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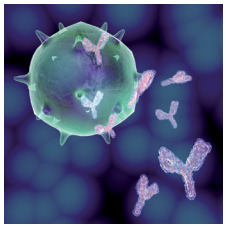
Raman Spectroscopy

Direct identification of clinically relevant microorganisms on solid culture media by Raman spectroscopy



Application Note

Biology RA62

I. Espagnon[a], D. Ostrovskii[b], R. Mathey[b], M. Dupoy[c], P. Joly[c], A. Novelli-Rousseau[b], F. Pinston[b], O. Gal[a], F. Mallard[b], D. Leroux[d]
[a]CEA, LIST, Département Métrologie, Instrumentation et Information, Gif-sur-Yvette, France, [b]bioMérieux, Technology Research Dept. Grenoble, France, [c]CEA, LETI, MINATEC, Grenoble, France, d bioMérieux, Technology Research Dept., Marcy l'Etoile, France

Abstract

Decreasing turnaround time is a paramount objective in clinical diagnosis. We evaluated the discrimination power of Raman spectroscopy when analyzing microcolonies from nine bacterial and one yeast species directly on solid culture medium after a shortened incubation time of 6 h (compared to 24 hour for macrocolonies). This approach, that minimizes sample preparation and culture time, would allow resuming culture after identification to perform downstream antibiotic susceptibility testing.

Keywords

Raman Spectroscopy, Rapid microbiology, Bacteria colonies, Culture medium, *in vitro* diagnostics

Introduction

In vitro microbiological diagnostics are still heavily relying on time-consuming cultivation of microorganisms to identify infectious agents and to prescribe therapeutic antibiotics. Because of long turnaround time (TAT), clinicians prescribe broad-spectrum antibiotics prior to the availability of a more precise diagnosis. In addition, the fact that pathogens can evolve to acquire antibiotic resistance traits, while at the same time fewer new antibiotics are being discovered, constitutes a major public health problem.

In order to reduce the TAT, we propose to shorten the time of culture and to minimize the number of sample preparation steps by proceeding with an identification of colonies directly onto the culture medium, using Raman spectroscopy. In addition to clinical benefits this approach [1, 2] would reduce the cost of running a clinical laboratory and increase process traceability and robustness, as it would allow for further cultivation or immediate abrogation of the process when no further characterization is needed.

Raman spectroscopy is a good candidate for this application as it is sensitive (good quality Raman spectra can be acquired from a single bacterium [3]), confocal, amenable to automation, non-intrusive and possibly non-destructive (a key point as resistance and susceptibility testing of pathogens are most often conducted after identification).

Choice of species and culture medium

For this study, we chose nine bacterial and one yeast species, the most frequently encountered in clinical microbiology (table 1). It includes Gram-negative species inside the Enterobacteriaceae family (*Escherichia coli, Enterobacter aerogenes, and Enterobacter cloacae) and outside (Acinetobacter baumannii, A. johnsonii, and Stenotrophomonas maltophilia*); Gram-positive bacterial (*Bacillus cereus, Staphylococcus aureus, and S. epidermidis*) and one yeast species, selected as a eukaryotic outlier (Candida *albicans*). Eight well-characterized strains were selected per species.

As a medium, we chose Trypcase Soy Agar (TSA) as it is a very generic medium that expresses a weak fluorescence. External studies conducted on a variety of culture media (Sabouraud agar [3, 4] and even Columbia Blood agar [5] despite the presence of hemoglobin) lead us to believe that our results can be extrapolated to a variety of media.

Species	Code	Nb of strains	Nb of spectra Macrocolines	Microcolones
Acinetobacter baunnanii	ACN-BAU	8	257	182
Acinetobacter johnsonii	ACN-JOH	8	236	192
Bacillus cereus	BAC-CEU	8	190	180
Candida albicans	CAN-ALB	8	261	143
Enterobacter aerogenes	ENT-AER	8	260	213
Enterobacter cloacae	ENT-CLC	8	264	191
Escherichia coli	ESH-COL	8	288	193
Staphylococcus aureus	STA-AUA	8	235	202
Staphylococcus epidermidis	STA-EPI	8	288	180
Stenotrophomonas maltophilia	STE-MLT	8	254	137
TOTAL		80	2533	1813

Table 1: Nine bacterial and one yeast species used in this study.

Classification of species

Raman spectra were recorded in the conditions optimised for micro- and macrocolonies, respectively, using 532 nm wavelength to improve the axial resolution of probing. These conditions, established experimentally are described in more details in section Experimental and data treatment details. Several thousands of spectra were taken over 80 strains studied in this model (results of the correct identification rates (CIR are shown in table 1) to account the possible inter- and intracolony variation. The number of the spectra and the acquisition time were optimised to obtain the best signal-to-noise ratio (SNR) in the shortest possible time.

To identify the colonies, five classification methods were tested: Euclidean distance, k-nearest neighbours (KNN), linear discriminant analysis (LDA), regularised quadratic discriminant analysis (rQDA) and support vector machine (SVN) using two types of cross-validation: in the so-called "stringent" and "non-stringent" modes. "Stringent", also referred as the leave-one-out validation method, signifies that spectra from the tested strain are removed from the reference database during classification. In which case, identification is performed by looking at similarities with other strains from the same species. The alternative "non-stringent" keeps the tested strain in the reference database.

	Stringent		Non-stringent	
	Macro-colonies	Micro-colonies	Macro-colonies	Micro-colonies
Raw spectra	93.3 ± 0.4	90.8 ± 0.3	99.8 ± 0.1	98.0 ± 0.1
1st derivative	91.6 ± 0.7	91.5 ± 0.4	99.7 ± 0.1	97.8 ± 0.2

Table 2: Correct identification rates (CIR) obtained for macro- and microcolonies in stringent and non-stringent cross validation modes

using SVM classification method and two sets of pre-processed spectra.

Prior to the classification, data pre-procession was done as described further in the section "Experimental and data treatment details"
The best results obtained using support vector machine (SVN) classification algorithms in a stringent mode as reported in [2]. Classification results of SVN method are summarized by the mean of the correct-identification rates (CIR) of all species and presented in table 2.

The results are presented graphically in a figure 1 in the form of a confusion matrix which consists in a cross-table of actual and found species membership, with classification rates expressed in percentages of the number of tested spectra in the actual species. Sensitivity for a given species can be obtained from this confusion matrix, since sensitivity simply is the corresponding CIR. The details of data preprocessing and analysis can be found in the section Experimental and data treatment details.

Misclassifications and taxonomy

Species that are the most difficult to differentiate by Raman spectroscopy are also the ones being very close in their taxonomic position, as defined using conventional phenotypic and molecular methods. With macrocolonies as well as with microcolonies, the lowest CIRs were observed inside the *Enterobacteriaceae* family (for *E. aerogenes*, *E. cloacae*, and *E. coli*) and inside the *Acinetobacter* genus (*A. johnsonii* and *A. baumannii*). Other significant errors occurred with *E. cloacae* instead of *A. johnsonii* for macrocolonies, and *S. maltophilia* instead of *E. cloacae* for microcolonies. Confusions confined under the genus level accounted for 89 % of all errors for macrocolonies and 44 % for microcolonies. When errors inside the *Enterobacteriaceae* family are included, the proportion increased to 93 % of all errors with macrocolonies and 85% with microcolonies.

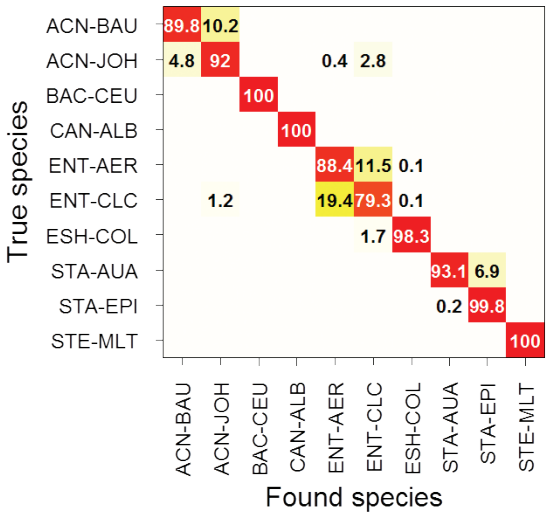


Figure 1: Confusion matrix for microcolonies with SVM method in stringent mode on first derivative spectra (CIR = 91.5 ± 0.4 %).

Comparison between macrocolonies and microcolonies and influence of the agar signal

For any given species, spectra acquired on microcolonies (incubation of 6h) were very different from spectra acquired on macrocolonies (incubation of 24h) ([2]). This clearly implies that identification is only possible if the spectra of the tested sample are acquired at the same culture age as the reference spectra forming the database, at least for times of culture where the growth stage is expected to be very different as is the case in this study. We suggest that the large differences observed between micro- and macrocolonies are indeed due to biological differences and not to the underlying growth medium (a result to be expected for microorganisms at different growth stages).

Indeed the microcolony average spectrum shows some characteristic peaks (664, 781, 808, 1095 and 1569 cm^{-1}) that match the position and tendency (higher nucleic acid content observed in exponential phase compared to stationary phase) of the metabolic activity markers identified by Moritz [6] and assigned to DNA and RNA nucleic acid bands (at 668, 783, 811, 1099, and 1578 cm^{-1}).

Conclusions

Our original intent was to evaluate spectral classification performance while minimizing data preprocessing to establish a benchmark for performance of identification. We have shown that it was possible to discriminate, at the species level, 80 strains belonging to 10 different bacterial and yeast species, with a correct identification above the 90% cut-off limit routinely accepted in IVD identification via direct measurements on the culture medium. Importantly, these numbers were obtained in a stringent cross-validation analysis. This opens the door to an innovative clinical diagnostic workflow, allowing the possible interrogation of cultures as early as 6 h from culture start with the possibility of resuming the culture after Raman spectroscopy in order to facilitate other forms of downstream microbiological analysis.

The simplicity of the pre-processing method used in this study as well as the absence of any sample preparation after culture, coupled with low biomass requirements, low invasiveness, and real time measurement make Raman spectroscopy an outstanding technology candidate for rapid and automated in vitro diagnostics.

Experimental and data treatment details

Sample preparation and manipulation

Petri dishes with bacterial cultures were directly transferred from the incubator to the spectrometer and Raman spectra were recorded directly from the grown colonies without any additional sample processing.

Spectroscopic device and measurements

Raman spectra were acquired using HORIBA Raman spectrometer equipped with a 532 nm laser. The acquisition spectral window ranged from 395 to 3075 cm^{-1} , the spectral resolution was about 12 cm^{-1} , the dispersion about 3 cm^{-1}/px. The parameters used for spectral acquisition are summarised in Table 3.

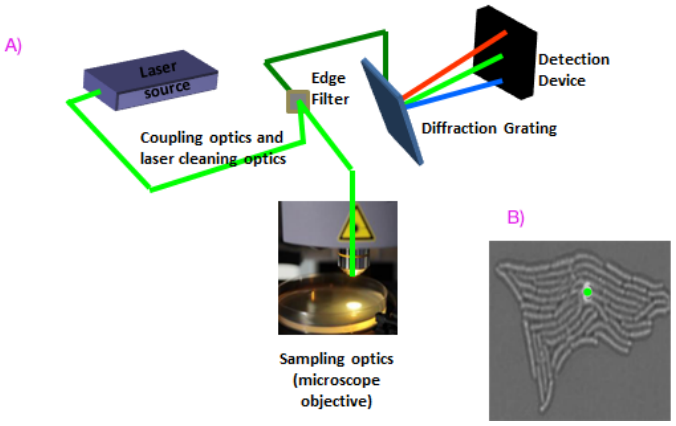


Figure 2: (A): schematic of the Raman setup showing the direct measurement of scattered light from the colonies (petri lid was removed); (B): Image of the laser projection on the E. coli micro-colony in reflection microscopy.

Colony type	Time of growth (h)	Microscope objective (x/NA)	Axial confocal thickness (µm)	Laser power (mW)	Acquisition Time (sec.)
Macro	24	50/0.5	60	11	5×20
Micro	6	100/0.8	5	36	5×15

Table 3: Parameters and conditions of Raman spectra acquisition for microbial macrocolonies and microcolonies.

Data preprocessing

Four pre-processing steps were used in this study consisting in (i) suppression of "cosmic" spikes, (ii) correction of possible wavenumber shift of the spectra, which has instrumental origin; (iii) extraction of the signal of interest (by deriving, or subtracting background, or the raw signal itself), accompanied with smoothing to reduce random spectral noise; and (iv) normalization of spectral intensities to exclude the effect of varying laser power, focusing grade, sample density, etc.. Figure 3 shows the same set of raw and pre-processed spectra, also with the corresponding PCA plots, here for the microcolonies.

Classification

Two types of cross-validation were carried out, in the so-called "stringent" (also referred as LOOCV for leave-one-out cross-validation) and "non-stringent" modes. When performing a classification at the species level in the stringent mode, all spectra belonging to the strain being classified were previously removed from the reference database, thus preventing an artificially almost perfect match. On the contrary, in the non-stringent mode, all strains were represented in the reference database.

In the non-stringent mode, the best CIR is 99.8 ± 0.1 % (interestingly, obtained for the raw spectra) while it is of 91.5 % in the stringent mode (versus 93.3 % for macrocolonies). Detailed descriptions of the methods published elsewhere [2].

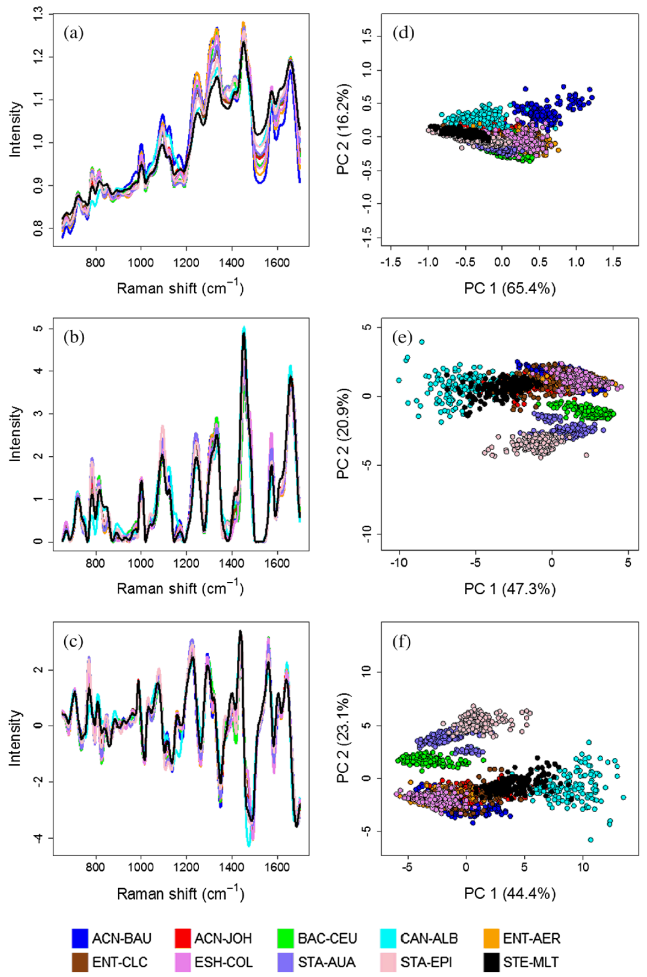


Figure 3: Mean spectra per species for microcolonies, for (a) raw spectra, (b) corrected spectra, (c) first derivative, and (d, e, f) corresponding PCA.

Further reading

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info.sci@horiba.com

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USA: +1 732 494 8660
UK: +44 (0)20 8204 8142
China: +86 (0)21 6289 6060
France: +33 (0)1 69 74 72 00
Italy: +39 2 5760 3050
Brazil: +55 (0)11 5545 1500
Germany: +49 (0)89 4623 17-0
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