (S1-S4) [12, 34-38].

E.coli ATCC 10536 (S1)	*E. coli* MTCC 1687 (S2)	*E. coli* O157:H7 DFRL 302 (S3)	*E. coli* O157:H7 DFRL 303 (S4)	Peak Assignments
622	622	622	622	Phenylalanine
637	644	644	643	Tyrosine
724	724	724	724	Adenine
782	783	783	783	Thymine, Cytosine
829	828	829	829	Protein Fermi Doublet
853	852	853	853	
894	893	897	897	v(CN), v(CON) symmetric, δ (CCH) aliphatic
961	961	959	960	δ (C=C)
1003	1003	1004	1004	v(CC) aromatic ring Phenylalanine
1034	1038	1034	1033	Phenylalanine
1087	1089	1093	1093	Carbohydrates, C-C, C-O, -C-OH
1128	1127	1127	1127	C-N, C-C stretch Proteins
1157	1158	1157	1158	δ (CH2) amide III
1173	1173	1174	1173	Phospholipid
1207	1208	1210	1208	Phenylalanine & Tyrosine
1236	1236	1247	1238	CN in plane bend Amide III
1338	1336	1338	1336	Tryptophan, CH2 twist
1451	1451	1451	1451	CH2 scissoring from Lipids, CH2 assym def of amino acid side chains
1557	1557	1558	1557	Indole ring of Tryptophan
1582	1582	1580	1582	Nucleic acids
1605	1604	1605	1607	Tyrosine, Phenylalanine, Tryptophan
1667	1669	1669	1668	C=O stretch of Amide I

2.3 Results & Discussion

2.3.1 Univariate Analysis

Figure 1 (left panel) shows the white light images of the spot of the bacteria from which the Raman spectra were acquired. The right panel of fig. 1 shows the plot of averaged Raman spectra (average of ~60 spectra) of the four *E.coli* strains S1-S4. Table 1 summarises the assignments of the observed Raman peaks for the four *E. coli* strains. Raman peaks observed around 622 cm^{-1} and 643 cm^{-1} are attributed to amino acids (phenylalanine and tyrosine) for all the strains. The peaks at 724 cm^{-1} and 783 cm^{-1} can be attributed to the nitrogenous bases *i.e.*, the purines and pyrimidines of DNA present within the bacteria. 720 cm^{-1} has been assigned for adenine, whereas the peak around 783 cm^{-1} represents thymine and cytosine[33]. The vibrational modes centred at 829 cm^{-1} and 853 cm^{-1} have been assigned for protein Fermi doublet. The strongest Raman peaks were observed for proteins at 1004 cm^{-1}, 1034 cm^{-1} and 1668 cm^{-1}. These peaks are characteristics of proteins in biological systems. The band around 1093 cm^{-1} was assigned as carbohydrate. The 1127 cm^{-1} band can be ascribed to C-N and C-C stretch in the proteins. The peak around 1451 cm^{-1} appears due to C-H deformation and CH_2 bending mode and could be attributed either to carbohydrate or to lipids. Another characteristic peak for biological samples is the distinct Amide I peak which appears at 1668 cm^{-1}. These spectral features were found to be consistent with the earlier reported literature and have been compiled inTable 1[12, 34-38]. The Raman shifts for all the four strains closely resembles each other and therefore, it is quite challenging to identify strains based on merely comparing the spectral frequencies or amplitude. Therefore,

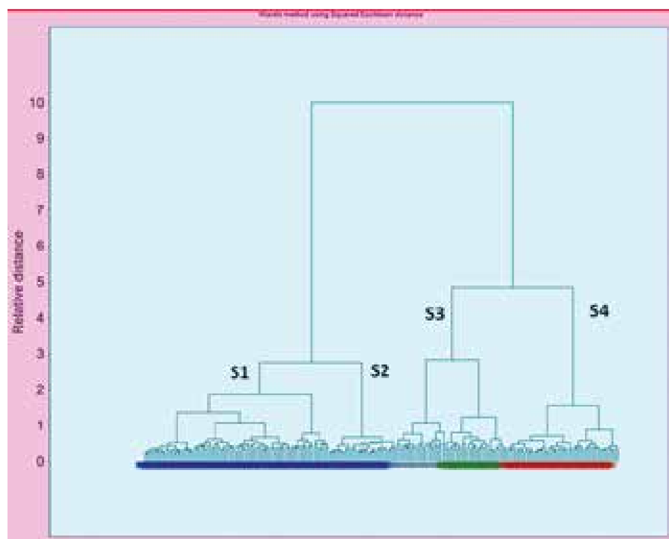

Figure 2. Dendrogram obtained from heirarchical cluster analysis performed on Raman spectra of four strains of *E.coli* using Wards Method. The dendrogram shows a clear separation of the four strains of *E.coli*.

in order to discriminate the bacteria unequivocally, one has to resort to multivariate analyses tools. The next section would provide details on the multivariate approaches employed for the same.

2.4 Multivariate Analysis

The first chemometric algorithm that was applied to the Raman spectra of the four *E.coli* strains was the unsupervised hierarchical cluster analysis (HCA). HCA is an unsupervised method for obtaining information and it clusters the spectra by spectral similarity. Since HCA is a distance based method, any distance metrics such as Euclidean or Mahalanobis can be employed[21]. In the present study, the dendrogram was generated using Ward's clustering algorithm and the squared Euclidean distance. In this case two clusters that yield a minimum increase in total within-cluster variance are merged. The entire hierarchical process is depicted in the form of a dendrogram or a hierarchical tree to illustrate the merging process and the distances involved. Figure 2 shows the dendrogram resulting from HCA performed on a total of 241 Raman spectra (~60 for each species) of the four strains of *E.coli.* In Figure 2, the horizontal axis represents the 4 bacterial strains, while the vertical axis represents the dissimilarity distance between two bacterium or clusters. The optimal distance observed from the dendrogram shows four major clusters. Additionally, it can also be observed that S1 and S2 branch away from S3 and S4 indicating a clear separation between the pathogenic and non-pathogenic E. *coli*. The results that we obtained show that this measure of overall signal variance within a set of spectra obtained from different strains belonging to the same species can be as large as the interspecies signal variance[22].

Principal component analysis in the mean centred data, using NIPALS algorithm was performed on the Raman spectra

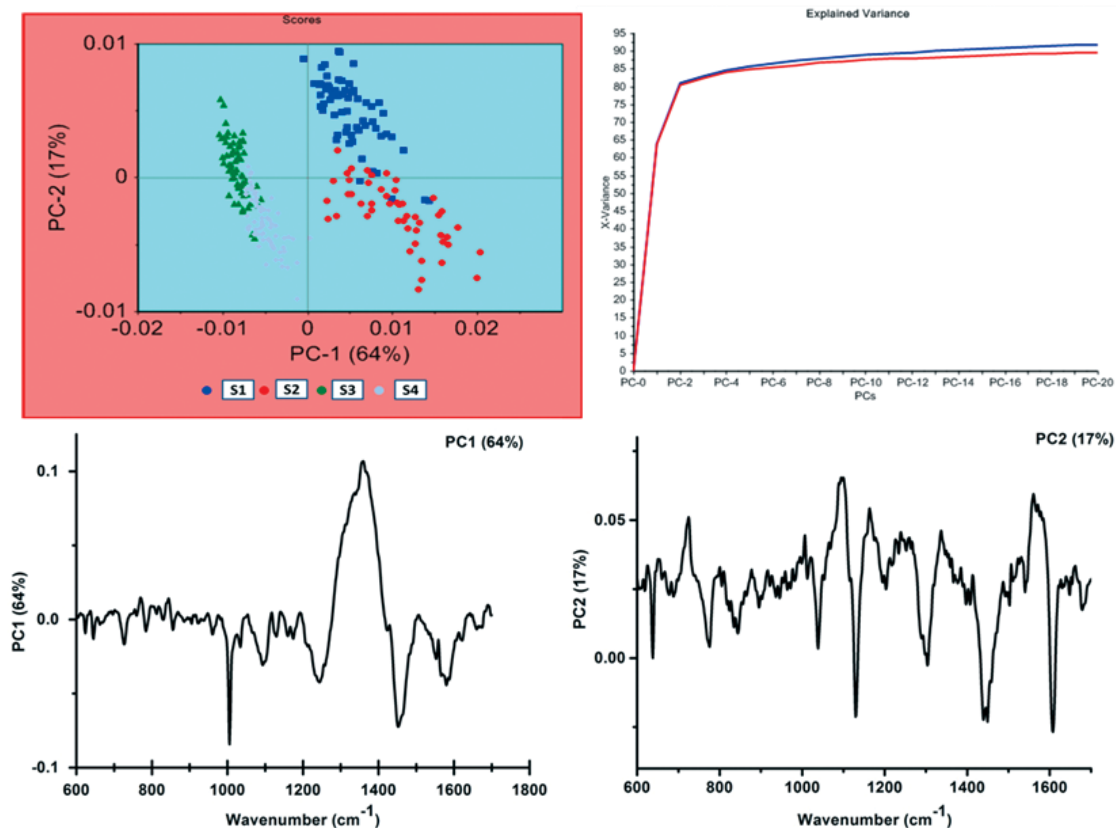

Figure 3. Principal Component Analysis (PCA) of E. *coli* (S1-S4). The top left panel depicts the scores plot. The plot of PC1 vs PC2 can clearly discriminate between pathogenic *E. coli* & non-Pathogenic *E.coli.* The top right panel shows the explained variance. The bottom two panels depict the PC1 & PC2 loadings respectively.

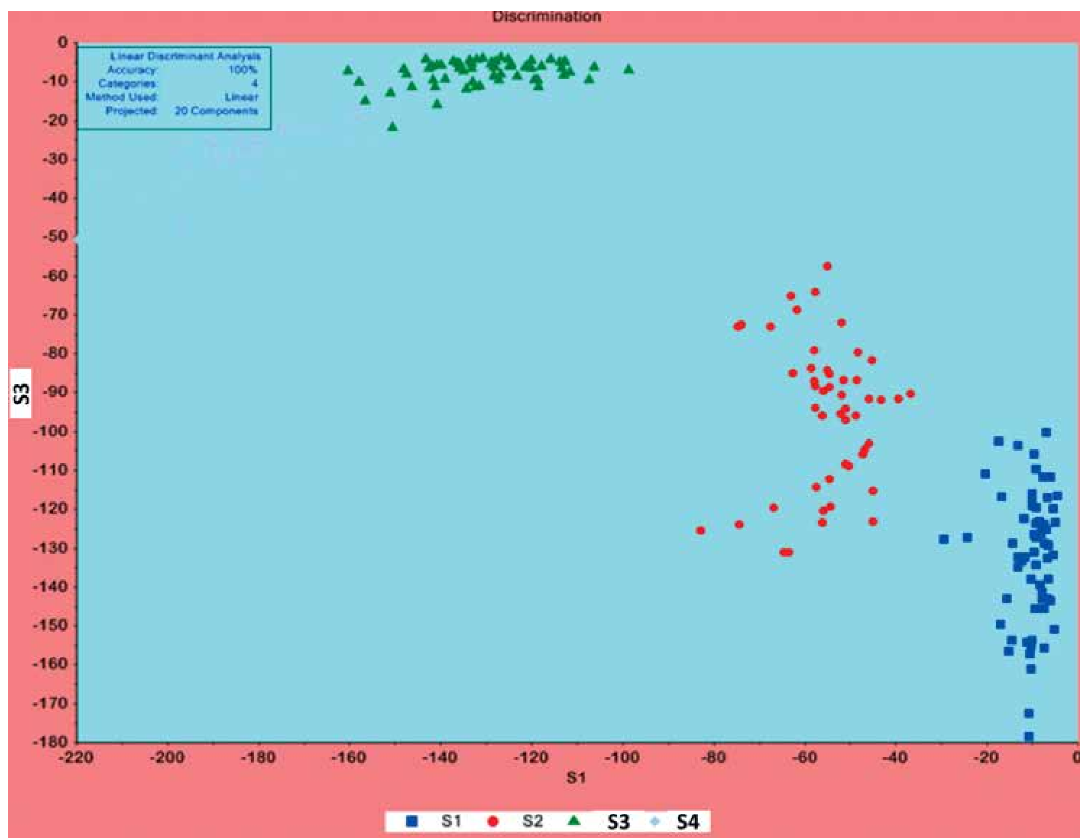

Figure 4. Linear Discriminant Analysis plot shows an accuracy of 100 per cent to discriminate the E. coli samples based on their Raman Spectral signature.

obtained from the four strains of *E.coli* and the results are discussed in this section. Twenty principal components (PC 20) were chosen for the analysis of *E.coli* (dimensionality reduction from 1100 to 20). Figure 3 shows the PCA of the four *E.coli* strains. Four distinct clusters could be seen in the scores plot as shown in the upper left panel of fig. 3. The four colours in the scores plot represent the four strains, solid blue square corresponds to *E.coli* ATCC 10536 (S1), red circle corresponds to *E.coli* MTCC 1687 (S2), green triangle and cyan diamond symbols represent *E.coli* 0157:H7 DFRL 302 (S3) and *E.coli* 0157:H7 DFRL 303 (S4) strains respectively. The top right panel depicts the explained variance. PC1 represents 64 per cent of the explained variance and allows discrimination between S1, S2 against S3, S4. The clusters are almost formed in the four quadrants of the scores plot. The S1 and S2 clusters are grouped on the positive side of the PC1 axis whereas the S3 and S4 clusters are grouped on the negative side of the PC1 axis. PC1 is able to discriminate between pathogen *vs* non-pathogenic *E.coli*. PC2 represents 17 per cent of the variance and it depicts the intra differentiation *i.e.* differences within the two types of strains. It is clear from the PCA that the avirulent *E.coli* (blue and red) groups are distinctly separated from the pathogenic strains (green and cyan). Furthermore, from the scores plot it is also indicated that there is good separation between the pathogenic and the non-pathogenic strains. To investigate the origin of the separation of the scores plot, the loading plots were analysed. The loadings plot of PC1 revealed that the changes in phenylalanine peak (~1004 cm^{-1}) and the carbohydrate/lipid band (1451 cm^{-1}) showed negative features. This suggests that the changes of these biochemicals were prominent in the pathogenic *E.coli* which could have been the basis for the discrimination of the *E.coli*. However, the loadings plot of PC2 is much more complex to decipher. PC2 depicts the changes responsible for the differences within the strains *i.e.* between S1 and S2 and S3 and S4. It is mention worthy that the intra variance of the Raman spectra in the scores plot is large i.e. the clustering is not too condensed/tight. The variance may be avoided by using difference spectra or by a different sampling approach for acquiring the Raman signals.

After the dimension reduction with unsupervised method PCA, the dataset was subjected to analysis with supervised method in an attempt to build a rigid classification model for this particular problem. In order to accomplish this task, PCA-LDA method was selected. PCA-LDA can be an efficient algorithm for classification of closely related pathogens. Linear discrimination analysis was performed using PC scores projecting 20 PCs (giving equal priorities to all). All the spectra were classified in four groups with 100 per cent accuracy with PC-LDA model using cross validation method (using random segments, number of segments was 20). The plot clearly shows demarcation between pathogenic and non-pathogenic bacteria along the diagonal line. Confusion matrix, although not shown in the results, showed no misclassification in the dataset. Thus, this model has proved to be very effective in discriminating pathogenic and non-pathogenic bacteria (even different strains of bacteria, to be precise). This model can be used further to classify blind or unknown samples.

3. CONCLUSIONS

In the present study, Raman microspectroscopic technique coupled with multivariate analyses approaches were applied to discriminate between four strains of *E.coli*, two of which were pathogenic. Raman spectra could be successfully acquired from the drop of *E.coli* samples cast on the MgF_2 slide. The Raman peaks of the four strains of *E.coli* were indistinguishable and appeared very similar. The frequencies and the amplitudes did not yield useful information to discriminate the strains from each other. HCA and PCA could cluster/ group the spectra well. Pathogenic *E.coli* could be distinguished from the non-pathogenic *E.coli*. Furthermore, supervised technique such as LDA yielded 100 per cent accuracy. These initial results are encouraging and it reflects that Raman spectroscopy combined with multivariate techniques will be an ideal combination for the detection and discrimination of pathogens. Further experiments are being conducted in the laboratory with other pathogens. Sensitivity studies shall be carried out to evaluate the limit of detection using Raman spectroscopy based approaches. It is pertinent to note that in order for any technology to succeed in the field condition, robust instrumentation along with suitable algorithm needs to be developed for pathogen detection. We anticipate that Raman spectroscopy based approaches combined with multivariate analyses hold a potential to be developed as a field deployable instrument for pathogen detection in the future.

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