Raman spectroscopy enables rapid identification of clinically relevant bacteria and yeasts. Limiting the time between receiving a patient sample and providing the results of a microbiological evaluation (identification of the microorganism which causes an infection and determination of an effective treatment) is crucial for correct and timely treatment of the patient. Using Raman spectroscopy identifications were obtained faster and with less sample handling, than with conventional methods, which makes the technique a potentially powerful alternative for conventional phenotypical identification methods.

Microbes and light, a vibrant interaction

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The work presented here is supported by the Dutch Technology Foundation (STW) grant number RPG.6311.

Clinical problem

The time required for the identification of pathogens is an important determinant of infection-related mortality rates of hospitalized patients. Rapid identification techniques significantly reduce mortality and costs associated with infectious diseases. Most commercially available identification systems in routine use in hospitals are based on the physiological and nutritional characteristics of microorganisms, from which the principles date back to Koch and Pasteur. These systems require a pure microbial culture and a large inoculum. Consequently, a turnaround time of 24 h (e.g., for ***Staphylococcus aureus***) to up to 5 days (for ***Candida*** species) between receipt of patient material and presentation of identification results to the clinician is common. Therefore, empirical treatment with broad-spectrum antibiotics is often started while awaiting further identification of the pathogens. Apart from the risk that the empirical treatment may not be effective at all, this practice may lead to adverse toxic side effects and is known to aggravate problems with resistance to antimicrobial agents. Early identification enables the clinician to precisely target a pathogen with the most effective antimicrobial agent.

Technical solution

A quick scan of the recent literature on the rapid identification of microorganisms identifies an ever-expanding number of polymerase chain reaction (PCR) assays. However, during the last few years more and more applications of biophysical methods for characterising and typing of microorganisms are presented. The most prominent ones are those based on mass spectrometry and vibrational spectroscopies, but also methods based on nuclear magnetic resonance (NMR) gain in popularity. Biophysical techniques have several clear advantages over conventional identification approaches:

1. One protocol for a wide range of organisms
2. Limited sample handling
3. No labels or dyes required
4. Rapid
5. Easy to automate

In our laboratories we are optimising Raman spectroscopy for routine diagnostic microbiology. Our primary goal was to facilitate rapid identification of bacteria and yeasts with simple modifications of the protocols however, detailed biochemical information can be obtained to use in other applications.

Sample preparation

The preparation of a microbiological sample, before a Raman spectrum can be obtained from it, is quite straightforward. Two basic applications are most often used which can easily be fitted into a traditional culturing protocol (figure 1). First, some biomass from a solid culture medium, or the pellet after centrifugation from a broth culture, can be directly transferred onto a glass slide (mostly CaF_2). Measurements can than be directly performed on this sample. Second, measurements can be performed on colonies, directly on a solid culture medium. Since the measurement volume of a Raman spectrometer is very small, rapid identification can be achieved by using microcolonies which will usually develop in only several hours incubations time. Without using labels or dyes, Raman spectra are obtained from the native sample, without altering or destroying it. Typical data collection times are in the order of

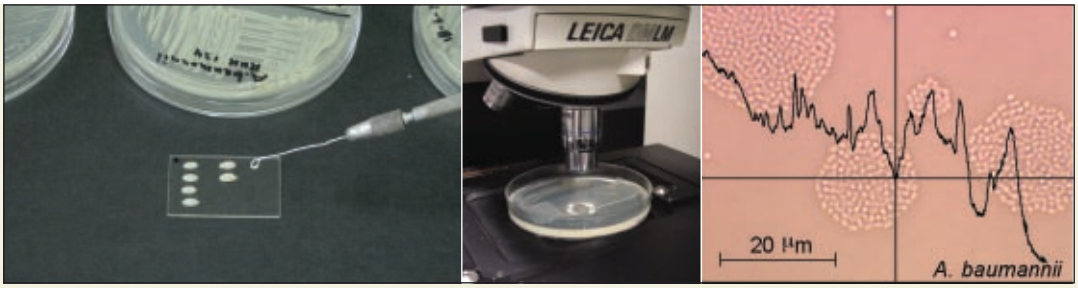

Figure 1. *Sample preparation for Raman measurements. Samples can be transferred e.g. from a culture medium, onto a glass slide (a), these smears can than be directly measured. For rapid identification, microcolonies can be measured directly on the solid culture medium by placing the petri dish under the microscope (b). The laser is aimed by a crosshair on the location in a microcolony of interest, after which a spectrum is collected.*

minutes (1 to 5 min.), and calibration times depend on the specifications of the PC used. After calibrating the spectra, so that the shift of the different peaks relative to the excitation wavelength is known, the data is ready for further analysis.

Spectral analysis

Fig.1 Sample preparation for Raman measurements. Samples can be transferred e.g. from a culture medium, onto a glass slide (a), these smears can than be directly measured. For rapid identification, microcolonies can be measured directly on the solid culture medium by placing the petri dish under the microscope (b). The laser is aimed by a crosshair on the location in a microcolony of interest, after which a spectrum is collected.

The position, intensity and width of a peak in a Raman spectrum all contain information about a specific molecular vibration. Different microorganisms will have a different biochemical make-up, which is reflected in their Raman spectra (figure 2). Biochemical differences between organisms can therefore be studied by comparing the differences in spectral features. However, spectra can also be evaluated as spectroscopic fingerprints of the sample studied. Chemometric or multivariate statistical techniques can be employed for the analysis of the complex spectra, in a similar manner as used in pattern recognition. Some examples of techniques often used are principal component analysis, hierarchical cluster analysis, linear discriminant analysis and artificial neural networks.

Applications of Raman spectroscopy in microbiological analyses

One of the major advantages of Raman spectroscopy is the possibility to collect spectra from defined positions within a sample (i.e. with high spatial resolution). It was shown earlier that when using a confocal measurement set-up, it is possible to study biochemical differences within microbial colonies. Reproducible layers of cells in different growth phases could be observed in colonies of ***S.aureus, E.coli*** and ***C.albicans*** (figure 3). This approach offers a way of examining microbial communities in situ, e.g. the various layers of physiologically different cells in a biofilm. In doing so, fundamental knowledge can be acquired on when the different layers develop, and what other biochemical gradients exist in biofilms. Using this technique offers the unique possibility to study the development of biofilms over time. Employing Raman spectroscopy for the rapid identification of microorganisms, measurements could be performed on microcolonies after 6 hours of growth on a solid culture medium. Using young cultures of ***Candida*** species for instance, allowed their discrimination on a regular culture medium with 97-100% accuracy. Conventional approaches like the API system from bioMerieux require 24 to 48 hours. Moreover, identifications from a positive blood culture also required 6 hours of culturing prior to Raman measurements. In a diagnostic laboratory, using conventional methods this would require 1-4 days after the blood was found positive for microbial growth.
Recently, Hutsebaut et al studied the influence of culture conditions on the identification of ***Bacillus*** strains by Raman spectroscopy .

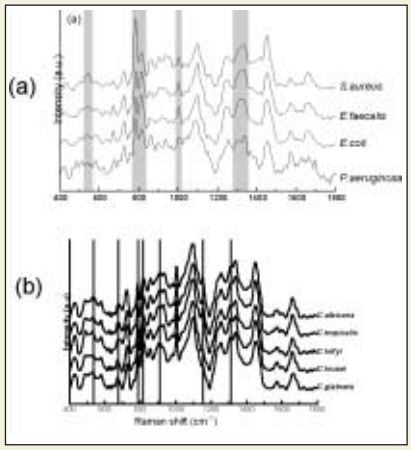

Figure 2. *Clear differences are visible in the Raman spectra of different microorganisms (a). The Raman species of more related microbial species show a higher degree of similarity, although characteristic features are still present as indicated by the dotted lines (b).*

Thirty strains from 3 species were cultured under 12 different conditions. This still left sufficient discriminatory information in the spectra to allow adequate separation at the species level (92% accuracy). This shows that Raman spectroscopy is a robust method and that small variations in the standard operating protocol in a diagnostic laboratory are not likely to hinder the identification results.

Identifications using vibrational spectroscopies is not limited to the species level. Analysis of ***Acinetobacter*** strains showed promising results for epidemiological studies (manuscript submitted). A set of isolates from 5 different outbreaks was used in this study. Hierarchical cluster analysis of the Raman spectra turned out to be highly similar to a cluster analysis on amplified fragment length polymorphisms (AFLP). Raman spectra measured in 30 seconds per isolate, when some biomass from an overnight culture was analyzed, seemed to be sufficient.

Conclusion

The Center for Optical Diagnostics and Treatment has a worldwide leading position in the field of microbial identification using Raman spectroscopy. The close cooperation with various disciplines in the Erasmus MC has proved very useful and will guarantee a constant flow of new ideas in the future. We have shown that the identification is rapid, accurate and relatively simple in terms of sample handling when compared to conventional methods. We believe that upon further improvement of the instruments and in view of the wealth of information in the spectra, Raman spectroscopy will eventually find its way to the clinical microbiology laboratories as an alternative to current automated phenotypical identification methods.

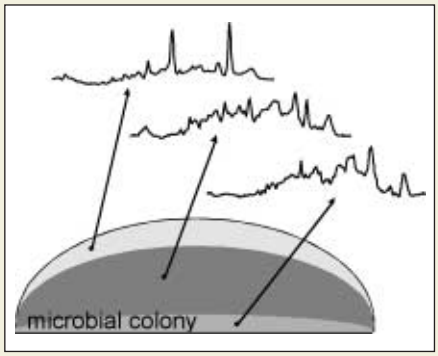

Figure 3. *Raman enables the analysis of biochemical variance in microbial communities. Here the different layers of a colony from S. aureus are shown. Choo-Smith et al. showed that differences could be ascribed to e.g. RNA and carotenoid levels in the cells, which correlates with the different growth phases of the cells in these layers.*