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FEMS Microbiology Letters 232 (2004) 127–132

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Ultra-violet resonance Raman spectroscopy for the rapid discrimination of urinary tract infection bacteria

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Received 3 November 2003; received in revised form 18 December 2003; accepted 13 January 2004

First published online 11 February 2004

Abstract

The ability to identify pathogenic organisms rapidly provides significant benefits to clinicians; in particular, with respect to best prescription practices and tracking of recurrent infections. Conventional bioassays require 3–5 days before identification of an organism can be made, thus compromising the effectiveness with which patients can be treated for bacterial infections. We analysed 20 clinical isolates of urinary tract infections (UTI) by ultra-violet resonance Raman (UVRR) spectroscopy, utilising 244 nm excitation delivering ~0.1 mW laser power at the sample, with typical spectral collection times of 120 s. UVRR results in resonance-enhanced Raman signals for certain chromophoric segments of macromolecules, intensifying those selected bands above what would otherwise be observed for a normal Raman experiment. Utilising the whole-organism 'fingerprints' obtained by UVRR we were able to discriminate successfully between UTI pathogens using chemometric cluster analyses. This work demonstrates significant improvements in the speed with which spectra can be obtained by Raman spectroscopic techniques for the discrimination of clinical bacterial samples.

Keywords: Raman microscopy; Ultra-violet resonance Raman spectroscopy; Chemometrics; Urinary tract infection

1. Introduction

In the effort to develop techniques by which micro-organisms can be rapidly and inexpensively identified there has been much interest in the application of physico-chemical spectroscopic methods. Such tools provide a means of generating 'whole-organism fingerprints' [1,2] or 'metabolic fingerprints' [3] from which, using chemometrics techniques, mathematical models can be generated that allow for the identification of micro-organisms. For this purpose attention has been centred on the vibrational spectroscopic techniques of Fourier transform infra-red and Raman spectroscopies [4–7], with an increasing interest in mass spectrometry [8,9]. Most importantly, any technique used for the purpose of microbial discrimination needs to be suitably reproducible in order for the derived mathematical model to hold for subsequent use on unknown samples. Deep ultra-violet resonance Raman (UVRR) spectroscopy is a potentially powerful tool since at this frequency Raman spectra of biological samples are not subject to the fluorescence background observed in experiments using infra-red or visible excitation [10].

Normal Raman spectroscopy has been applied to the problem of bacterial discrimination with great success [4–6]. However, the drawback of the technique lies in the low probability of a Raman scattering event occurring, with typically only 1 in 10^8 photons Raman-scattered. To a great extent the use of lasers, modern optics and CCD cameras in Raman instruments can offset the underlying weakness of the effect, although in spite of such developments, collection times for these Raman experiments of biological materials can still be many minutes. Alternatives to the normal Raman technique exist in the form of a variety of Raman enhancement methods, amongst which are surface-enhanced Raman scattering (SERS) [11] and UVRR. SERS is a sensitive technique, widely used for single-molecule studies, that relies on a metal substrate in close proximity to the analyte, to produce a resonance enhancement when illuminated by incident laser light. This enhancement can be of the order 10^6–10^8 [11] or even 10^{14} for some studies [12]. The obvious benefits of such increased signals are offset some-

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0378-1097/04/$22.00
doi:10.1016/S0378-1097(04)00040-0

what by the unpredictability of the technique and the difficulties posed by ensuring that the experimental conditions with regard to the analyte and metal substrate allow for the observation of SERS spectra, although with suitable experimental design these limitations can be overcome [13].

Enhancement of Raman bands at UV frequencies is the result of a resonance between the wavelength of excitation and the electronic transition of a molecule [14]. The effect is selective for certain chromophoric segments of macromolecules and depends greatly on the excitation energy used [15]. For example, at the 244 nm excitation used in this study the spectra will be particularly rich in nucleic acid bands [15,16]. In contrast, a shift to 229 nm excitation will result in spectra dominated by aromatic amino acids [17]. However, that is not to say that the spectra at 244 nm will not be rich in bands from a multitude of bacterial cell components. Despite the benefits of UVRR with respect to background fluorescence over the normal Raman technique, photochemical or 'burning' effects due to the highly energetic nature of UV photons can present problems with regard to sample preparation and presentation [18].

The aim of this study was to develop UVRR as a means of discriminating between the common causal agents of urinary tract infection (UTI), a debilitating and globally important disease.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The typical causal agents of UTI are the Enterobacteriaceae, predominantly *Escherichia coli* (a contributing pathogen in $>50\%$ of cases) and *Klebsiella* spp., in addition, the Gram-positive enterococci are also represented in $\sim 10\%$ of cases [19].

Twenty clinical bacterial isolates from patients with UTI were obtained from Bronglais Hospital, Aberystwyth as previously reported [20]. Identification by API20E and subsequent sequencing by FAFLP showed the isolates to belong to: *E. coli* (five strains coded: Eco7, Eco13, Eco17, Eco41, Eco48), *Klebsiella oxytoca* (one strain coded: kox108), *Klebsiella pneumoniae* (four strains coded: kp51, kp52, kp59, kp61), *Enterococcus* spp. (five strains coded: EntC82, EntC85, EntC90, EntC92, EntC93) and *Proteus mirabilis* (five strains coded: pm65, pm66, pm69, pm70, pm73). All isolates were cultivated axenically and aerobically for 12 h at 37°C on LabM blood agar base (IDG, Lancashire, UK). After subculturing three times biomass was carefully emulsified on the plate using 100-μl aliquots of distilled water and then transferred to UV quality calcium fluoride windows (Crystran, Dorset, UK). All isolates were analysed in quadruplicate from four separate cultures (that is to say four 'biological' replicates per UTI strain).

2.2. Raman microscopy

A Renishaw Raman microscope (Renishaw, Wotton-under-Edge, Gloucestershire, UK) was used to collect UVRR data. Approximately 0.1 mW power was delivered to the sampling point using a Lexel 95-SHG intracavity frequency doubled argon ion laser emitting at 244 nm. For wavelength calibration a diamond chip was focused under the 40× objective and collected as a static spectrum centred at 1332 cm^{-1} for 1 s.

In order to eliminate the photochemical destruction of the bacteria, samples were presented dried on to the CaF_2 windows then placed on a rotating aluminium sample holder and spun at ~ 30 rpm. Each sample was focused under a 40× objective, and a 60-μm focussing offset applied (to account for the focal difference between visible and UV light) before a single spectrum was collected for 120 s. The next sample was then focused under the objective and the procedure repeated.

Instrument control and data capture was performed using the GRAMS WiRE software package (Galactic Industries, Salem, NH, USA) running under Windows 95. UVRR spectra were collected as static spectra centred at 1300 cm^{-1}, equating to a wavenumber shift range of 780 cm^{-1} to 1910 cm^{-1}. The resolution was ~ 8 cm^{-1} and the bin size used 1.015 cm^{-1}. Each sample was represented by a spectrum containing 574 points and spectra were displayed in terms of the Raman scattered photon count (see Fig. 1 for examples).

ASCII data were exported from the GRAMS WiRE software into Matlab version 6 (The Math Works, Natick, MA, USA). The spectra were optimised prior to cluster analysis by extracting the most information-rich region of the spectra from 780–1910 cm^{-1} which was then smoothed using a Savitsky–Golay polynomial filter of order 3, over a frame size of 15 points [21].

2.3. Cluster analysis

Cluster analysis was performed in Matlab as previously described [1–22]. Briefly, a PC-DFA classification model was constructed using quadruplicate spectra of four isolates from each genus under study. Firstly, principal components analysis (PCA [23]) was used to reduce the dimensionality of the UVRR data prior to discriminant function analysis (DFA [24]). The DFA was programmed to maximise the Fisher ratio (i.e. the within-class to between-class variance) and the spectral similarity between different classes reflects the optimal number of PCs that are fed into the DFA algorithm. To fully validate the PC-DFA clustering an isolate of each genus was held back at the model prediction stage and then projected into the resultant PC-DFA model, as detailed fully elsewhere [25].

To further clarify the relationships between the clusters and those individuals forming the clusters, a dendrogram was generated by hierarchical cluster analysis (HCA [24]),

whereby the Euclidean distance between a priori group centres in PC-DFA space was used to construct a similarity measure and these distance measures were then processed by an agglomerative clustering algorithm to construct the dendrogram.

3. Results and discussion

Examples of raw UVRR spectra from each of the bacterial groups studied are shown in Fig. 1A. There seems to be little difference between these spectra and the processed spectra depicted in Fig. 1B. This reflects the difficulties faced when attempting to interpret multivariate hyperspectral datasets with the naked eye; since although the quality of the analysis is improved by the pre-processing steps, determining the salient characteristics of the spectra and how they have been altered is problematic. The spectral collection time and laser power setting selected to collect the spectra in Fig. 1A were 120 s and 0.1 mW respectively. Preliminary experiments performed to determine these parameters suggested that increasing the power in order to reduce acquisition time led to a reduction in spectral quality. The obvious conclusion to draw would be that the minimum measurement time for these experiments would therefore be 120 s. However, the resilience of micro-organisms under UV light is dependent on their cellular structure; for instance, we observed that Gram-negative organisms degraded more rapidly than Gram-positives under the same conditions, so for a different culture collection it may be possible to adopt more rapid measurement parameters. Another possibility for increasing measurement times for these experiments could be to apply different methods of sample presentation such as flow cells. Finally, if one were to use a pulsed laser it would be possible to deliver radiation of higher energy density to the sample, increasing collection times whilst providing enough time between pulses for the sample to relax and consequently avoid photodegradation.

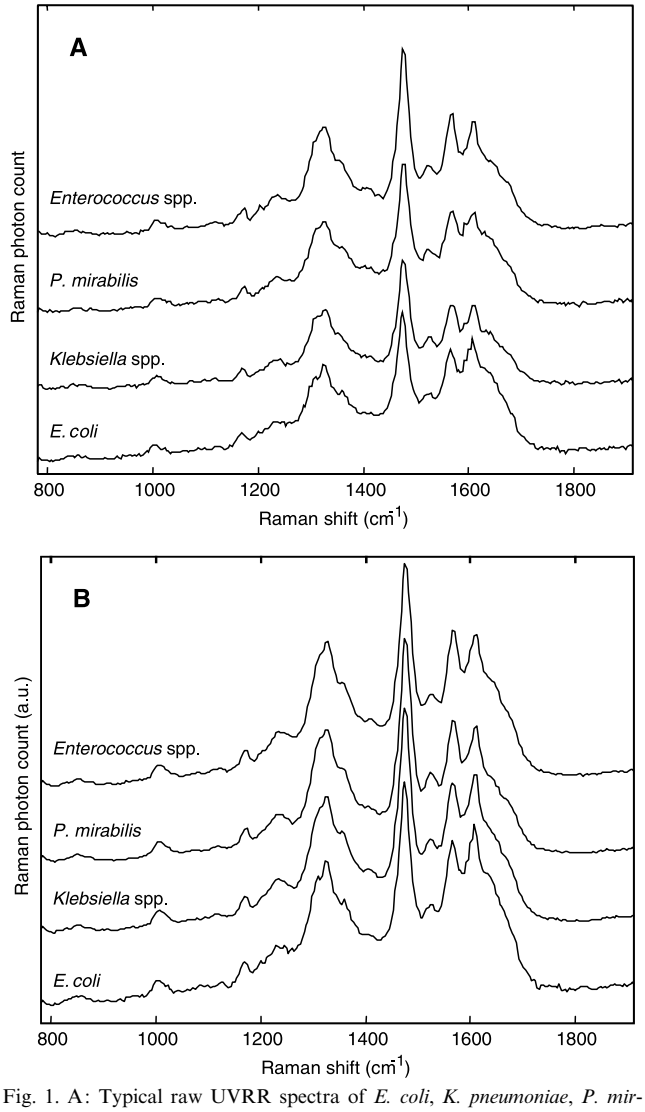

Fig. 1. A: Typical raw UVRR spectra of *E. coli*, *K. pneumoniae*, *P. mirabilis* and *Enterococcus* sp. B: The spectra in A following the pre-processing steps described in the text.

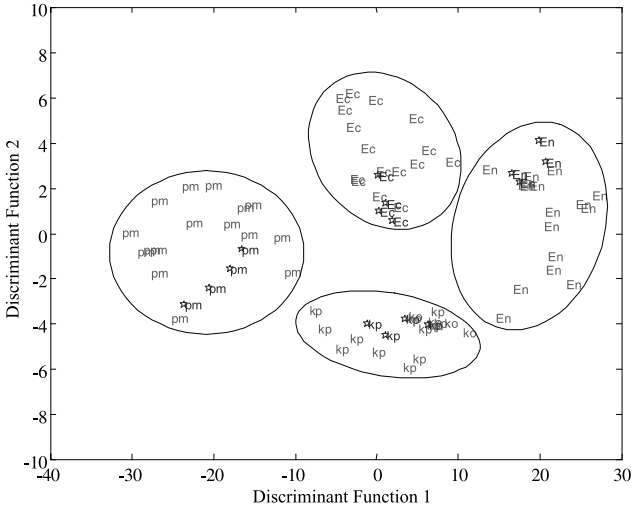

Fig. 2. PC-DFA ordination plot (DF1 v. DF2) using a priori knowledge of the UTI genus classification. The isolates are coded as follows: Ec-*E. coli*; En-enterococci; kp/ko-*K. pneumoniae*/*K. oxytoca*; pm-*P. mirabilis*. The projected validation data are identified by pentagram markers. Boundaries drawn around the clusters are given for clarity only.

UVRR spectra of micro-organisms and associated biological material have already been researched in some detail [15–17,26–28]. From these studies it is clear that for excitation between 222 nm and 257 nm the spectra will mostly be dominated by nucleic acids and aromatic amino acids, which are strong absorbers of UV light at these wavelengths. The peak assignments in Table 1, determined by analysis of aromatic amino acid and nucleic acid base UVRR spectra obtained in this laboratory (data not shown), agree with previously published data at 244 nm excitation [17]. As can be seen from Fig. 1A, the most striking feature is the 1475 cm^{-1} band, a contribution

Table 1
Principal resonance Raman bands with their associated peak assignments, encoded as follows: Tyr-tyrosine; Trp-tryptophan; A-adenine; C-cytosine; G-guanine; T-thymine; U-uracil

Raman band (cm^{-1})	1172	1247	1324	1475	1524	1567	1607
Peak assignment	Tyr	G+A+U	A+G+Tyr	G+A	C	G+A	Tyr+Trp

from the nucleic acids adenine and guanine, with the obvious doublet at c. 1600 cm^{-1} a result of the aromatic amino acids tyrosine and tryptophan (1607 cm^{-1}) and a further adenine and guanine contribution (1567 cm^{-1}).

The PC-DFA model shown in Fig. 2 was generated by using a priori knowledge of the genus classification (i.e. four groups). This shows four clusters clearly recovered from PC-DFA analysis, with the validation replicates falling within the boundaries of the test data. As described above the model is validated by leaving a strain from each genus out of the analysis, in Fig. 2 the projected validation data are identified by a pentagram marker.

Given the significant representation of nucleic acid components within the UVRR spectra, one might assume that any observed clustering will be heavily dependent on these (bio-)chemical species and would reflect the GC:AT ratio. However, if this were to be the case then the expected result would be clusters distributed linearly in DFA space which would be directly related to the GC content. As seen in Fig. 2 this is not the case as the four cluster

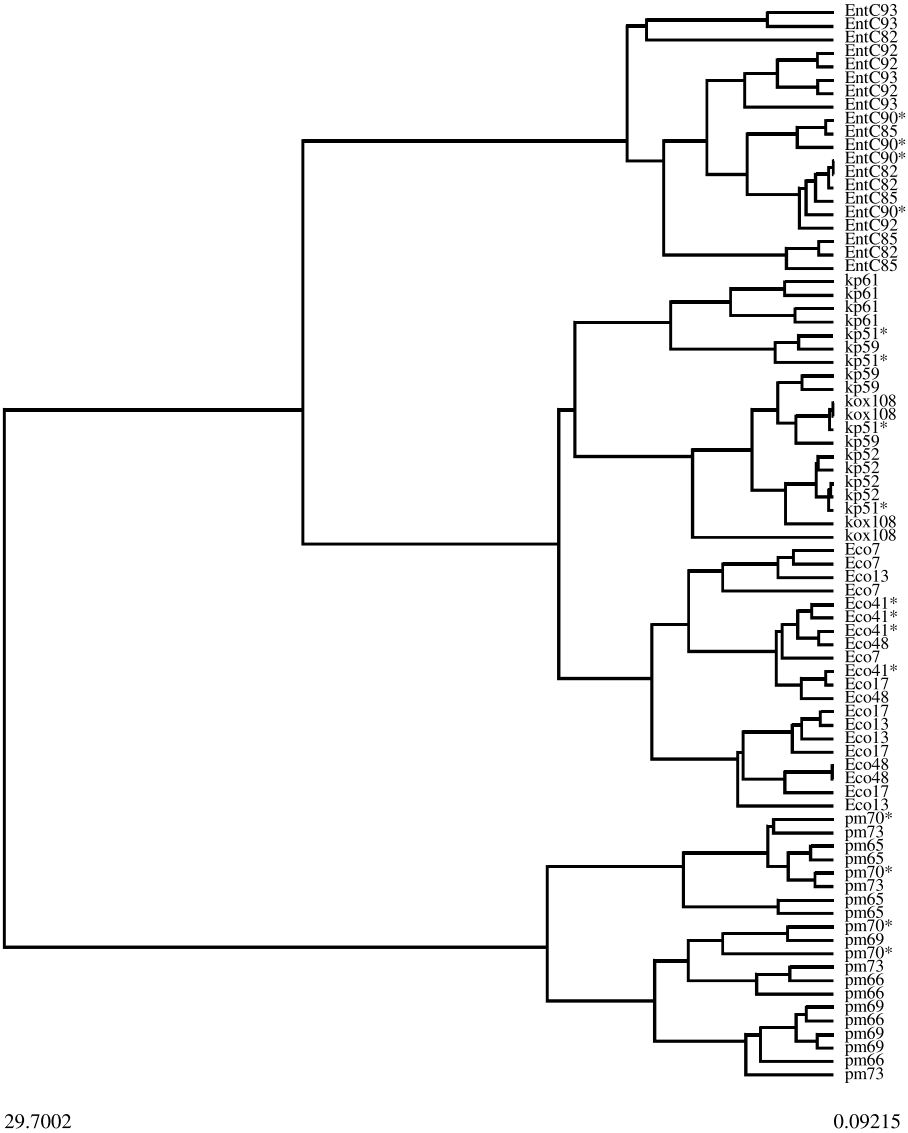

Fig. 3. A dendrogram generated by HCA from the PC-DFA model in Fig. 2, providing an additional perspective on the relationships between the clusters and the individual isolates being studied. The isolates are coded as defined in the text, with the validation data identified by asterisk.

mean centres are almost geometrically equidistant from each other.

In order to provide an additional perspective on this result, the dendrogram in Fig. 3 was generated from the PC-DFA space as described above. This clearly shows that each of the four classes of UTI micro-organism is correctly resolved into four separate clusters. The first branch in the dendrogram separates the two main clusters consisting of *Proteus* in one against *Klebsiella*, *E. coli* and the enterococci in another; subsequent branches differentiate all four causal agents of UTI. Note that as a result of using just four a priori groups in the DFA analysis the distribution of individuals within the dendrogram is heterogeneous, indicating that there is little resolution as yet at the strain level.

4. Conclusion

The work presented here clearly demonstrates that UVRR spectroscopy can be used, in conjunction with appropriate clustering algorithms, to discriminate between the main causal agents of UTI. We have shown that the technique could provide a more rapid, cost-effective diagnostic tool than the conventional biochemical assays currently used in clinical practice. However, to demonstrate the power of UVRR fully as a tool for bacterial discrimination, further work needs to be carried out to develop a platform for high-throughput automated analysis. With larger datasets more realistic models could be developed which would allow us to determine whether there is real potential for the use of this technique in the clinical environment.

Acknowledgements

We are very grateful to Dr E. Consuelo López-Díez for providing UVRR spectra of nucleic acid bases and aromatic amino acids. R.M.J. is indebted to Renishaw plc. and the UK EPSRC for his CASE PhD studentship. R.G. also thanks the UK BBSRC Engineering and Biological Systems Committee and EPSRC for financial support.

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