Confocal Raman Microspectroscopy

a novel diagnostic tool in medical microbiology

Confocale Raman-microspectroscopie

een nieuwe diagnostische techniek in de medische microbiologie

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Figure 1

Antoni van Leeuwenhoek
(1632-1723)

Figure 2

Louis Pasteur
(1822-1895)

Figure 3

Robert Koch
(1843-1910)

1. Introduction

In 1676 the Dutchman Antoni van Leeuwenhoek (figure 1) wrote a letter to the Royal Society in London describing his observation of "kleijne diertgens" using one of his famous self-made microscopes. With this finding, he was the first to ever see bacteria and consequently the day of his observation (24th of April 1676) is considered the birthday of bacteriology. However, it was not until 150 years later before any serious investigations into the nature of microorganisms began. One of the most prominent people in this period is Louis Pasteur (figure 2). This French chemist developed aseptic techniques that could be used to obtain pure microbial cultures. He was particularly interested in the chemical reactions/fermentations bacteria were able to perform and between 1855 and 1860 he devoted most of his research to this subject. Pasteur was able to show that the fermentation of various organic fluids was always associated with the presence of living cells. Furthermore, he found that different types of fermentation were associated with the presence of microscopic organisms which could be distinguished from one another by their morphology and their cultural requirements. Thus, at this early stage, the idea of specificity entered into bacteriology, which later developed into classification schemes based on differences in fermentation capabilities. Although, later Pasteur also described the relationship between microbes and certain illnesses such as anthrax, it was the German physician Robert Koch (figure 3) who laid the definitive basis for clinical microbiology. He was the first to demonstrate the relation between a single bacterial species and a specific disease; anthrax. From his research on anthrax, tuberculosis and wound infections, Koch deduced his 4 postulates, that would prove whether or not an infectious agent is the cause of a disease:

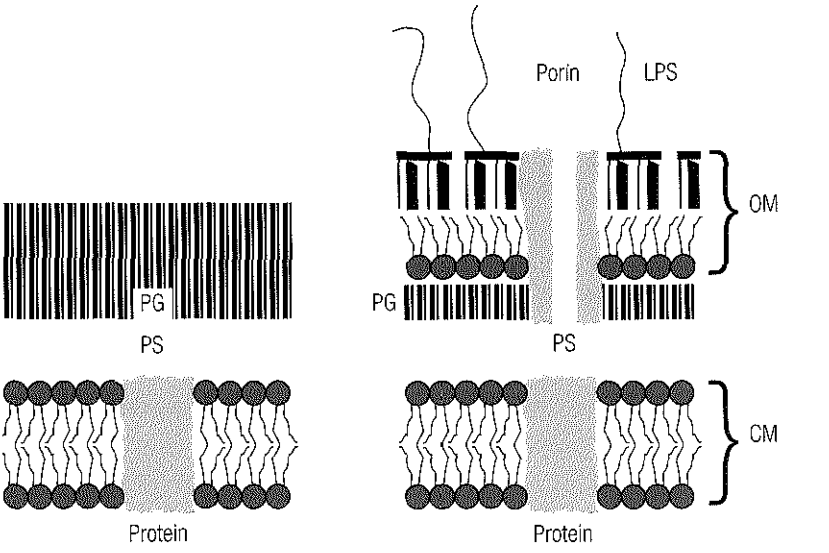
1. The causative agent of the disease must be present in all cases of the disease and must be absent from healthy individuals.
2. The agent of disease can be isolated from the diseased individual and can be cultivated in pure culture in the laboratory.
3. The disease can be reproduced by inoculating a portion of the pure culture into healthy individuals.
4. The agent of disease can be re-isolated from the infected individual and again cultivated in the laboratory.

Another development, still important, for modern microbiology, the solid culture medium based on agar-agar as a gelling agent, also came from the laboratory of Robert Koch. Before that, potato slices or gelatin were used to culture bacteria on, but neither are ideal for supporting bacterial growth, because of the lack of nutrients and the fact that gelatin easily liquefies, either due to bacterial enzymes or at temperatures around 37°C. It was the wife of the German physician Walter Hesse who suggested agar-agar, which she had been using for several years in preparing fruits and jellies. After reporting this successful observation to Koch, his associate Friedrich Loeffler devised a nutrient medium, composed of meat extract and a digest of protein. This nutrient medium is still the basis for a lot of the culture media currently in use. Contemporary clinical microbiological techniques are still largely based on the findings of Pasteur and Koch. The fermentation reaction of a wide range of microorganisms for large numbers of substances is now well documented and is the basis for bacterial identification schemes. Solid culture media are indispensable to the clinical laboratory, because of the ease of obtaining pure cultures and the possibility to inspect colony morphology.

Diagnostic microbiology In clinical practice it is important to identify the etiological agent of an infectious disease. Identification and characterization of the causative organism enable targeted intervention by the clinician. Several interventions are available to the physician. The most obvious one is treating the infection with antibiotics, but others such as specific immunization and hygiene measures are also at his/her disposal.

Traditional identification of bacteria starts by inspecting the colony morphology when the cells are cultured on solid media followed by a microscopic inspection of the cell morphology in a Gram-stained smear. The Gram-stain procedure is named after the Danish physician H.C.J. Gram who in 1884 discovered that bacteria can be separated in 2 major groups, Gram positive and Gram negative based on their ability to retain the dye crystal violet. This in turn depends on the molecular structure of

Figure 4

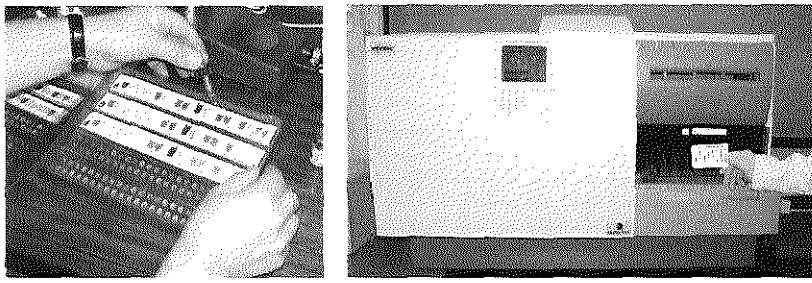


Schematic cross-sections of Gram-positive and Gram negative bacterial cell walls. The cytoplasmic membrane (CM) surrounds the cytoplasm, the other layers are exposed on the periphery of the CM. PS, *periplasmic space;* PG, *peptidoglycan;* OM, *outer membrane;* LPS *lipopolysaccharide.*

the cell wall (figure 4). Moreover, with microscopic examination the bacteria can be divided into groups based on their cell shape (e.g. rods, cocci and spirochetes) and cell formation, for example string or chain like structures (streptococci). Based on these morphological properties a series of tests is chosen to examine the biochemical, physiological and nutritional properties of an organism.

Traditionally these tests are combined in a series of solid and/or liquid media, which are inoculated with a suspension of bacteria and interpreted after a certain incubation period. Most tests are interpreted by changes in elaborate indicator systems, predicting changes in pH, presence of a certain compound or breakdown of others. Staphylococci for instance contain the catalase enzyme to break down hydrogen peroxide, whereas Enterococci do not have the enzyme[23]. The presence of the enzyme can be detected by bringing the bacterial cells into contact with hydrogen peroxide, where the production of gas indicates the presence of the enzyme ($2H_2O_2 \rightarrow 2H_2O + O_2$). A difference between the species *Staphylococcus aureus* and *Staphylococcus epidermidis* for example is the formation of acid

Figure 5



Examples of miniaturised identification systems, that are commercially available. LEFT: *the manual* API *system from bioMerieux, Marcy-l'Etoile, France. The strips with dehydrated reagents is inoculated with a bacterial suspension from a pure culture. After an incubation period the test results are interpreted using elaborate indicator systems, and compared to a database.* RIGHT: *the automated commercially available Vitek2 system (bioMerieux). Still based on the traditional methods but requiring less biomass, they are easier to operate and need shorter incubation times before the tests to be interpreted. The test cartridges (white card held before the instrument) are filled with dehydrated reagents, as in the* API *system. These cartridges are placed in the instrument together with a bacterial suspension to be examined. Inoculation, incubation and interpretation of test results is all performed automatically and the identification result can be stored in a computer.*

from the sugar D-mannitol, which the latter is incapable of[33]. Performing these traditional tests is laborious, hard to standardize and takes approximately 2 to 3 days to complete for most clinically relevant organisms. A substantial amount of this time is accounted for by the initial culturing steps, in order to obtain enough cells or biomass to perform the tests. Miniaturized identification systems have become available which simplify the process and increase the speed by which results can be obtained. Well known systems are API and Vitek (bioMerieux, Marcy-l'Etoile, France) which are still based on the traditional methods but require less biomass, are easier to operate and need shorter incubation times in order for the tests to be interpreted. The miniaturized identification systems can be divided into those of which the interpretation is done manually and those that are interpreted using automated systems (figure 5)[9, 13, 52, 61]. Also on the market are so-called rapid identification systems that can produce a result within 2-4 hours after the initial culture has yielded enough cells (usually 16-24 hours) for usage in these systems[28]. The methodology behind these rapid identification systems is based on the

ability to detect metabolic activity earlier and the tests can be inoculated with limited biomass.

Rapid identification of microorganisms Even though the total time required for the identification of microorganism, causing an infection, using a miniaturized system is decreasing, there are situations in the clinical setting in which a clinician cannot wait for the test results. For critically ill patients on intensive care wards, for example with life-threatening infections such as sepsis or meningitis, immediate intervention can mean the difference between life and death. It is common practice to start broad-spectrum empirical antimicrobial therapy based on experience with similar cases. As soon as the test results from the microbiology laboratory are known, the physician can, when indicated, modify and target the initial therapy to the microorganism that causes the specific infection. This strategy bears some inherent problems; there is a chance that the causative microorganism is resistant to the initially administered drug, implying that effectively no treatment is given, the antibiotic could affect the normal flora of the body (microbes permanently present on and in the body) and some antibiotics have toxic side effects. Doern *et al* conducted a study, in which patient material was processed with either a rapid test, producing results on the same day that the test was started, or a more traditional test in which the results were only known the next day[11]. Both identification schemes were started after the initial culture from the patient material was positive, which took approximately 24 hours for both groups. Besides advantages in reducing morbidity and mortality (8.8% versus 15.3%) it was found that the overall costs for the hospitalization were significantly lower for the group in which the rapid identification scheme was used. The authors therefore concluded that "*rapid same-day bacterial identification and susceptibility testing in the microbiology laboratory can have a major impact on the care and outcome of hospitalized patients with infection*".

Besides the miniaturized identification systems there are also possibilities for the rapid identification of clinically relevant microorganisms based on molecular biological methods. These techniques are currently being evaluated for their usefulness in clinical diagnostic microbiology[1, 12, 24, 36, 46, 55]. Molecular diagnostics are powerful tools for the identification and characterization of microorganisms. Most tests are targeted at specific DNA sequences enabling species-specific identification or demonstrating the presence of antibiotic resistance genes[5, 33, 48, 53, 56]. Major advantages of molecular diagnostics are the high sensitivity, specificity and potential speed (as little as 7 minutes have been reported for the detection of bacteria directly in a sample[3]). To detect a microorganism, theo-

retically only one copy of bacterial DNA is required in the DNA amplification technique called polymerase chain reaction (PCR). Because of this sensitivity it is possible to detect unculturable organisms or identify fastidious organisms at an earlier time[43, 44]. In the case of *Mycobacterium tuberculosis*, the causative organism for tuberculosis, detection can even be performed directly on the sputum obtained from the patient[55]. An exciting new approach in molecular diagnostics is the DNA-chip. On a small solid support, high numbers of molecular probes can be attached and a sample can be screened against all probes at once[14, 15, 50]. This technique offers new possibilities to the clinical microbiologist[4, 54, 60]. Fluorescent probes, targeted at specific gene sequences have also been used for the rapid identification of clinically relevant microorganisms[1, 26, 27]. With this technique called fluorescent *in situ* hybridization (FISH), identification of microbes in positive blood cultures have been reported within 45 minutes[26] to 2.5 hours[27], while traditional identification required 1 to 3 days.

Although the advantages of using molecular diagnostics are obvious, there are some problems that can hamper their usefulness in a clinical laboratory. First, the reagents that are required for molecular techniques are expensive[35]. Particularly when there has to be tested for a wide range of microorganisms, where each organisms requires it's own probe, the costs rise accordingly[36, 60]. A second more fundamental problem is that of false positive and false negative reactions[16, 25, 34, 46, 60]. Previously amplified DNA for example can contaminate a sample, leading to false positive reactions. False negative reactions can occur due to inhibitors of the polymerase chain reaction, which can be introduced during sample preparation. Therefore, most of the techniques are complicated with a lot of sample handling and consequently require highly trained personnel to be performed correctly[36, 46].

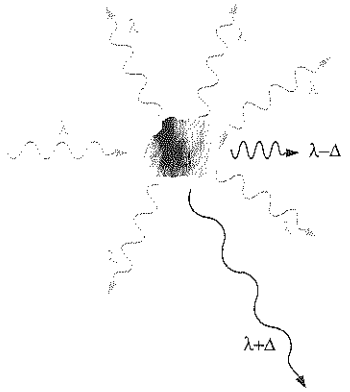
A different approach towards the rapid identification of microorganisms is based on spectroscopic techniques. Pyrolysis mass spectrometry (PyMS) and matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry are examples of "destructive" techniques that show potential for identification methods[2, 6, 10, 17-21, 57, 58, 62, 63]. Non-destructive (i.e. leaving the sample intact during the analysis) methods can be developed on the basis of vibrational spectroscopies (infrared and Raman spectroscopies). Of these techniques Fourier transform infrared (FT-IR) spectroscopy is starting to become an accepted technique in microbiology. For its vibrational spectroscopic counterpart, Raman spectroscopy, the potential in medical microbiology seems equally great. The development of Raman-based rapid and accurate identification methods for clinically relevant microorganisms is the subject of the study presented here.

History of vibrational spectroscopy In 1911 W.W. Coblentz was probably the first scientist suggesting that biological materials can profitably be analyzed by means of infrared (IR) absorption spectroscopy[45]. The use of IR-spectroscopy as a means of differentiating and identifying bacteria was extensively reported as early as in the 1950's and 1960's[31, 35, 45]. A critical review on this subject published in 1959 summarized that, although bacteria definitely exhibit IR-spectra that are unique for individual strains, the identification of bacteria via IR-techniques cannot be regarded as a practical scheme as it is a too time consuming and impractical procedure[35]. Because of the limited performance specifications of IR-spectrometers at that time (with regard to sensitivity, signal collection time, and reproducibility), reports on IR-applications in microbiology became less frequent in the 1960's and virtually ceased to appear in the mid 1970's. It was the development of modern interferometric IR-spectroscopy, the availability of low-cost mini-computers and powerful new algorithms of multivariate statistical analysis and pattern recognition methodologies that contributed to the revival of IR-spectroscopy as a means for characterizing microbial samples.

When compared to IR spectroscopy, Raman spectroscopy was neglected in the field of biological sciences. This has grown historically, ever since the IR techniques were improved. In the 50's and early 60's Raman spectroscopy gave similar information as IR spectroscopy but at higher cost, lower speed, much lower sensitivity and demanding relatively complicated instrumentation[32]. Coinciding with laser developments in the late 60's and early 70's, Raman spectroscopy was increasingly being applied in biological studies. It was, however, not until the late 80's that publications occurred in the literature reporting on the possibilities of Raman spectroscopy for identification purposes in microbiology[7, 8].

Because vibrational spectroscopies provide information about the overall molecular composition of cells, in a non-destructive manner, they offer numerous possibilities to study microorganisms. No labels, dyes or other contrast-enhancing exogenous compounds are needed. IR spectroscopy and Raman spectroscopy are complementary techniques, and different selection rules apply for IR absorption and Raman scattering by a molecule[59]. Together the two techniques provide a vibrational spectroscopic *"fingerprint"* of cells[22, 30, 31, 38, 40, 41]. In the last decade, the techniques have reached a level of sensitivity that enables spectra to be obtained of even one single living cell[29, 38-41, 47, 49]. Therefore only a small number of microorganisms will suffice to obtain spectroscopic data. When compared to routine clinical methods, culturing times could be reduced by a significant number of cell cycle periods. This will greatly speed up the microbiological analysis of patient material.

Figure 7



Schematic presentation of the interaction of light and matter. The major fraction of incident light with wavelength λ is scattered with the same wavelength (Rayleigh scattering). A small fraction of the incident light however, is scattered with an altered wavelength due to an energy exchange. The process in which scattered light has an decreased (λ−Δ) or increased (λ+Δ) wavelength is the so-called inelastic scattering of light.

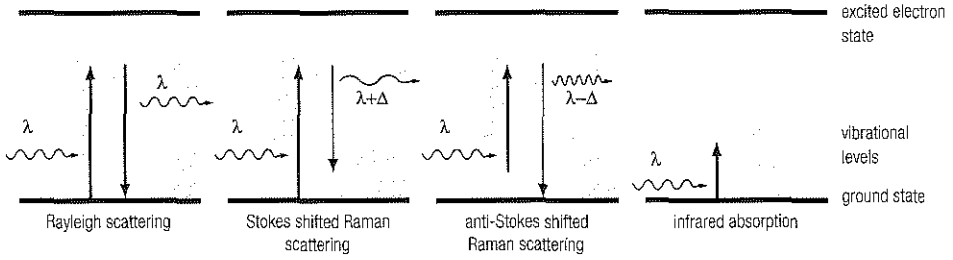
Figure 6

Chandrasekhara Venkata Raman (1888-1970).

Raman spectroscopy Raman spectroscopy is named after its discoverer, the Indian physicist Chandrasekhara Venkata Raman (figure 6). In 1928 Raman was the first to find experimental prove for the inelastic scattering of light from matter[42]. This interaction of light and matter had been predicted in 1923 by Adolf Gustav Smekal[51]. For his finding "*of extraordinary great importance for our knowledge of the structure of molecules*"[37], Raman received the Nobel Prize in Physics in 1930.

When light interacts with molecules, most of the incident photons are scattered from that matter with an identical wavelength (figure 7). This process is called Rayleigh scattering or elastic scattering. However, a very small portion of the light is scattered inelastically and will therefore have wavelength that differs from that of the incident light. In this last interaction, there will be an energy transfer between the incident photon and the molecules of that particular matter. Due to the resulting energy exchange, molecules will exhibit an altered vibrational level, hence the name 'vibrational spectroscopy'. In infrared absorption spectroscopy, the vibrational spectroscopic counterpart of Raman spectroscopy, a higher vibrational energy level is reached by absorption of certain frequencies of infrared light by a molecule (figure 8). As shown in figure 8, the Raman effect can be divided into Stokes and anti Stokes shifts. In case of a Stokes shift, a molecule is excited to a higher vibrational energy level and the energy of the scattered photon has decreased. Consequently, the scattered light has a larger wavelength.

Figure 8



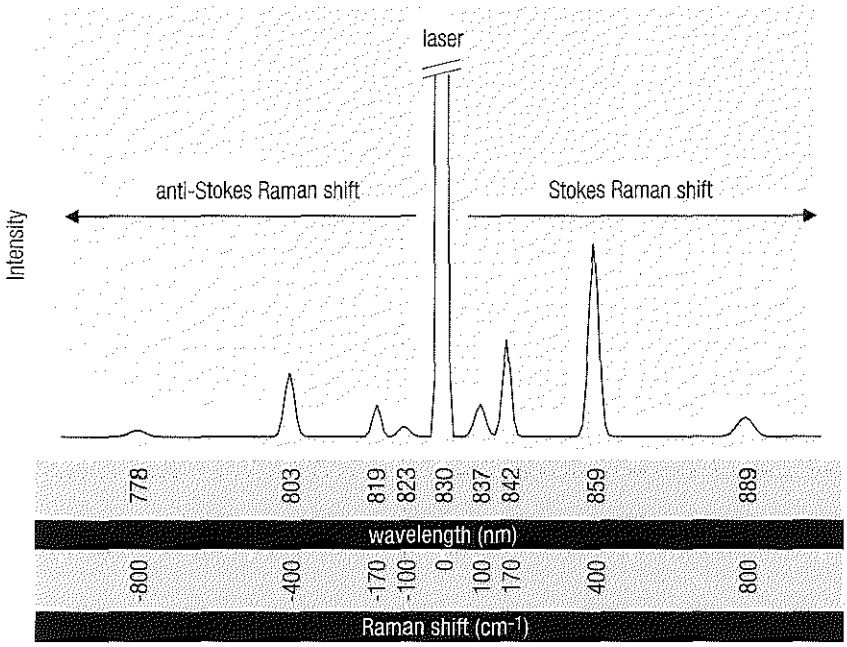
Energy diagrams of the processes referred to in this chapter. Rayleigh scattering: after the interaction with the photon, the molecule is in the same energy state as before. Stokes shifted Raman scattering: energy is transferred from the incident photon to the molecule, thereby promoting the molecule to higher vibrational energy level. Scattered light is detected at a higher wavelength than that of the incident light. Anti-Stokes shifted Raman scattering: energy is transferred from the molecule to the incident photon. The molecule ends up in a lower vibrational energy level, whereas the scattered photon has gained the same amount of energy. Scattered light is found at wavelengths smaller than that of the incident light. IR *absorption: infrared (*IR*) light with an energy corresponding to the energy needed to excite a molecular vibration is absorbed by the molecule.*

An anti-Stokes shift occurs when a molecule relaxes to a lower vibrational energy level when it passes part of its energy to the incident photon. This latter process, where the scattered light has a shorter wavelength than the incident light, is less efficient at room temperature. Stokes shifted Raman signals therefore are more intense and were exclusively used for the research described here.

In order to precisely monitor the changes in wavelength between incident and scattered light, monochromatic light (a single wavelength) from a laser is used to excite a Raman signal from a sample. Figure 9 is a schematic representation of a Raman spectrum, where the Stokes and anti-Stokes lines are symmetrically positioned around the Rayleigh scattered light from the laser. The x-axis corresponds to the wavelength of the different Raman peaks in a spectrum, and is normally given in relative wavenumbers or Raman shift units (i.e. the difference between scattered and incident light). Raman shifts are calculated according to the formula:

$$\Delta cm^{-1} = \left(\frac{1}{\lambda_0} - \frac{1}{\lambda_s} \right) * 10^{-2}$$

Figure 9



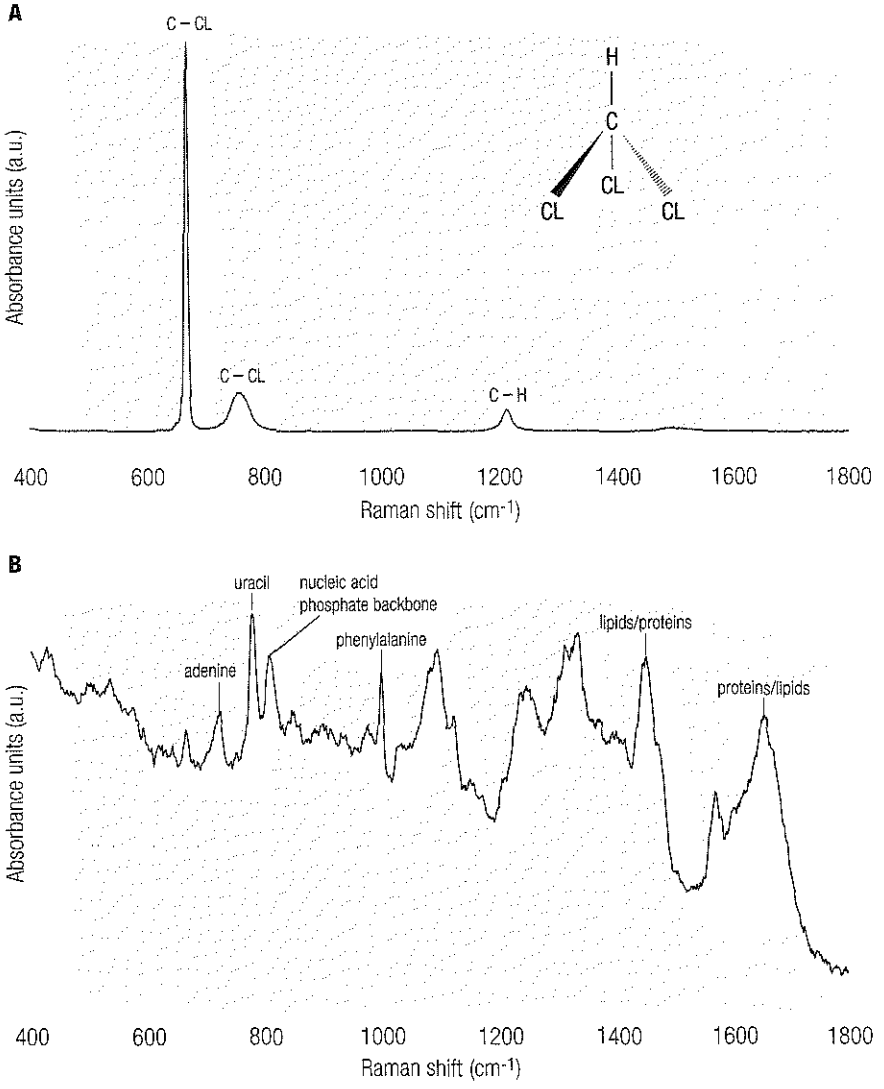
A theoretical Raman spectrum with the Stokes and anti-Stokes shifted Raman lines symmetrically distributed around the laser line (in this example 830 nm). Symmetry of the two types of Raman scattering is more clearly observed when expressing the wavelength changes in wavenumbers or Raman shifts (in cm^{-1}).

Where λ is in meters (m), λ_0 = excitation wavelength, λ_s = wavelength of scattered light.

The position of a line in the Raman spectrum corresponds to the energy that is required to excite a molecule to a certain vibrational energy level. A molecule can have several vibrational levels, the number of which are calculated by the formula $3N-6$, where N is the number of atoms in the molecule[59]. However, not all of these vibrations are Raman active. The Raman effect is caused by an oscillating induced dipole moment, which means that the molecular interaction with light is through the polarizability of the molecule. Those molecular vibrations that cause a change in the dipole moment are observed in infrared absorption spectroscopy.

Each peak in a Raman spectrum therefore corresponds to a usually well-defined molecular vibration and consequently, a Raman spectrum is highly specific for that particular molecule (figure 10a). A bacterial cell

Figure 10



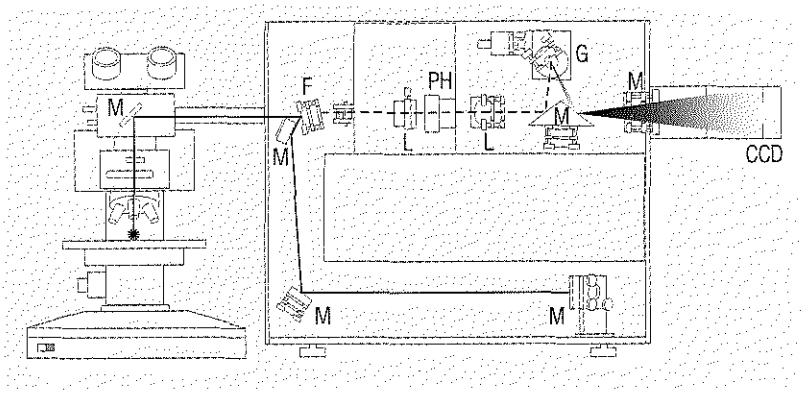
Examples of Raman spectra. (A) spectrum of chloroform, this small molecule produces a relatively simple Raman spectrum. The peaks in the spectrum can be attributed to specific vibrations within the molecule. (B) Raman spectrum of the bacterium Enterococcus faecium. Due to the complex molecular composition of the sample involved, a complex Raman spectrum is obtained. Based on the Raman spectra of purified compounds, spectral features can be assigned to specific molecular moieties in the bacterial cell. Some typical vibrations are indicated.

is composed of a multitude of complex biomolecules and therefore its Raman spectrum is very complex. However, based on the known Raman spectra of purified compounds, it is still possible to assign peaks in the bacteria spectrum to particular cellular constituents (figure 10B). A Raman spectrum of a bacterium is a representation of the overall molecular composition of a cell. The intensity of the features in a Raman spectrum is linearly dependent on the number of molecules in the measurement volume. With an increase of molecules in the measurement volume, the intensity of it's Raman lines also increase.

Instrumentation Nowadays, the instrumentation required for Raman measurements is relatively simple. High power monochromatic light is obtained from a laser. Since the intensity of Raman scattered light is much lower (by a factor of ~ 10^6) than that of Rayleigh scattered light, it is important to suppress the latter in order to prevent it from obscuring the Raman signal on the detector. In the spectrometer used for the experiments described here (figure 11), an optical filter was used for this purpose. The properties of this filters is such that shorter wavelengths, including the laser wavelength are minimally transmitted (<0.0001%), while longer wavelengths (Stokes shifted Raman light) are optimally (>99.9%) transmitted. Next, the different wavelengths in the Raman spectrum are dispersed on an optical grating and projected on a detector. A sensitive, infrared optimized charge-coupled device (CCD) camera served as a multichannel detector. The instrument used throughout the study presented here, consisted of a microscope attached to a spectrometer, the so-called microspectrometer. Via a microscope objective the laser light is focussed on the sample and the Raman scattered light is collected via the same objective. Applying a confocal signal collection geometry allows for very small measurement volumes to be used[39], figure 12 illustrates this approach. The sampling volume of the spectrometer was determined to be approximately cylindrical (1.5 μm in lateral direction and 7-8 μm along the optical axis of the microscope objective).

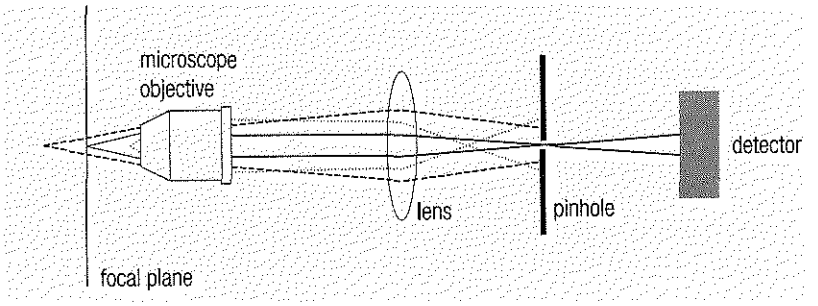
Applying confocal Raman microspectroscopy in (medical) microbiology A number of characterisitic feature of Raman spectroscopy make the technique particularly appealing for application in microbiology. The fact that no dyes, labels or other extraneous chemicals are required in order to obtain a Raman spectrum, means that microbes can be studied almost in their native state. Furthermore, with the small confocal measurement volume, Raman spectra can be collected from only a limited amount of microbial cells; even from a single cell[39, 49]. This means that the inoculum

Figure 11



Lay out of a confocal Raman microspectrometer. The laser light (solid line) is reflected by a series of mirrors (M) and an optical filter (F), through the microscope objective on the sample. The excited Raman signal (dashed line) is collected by the same microscope objective and passed through the optical filter, in order to filter out the remaining, more intense, laser light. Via a lens (L) the Raman signal is focussed on a pinhole (PH) for confocal detection. Through lenses (L) and a mirror (M) the different wavelengths of the Raman spectrum are separated on an optical grating (G) and projected on the CCD detector.

Figure 12



The confocal signal detection principle. Laser light is focussed on the sample, in the focal plane of a microscope objective. The light, collected by the same objective, is focussed by a lens on a pinhole, which only allows the lights from the focal plane to pass. Light from below (dashed line) and above (dotted line) the focal plane is blocked by the pinhole and does not reach the detector.

for measurements can be very small and culturing can be reduced to only several cell cycles.

The aim of the research described in this thesis was to develop confocal Raman microspectroscopy techniques for the rapid identification and characterisation of clinically relevant microorganisms. Chapter 2 describes a study in which the accuracy of the identification of *Enterococcus* spp., based on Raman spectroscopy, is exemplified by a comparison with identification results based on phenotypic and genotypic methods. This chapter clearly shows the high accuracy that can be obtained when applying vibrational spectroscopies for microbial identifications. Chapter 3 reports on the unique method that was developed to analyse microbial microcolonies, directly on the solid culture medium. With this approach it is possible to "record" Raman spectra from very young cultures, where the colonies are typically between 10 and 100 microns in diameter. Culturing times therefore can be decreased to several hours, allowing rapid identification schemes to be developed. Chapter 4 describes the possibilities of this technique to probe the heterogeneity of microbial colonies directly on the solid culture medium. It was shown that microbial colonies after 6 hours incubation were most homogeneous as compared to older (12 and 24 hours) colonies, and consequently these 6 hour old colonies are better suited for the composition of a database. From the results obtained in this study, standardized protocols were derived, based on which reproducible Raman spectra can be obtained. With the methodology for rapidly performing measurements now developed, and the culturing conditions optimized for obtaining reproducible Raman spectra, the potential of the technique to identify microbes by their Raman spectrum was evaluated in chapter 5. A collection of yeast strains from the genus *Candida* was used to arrive at the most appropriate manner of analysing large amounts of data. Based on a library of Raman spectra from known *Candida* strains, an identification method was developed to identify these strains based on their Raman spectrum. Finally, to test the methodology developed in all previous studies, a prospective study of clinical isolates is described in chapter 6. Parallel to the routine analysis of positive blood samples from patients in intensive care units and a random selection of other wards, Raman identification was performed of these samples. A summary of all results is provided in chapter 7.

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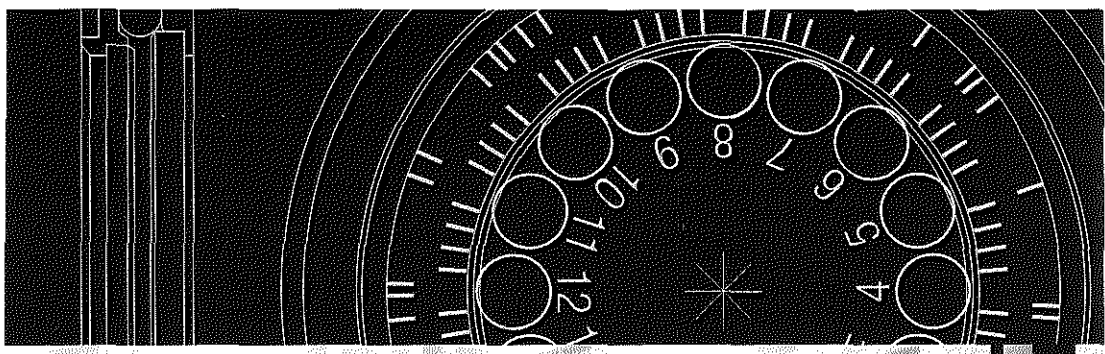
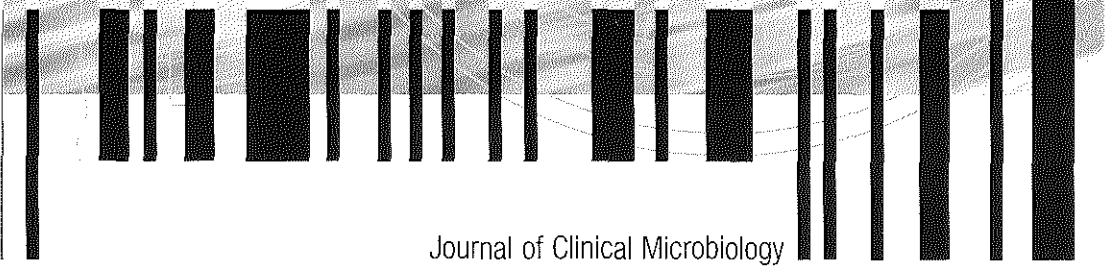


2. Classification and identification of enterococci: a comparative phenotypic, genotypic, and vibrational spectroscopic study



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2. Classification and identification of enterococci: a comparative phenotypic, genotypic, and vibrational spectroscopic study

Rapid and accurate identification of enterococci at the species level is an essential task in clinical microbiology since these organisms have emerged as one of the leading causes of nosocomial infections worldwide. Vibrational spectroscopic techniques (infrared [IR] and Raman) could provide potential alternatives to conventional typing methods, because they are fast, easy to perform, and economical. We present a comparative study using phenotypic, genotypic and vibrational spectroscopic techniques for typing a collection of 18 *Enterococcus* strains comprising 6 different species. Classification of the bacteria by Fourier transform (FT)-IR spectroscopy in combination with hierarchical cluster analysis revealed discrepancies for certain strains when compared with results obtained from automated phenotypic systems, such as API and MicroScan. Further diagnostic evaluation using genotypic methods - i.e., PCR of the species-specific ligase and glycopeptide resistance genes, which is limited to the identification of only four *Enterococcus* species and 16S RNA sequencing, the "gold standard" for identification of enterococci - confirmed the results obtained by the FT-IR classification. These results were later reproduced by three different laboratories, using confocal Raman microspectroscopy, FT-IR attenuated total reflection spectroscopy and FT-IR microspectroscopy, demonstrating the discriminative capacity and the reproducibility of the technique. It is concluded that vibrational spectroscopic techniques have great potential as routine methods in clinical microbiology.

Introduction

Enterococci are opportunistic human pathogens. The two most important species *E. faecalis* and *E. faecium*, which are considered part of the normal intestinal flora, are among the leading causes of nosocomial infections and may cause severe infections, including endocarditis and septicaemia[16]. These infections are often difficult to treat due to the increased antibiotic resistance associated with this organism[4, 27, 29]. Especially the recent increase of vancomycin resistant *E. faecium* (VRE) strains in clinical isolates is a cause of serious concern, because this glycopeptide-type antibiotic often remains the last treatment available in life-threatening infections[31]. The situation is further complicated by the fact that enterococci have developed a number of mechanisms for the transfer of resistance genes[1]. Therefore perhaps the greatest threat posed by VRE comes not from these organisms themselves, but from the potential that they could transfer their resistance genes to other more pathogenic gram-positive bacteria, thus creating a highly dangerous pathogen difficult to treat with currently available antibiotics[15]. Furthermore, recent studies have revealed that the incidence of more unusual species such as *E. durans*, *E. hirae*, *E. gallinarum* and *E. casseliflavus* has increased significantly in clinically isolated enterococci[32]. Overall, this has resulted in an increased need for rapid and accurate identification of enterococci at the species and subspecies level as a means of effectively assisting infection control and epidemiological studies.

For most clinical microbiological laboratories, their primary method of identifying *Enterococcus* strains, relies on phenotypic characterization. However, various studies have shown that an unequivocal species identification of enterococci by phenotypic means is a challenging procedure that can take several days to accomplish because of the phenotypic and biochemical similarities between many enterococci[5]. In addition, the automated systems currently in use, often fail to accurately identify rare species[3, 6, 11, 23, 26]. Molecular genetic techniques, such as randomly amplified polymorphic DNA analysis, intergenic ribosomal PCR, or other PCR based methods targeting various genes, have been successfully used to identify enterococci at the species level[6, 12, 13, 21, 22, 28]. Although these techniques are specific and sensitive, it is difficult to adapt them for use in routine laboratories due to their high costs and the requirement for highly skilled personnel. Infection control and epidemiological studies primarily require rapid and simple means of identifying and typing clinical isolates. As a consequence, a variety of approaches have been developed. The application of vibrational spectroscopic techniques (Fourier transform

infrared [FT-IR] and near-IR Raman spectroscopies) is such an approach which may provide a potential alternative to conventional methods. These techniques are rapid, because little biomass is needed, significantly reducing culturing time. Vibrational spectroscopies are also easy to use and may become very cost-effective, because they enable considerable reduction in sample handling and use of reagents and do not require highly skilled personnel. These methods allow the discrimination of intact microbial cells without their destruction and produce complex biochemical fingerprint-like spectra which are reproducible and distinct for different microorganisms. IR and Raman spectroscopy measure molecular vibrations on the basis of the absorption (IR) or scattering (Raman) of IR or near-IR radiation interacting with a sample. The observed microbial IR or Raman spectra are a complex composition of many different vibrational modes of all the cell components, i.e. DNA, RNA, proteins, and membrane and cell wall components. The applicability of FT-IR spectroscopy in the field of microbiology has already been persuasively demonstrated[2, 8-10, 17-19, 25]. Various studies have shown that vibrational spectroscopy provides sufficient resolution power to distinguish microbial cells at different taxonomic levels, even at the strain level[10]. Raman spectroscopy of microorganisms is a relatively new and promising approach, since recent studies revealed, that it is possible to discriminate among various microorganisms at the species level based on Raman spectra of 6 hour old microcolonies[14]. The two vibrational spectroscopic techniques provide complementary information. The combined use of IR and Raman spectroscopy could therefore offer a more complete approach for analyzing intact bacterial cells.

The purpose of this study was to evaluate the discriminatory power of vibrational spectroscopic techniques for accurately typing enterococci at the species level in direct comparison with phenotypic and genotypic methods.

Materials and Methods

Strains and growth conditions A collection of 18 *Enterococcus* strains was used in this study. Strains were either food isolates, clinical isolates, or from the collection of the Pasteur Institute (CIP, Paris, France) as summarized in Table 1. The strains were stored in cryovials containing a cryopreservative (MICROBANK [MAST DIAGNOSTICA, Reinfeld, Germany]) at −70°C until use. Strains were streaked onto agar plates using a three-quadrant streak pattern. All strains were subcultured on casein peptone-soy meal peptone (CASO) agar plates (MERCK, Darmstadt, Germany) for 24 hours. The growth temperature was 37° ± 2°C.

Table 1

***Enterococcus* strains used in this study**[a]

No.	API identification	Specimen source	Ward[c]
1	*E. faecium*	urine	MED
2	*E. hirae*[b]	urine	MED
3	*E. hirae*[b]	hemoc	ICU
4	*E. faecalis*	urine	MED
5	*E. faecium*	urine	SUR
6	*E. durans*[b]	food	IDC
8	*E. durans*[b]	food	IDC
9	*E. faecalis*	carriage	PED
10	*E. faecalis*	food	IDC
11	*E. faecalis*	CIP	–
12	*E. faecium*	CIP	–
13	*E. faecalis*	CIP	–
14	*E. gallinarum*	carriage	ELD
15	*E. faecium*	carriage	ELD
16	*E. casseliflavus*	carriage	ELD
17	*E. hirae*[b]	carriage	MED
18	*E. faecalis*	carriage	PED
19	*E. faecium*	food	IDC

[A] *Strains were isolated from different sources, such as food, clinical isolates, or strain collections.* [B] *Strain was reidentified.* [C] *Abbreviations:* MED, *medical;* ICU, *intensive care unit;* SUR, *surgery;* PED, *pediatric;* ELD, *elderly.*

Sample preparation For the IR absorbance measurements, bacterial cells from 24 hour old cultures were harvested and prepared as described earlier [9, 10, 17]. Briefly, small amounts of late exponential-phase-cells (~10-60 µg [dry weight]) were carefully removed with a platinum loop from regions of confluent colony growth in the third quadrant of the culture plate and suspended in 80 µl distilled water. An aliquot (35 µl) of the suspension was transferred to a ZnSe (zinc selenide) optical plate in a multi-sampling cuvette and dried in a desiccator over a drying agent (P_4O_{10}, [Sicapent]; MERCK) with the application of a moderate vacuum (2.5 to 7.5 kPa) to form a transparent film suitable for FT-IR measurements. Prior to spectral measurements, the sample holder was sealed with a KBr cover plate to control the humidity and to prevent the instrument from contamination.

For Raman studies, a loopful of biomass from 24 hour cultures was transferred onto CaF_2 substrate. From each strain, duplicate smears were made. The smears on CaF_2 substrate were dried in a desiccator over drying beads for at least 15 min, prior to Raman measurements.

For the IR attenuated total reflectance (ATR) measurements, cells from 18 hour old cultures were carefully harvested from the solid agar plate and homogeneously spread over the whole ATR crystal surface. The substrate used for ATR measurements was a ZnSe crystal (50 by 10 by 1.5 mm; Specac, Orpington, United Kingdom) with a refractive index of 2.4 and an incidence angle of 45°, yielding a total of six internal reflections at the sample.

For the IR microspectroscopic studies, the bacterial cultures were incubated for 8 to 10 hours and produced colonies of approximately 100 to 250 µm in diameter. The microcolonies were transferred manually from the agar plate to an IR transparent ZnSe optical plate by gently pressing the plate onto the agar surface. Imprints were allowed to air dry prior to spectral measurement. Spectra were acquired from the dried microcolony imprints on this substrate.

Recording of spectra and data evaluation:

FT-IR *spectroscopy*

Spectra were recorded between the region 500 and 4000 cm^{-1} on an IFS 28/B spectrometer (BRUKER OPTICS, Karlsruhe, Germany) specially designed for the measurement of microorganisms and equipped with a deuterated triglycerine sulfate (DTGS) detector. For each FT-IR spectrum, 64 scans were co-added and averaged. Fourier transformation was done using a Blackmann-Harris 3 term apodization function, and a zerofilling factor of 4 to give a nominal resolution of 6 cm^{-1}. The spectrometer was continuously purged by dry air to reduce contributions from water vapor and CO_2.

Evaluation of IR spectral data (calculation of derivatives, normalization, etc.) was performed using the OPUS software (version 3.0; Bruker). First and second derivatives of the original IR spectra were calculated using a 9-point Savitzky-Golay filter to enhance the resolution of superimposed bands and to minimize problems from unavoidable baseline shifts. Multivariate statistical analysis was carried out using the cluster analysis module of OPUS (version 3.0). To compare spectra of the six different species, cluster analyses using the first derivatives of the original spectra as input were carried out for different wavenumber regions. Spectral distances, providing a measure of the similarity of the spectra, calculated from Pearson's correlation coefficient and Ward's algorithm, were used for hierarchical clustering analysis.

Raman spectroscopy

Raman measurements were performed as described earlier[14], using a confocal Raman microspectrometer. Briefly, bacteria smears were placed under a microscope objective and excited with 100 mW laser power (830 nm). At random locations in each smear, 10 spectra, each with a 30-s signal integration time, were collected. The 10 spectra thus obtained were averaged before being used in further analysis.

Evaluation of Raman spectra was accomplished as already described for the IR data. First derivative spectra consisting of the spectral region 400 to 1800 cm^{-1} were used. Cluster analysis was also performed, considering four spectral regions (400 to 980, 1020 to 1140, 1190 to 1500 and 1550 to 1800 cm^{-1}) in order to exclude the intensive spectral features that are caused by the carotenoids of the pigmented *E. casseliflavus* strain 16 and *E. hirae* strain 6.

FT-IR *attenuated total reflectance (ATR) spectroscopy*

Spectra were recorded using a Bomem MB-100 (Vannier, Quebec, Canada) FT-IR spectrometer equipped with a KBr beamsplitter and a DTGS detector. One-hundred interferograms were averaged per spectrum, at a resolution of 4 cm^{-1}. For each strain, 10 spectra were recorded and averaged.

FT-IR *microspectrometry*

FT-IR absorption spectra were collected using a UMA 500 infrared microscope coupled to a FTS-40A spectrometer (Spectroscopy Division, Bio-Rad, Cambridge, Mass.) equipped with a mercury cadmium telluride narrowband detector. A microscope diaphragm size of 80 by 80 μm was used for spectral data acquisition. Measurements were performed in transmission mode using the following parameters: 4 cm^{-1} resolution, 5-kHz scan speed, 32 to 64 scans of coaddition, triangular apodization, and spectral range of 800 to 4000 cm^{-1}. No baseline correction or smoothing was

applied to the data. First derivative spectra were subjected to cluster analysis using Matlab's Statistics Toolbox (The Math Works, Inc., Natick, Mass.) employing Ward's algorithm and Euclidean distance measure.

Phenotypic and genotypic methods:

Phenotypic methods:

API AND MICROSCAN

Phenotypic identification of all strains was performed using the automated API (bioMérieux, Marcy l'Etoile, France) and the MICROSCAN (Dade International, MICROSCAN Inc., West Sacramento, Calif.) systems.

Genotypic methods:

PCR

PCR analyses of species specific ligase genes (ddl) and related glycopeptide enzymes were performed according to Dukta-Malen et al[7] for all *Enterococcus* strains investigated in this study.

16S RNA SEQUENCING

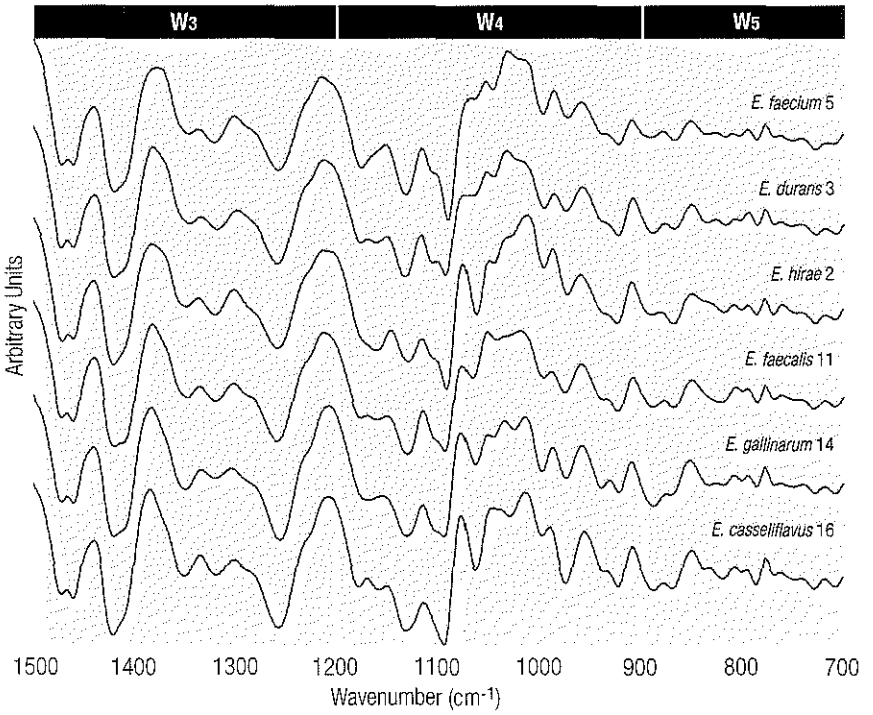
16S RNA sequencing was performed for the 5 equivocally typed strains, i.e., strains 2, 3, 6, 8 and 17, as well as for strains 10, 15, and 19. 16S RNA sequencing was performed using the MicroSeq™ 500 sequencing kit (Perkin-Elmer). The sequence data were analyzed with a Genetic ABI PRISM 310 Sequencer (Perkin-Elmer).

Results and Discussion

Phenotypic identification by the API test system Conventional identification was performed for all 18 strains used for vibrational spectroscopic analyses. Of the 18 isolates studied, five were identified as *E. faecium* (strains 1, 5, 12, 15, and 19) and six as *E. faecalis* (strains 4, 9, 10, 11, 13, and 18). Three isolates were identified as *E. hirae* (strains 2, 3, and 17) and two were identified as *E. durans* (strain 6, and 8). Of the remaining two isolates one was identified as *E. gallinarum* (strain 14) and one as *E. casseliflavus* (strain 16) (Table 1).

Comparison between phenotypic identification and FT-IR analysis Seven repetitive measurements over a time period of 6 months from independent sample preparations of all strains were performed, resulting in 126 spectra. The repetitive measurements, performed to judge the reproducibility of the IR technique, yielded strain specific subcluster (data not shown for brevity), indicating that this method is highly reproducible and specific at the strain level. A representative dataset consisting of three repetitive measurements including all strains was subjected to multivariate statistical analysis to explore the discriminative potential of the spectral information for this taxonomic task. Typical first derivative IR spectra of the 6 different species plotted for the spectral ranges used to calculate spectral distances are displayed in figure 1. This figure reveals the spectral differences responsible for the separation of the 6 *Enterococcus* species, which, however, are not yet interpretable in terms of biochemical and/or chemical structures. Hierarchical clustering based on spectral information contained in three different spectral ranges – 1200 to 900 cm^{-1}, the polysaccharide region; 900 to 700 cm^{-1}, the fingerprint region and 1500 to 1200 cm^{-1}, the mixed region - was used as classfication method, resulting in the dendrogram displayed in figure 2. A clear discrimination could be observed and 6 distinct clusters were produced. However, the grouping of some strains was in contradiction to the routine phenotypic classification. Most noticeable is the separation between the two major enterococcal species into two clusters with all but one *E. faecium* strain (strain 15) in one group and all *E. faecalis* strains (strains 4, 9, 10, 11, 13, and 18) in the other group. Upon closer inspection the inconsistencies with the phenotypic identification become apparent. The dendrogram suggests a close relatedness between the strains 2 and 6 and also between the strains 3 and 8, which is in contradiction to the phenotype-based identification results. Accordingly, the findings

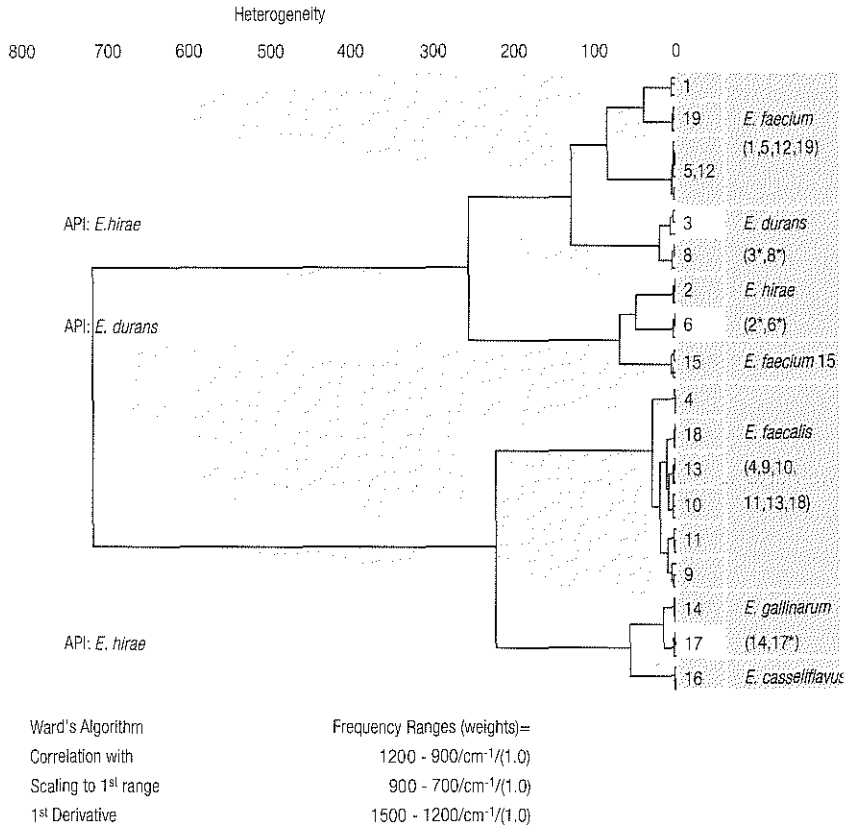
Figure 1



Typical first derivative IR *spectra of the six different Enterococcus species depicted in the most discriminatory spectral windows. The spectral windows are defined according to Helm et al*[10] *as follows: W_3, the window between* 1500 *and* 1200 *cm^{-1} (the mixed region), a spectral region containing information from proteins, fatty acids, and phosphate-carrying compounds; W_4, the window between* 1200 *and* 900 *cm^{-1} (the polysaccharide region), a spectral region dominated by the fingerprint-like absorption bands of the carbohydrates present within the cell wall; W_5, the window between* 900 *and* 700 *cm^{-1} (the true fingerprint region), showing some remarkably specific spectral patterns, which are as yet unassigned to cellular components or to functional groups.*

indicate either that the FT-IR classification is not useful for the differentiation of enterococci or that the four strains have not been typed accurately by the conventional methods. Further discrepancies between FT-IR and conventional methods were also observed for strain 17, which was typed as *E. hirae* by the API method but clustered together with *E. gallinarum* (strain 14) in the FT-IR analysis. From these findings we hypothe-

Figure 2



Classification scheme based on the FT-IR *spectra of six different Enterococcus species. Cluster analysis of three repetitive measurements was performed using the first derivatives of the spectra, considering the spectral ranges between* 1200 *to* 900, 900 *to* 700, *and* 1500 *to* 1200 cm^{-1}. *All spectral ranges were equally weighted. Ward's algorithm was applied. The strains marked with an asterisk were not in accordance with the phenotypic identification by the* API *system. The highlights mark the identity of certain strains by the* API *system.*

sized that the identification of rare *Enterococcus* species by conventional identification systems like the API system might not be reliable, as described before[11,27]. In fact, studies evaluating the commonly used commercially available bacterial identification systems have repeatedly encountered problems associated with enterococcal species identification[23,27]. Error rates for enterococcal species identification of 2 to 21% for

E. faecalis, 5 to 9% for *E. faecium*, and 14 to 79% for other species have been found for these systems[32]. To give an example, Singer and coworkers reported a high percentage of misidentifications for the analysis of isolates from a VRE outbreak in a hospital after the introduction of an automated identification system software update (Vitek gram-positive identification card)[24]. He concluded from the study that "automated microbial analysis is a potential source of error that is not easily recognized"[24].

Species identification by Microscan, PCR, and 16S RNA sequencing To clarify the discrepancy between FT-IR spectroscopic and phenotypic classifications, further diagnostic evaluation, using another phenotypic based automated test (namely, MICROSCAN) and a species specific PCR approach, were undertaken for all 18 strains. Only the identification results of the 5 equivocally typed strains (strains 2, 3, 6, 8, and 17) are summarized in Table 2. All the other strains yielded uniform identification results by the various methods used. As for the first automated test system (API), the MICROSCAN system also failed in identifying these obviously rare species unequivocally. All the analyzed strains were identified as *E. durans* producing results in contradiction to both the API and the FT-IR classification. Subsequently, PCR analyses of the species specific genes encoding for the D-alanine:D-alanine (D-Ala:D-Ala) ligases and related glycopeptide resistance enzymes was performed for all 18 strains. This technique, although reliable, identifies only four species, i.e. *E. faecium*, *E. faecalis*, *E. gallinarum*, and *E. casseliflavus*, leaving the other *Enterococcus* species unidentified[7]. Therefore only 14 of the 18 strains investigated could be iden-

Table 2

Identification of five equivocally typed strains based on phenotypic, genotypic, and vibrational spectroscopic data.

Strain			Identification by:		
no.	API	FT-IR and Raman Clustering[a]	MICOSCAN	PCR	16S RNA Sequencing
2	*E. hirae*	6	*E. durans*	–[b]	*E. hirae*
3	*E. hirae*	8	*E. durans*	–	*E. durans*
6	*E. durans*	2	*E. durans*	–	*E. hirae*
8	*E. durans*	3	*E. durans*	–	*E. durans*
17	*E. hirae*	14 (*E. gallinarum*)	*E. durans*	*E. gallinarum*	*E. gallinarum*

[A] *Numbers indicate with which strain the indicated strain clustered.* [B] –, *not identifiable by* PCR.

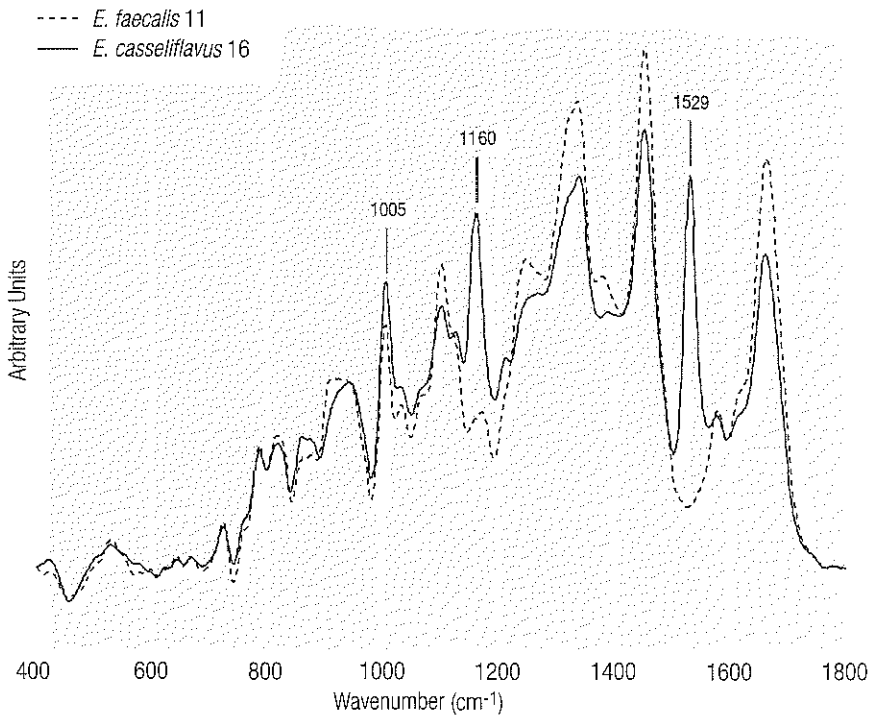
tified. The analysis produced an outcome in good agreement with the FT-IR classification results. Particularly interesting is the identification of one of the five equivocally typed strains (strain 17) as *E. gallinarum* by the PCR approach in accordance with the FT-IR analysis (strain 17 is grouped in one cluster with strain 14, an *E. gallinarum* species) (figure 2).

For the five *Enterococcus* species whose phenotypic and genotypic identification differed from the FT-IR identification, 16S RNA sequencing was performed to assess the accuracy of all methods involved. The 16S RNA sequencing is considered to be the "gold standard" for microbial identification. It is particularly encouraging that of all methods used in this study only the FT-IR result was in accordance with the identification by the gold standard (Table 1). Specifically, the 16S RNA sequencing identified strains 2 and 6 as *E. hirae* and strains 3 and 8 as *E. durans*, providing strong support for the FT-IR classification, which yielded two distinct clusters for these species. Furthermore, strain 17, originally classified as *E. hirae*, was determined to be *E. gallinarum* again confirming the FT-IR analysis and the PCR result. Therefore, these findings demonstrate the superior discrimination ability of the FT-IR technique on the one hand and on the other hand demonstrate the weakness of the API and the Microscan systems.

Classification by Raman spectroscopy

The classification results shown in figure 2 could be reproduced by three other laboratories using confocal near-IR-Raman microspectroscopy, FT-IR ATR spectroscopy and FT-IR microspectroscopy. Raman spectroscopy produced correct species differentiation for all but one strain (*E. hirae* strain 6) when the analysis was performed considering the whole spectral range from 400 to 1800 cm^{-1} (data not shown). Interestingly, this Raman dendrogram suggested in contrast to the FT-IR dendrogram, that the *E. casseliflavus* strain shows only little similarity to the other strains. This finding can be attributed to "additional" peaks that occur at 1005, 1160, and 1529 cm^{-1} in the Raman spectrum of the strain as shown in figure 3. Specific bacterial constituents such as pigments give rise to these additional Raman signals which gain considerable intensity due to a pre-resonance effect. According to the diagnostic bands near 1160 cm^{-1} [ν(C—C)] and 1529 cm^{-1} [ν(C=C)] (ν, stretching vibrations), the yellow to orange pigment compound of *E. casseliflavus* strain 16 can be assigned to a carotenoid structure[30]. Upon closer spectral inspection, the different clustering of the *E. hirae* strain 6 by the Raman approach could be ascribed to the fact that this strain expresses low levels of carotenoid as well. On the basis of these findings we performed a cluster analysis of the Raman spectra, excluding the carotenoid specific regions. This could be achieved by using the spectral information encoded in four spectral regions (400 to

Figure 3

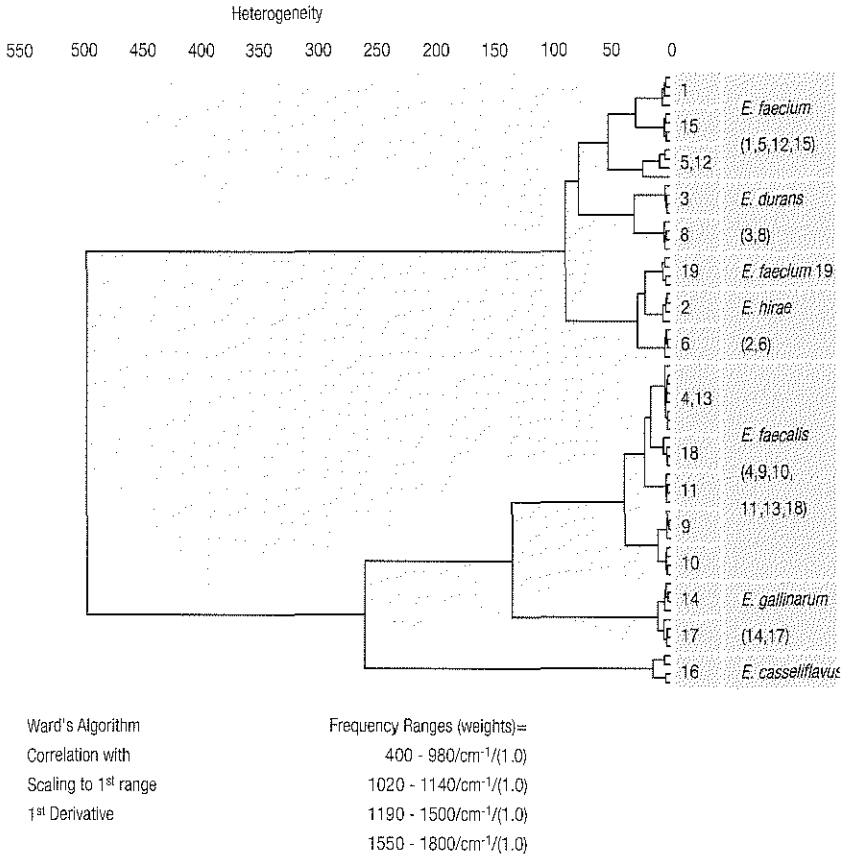


Raman spectra of E. casseliflavus strain 16 and E. faecalis strain 11 are depicted for spectral comparison. The Raman spectrum of the pigmented E. casseliflavus exhibits pre-resonance enhanced bands of carotenoids. Three characteristic bands near 1005, 1160, and 1529 cm^{-1} are clearly visible.

980, 1020 to 1140, 1190 to 1500, and 1550 to 1800 cm^{-1}) as input data. The dendrogram (figure 4) obtained on the basis of this wavelength selection is comparable to the FT-IR dendrogram. It was also very encouraging that replicate cultures of all strains turned out to be in strain specific subclusters illustrating the excellent reproducibility and strain specificity of the Raman technique as was found with the infrared measurements.

However, both spectroscopic techniques did not clearly classify all five *E. faecium* species. A closer inspection of the *E. faecium* species group[20] shown in figure 2 demonstrates that the *E. faecium* strains cluster in two groups consisting of all but one strain (strain 15) for the IR data. This strain seems to be more closely related to the *E. hirae* strains (strains 2 and 6) than to the other *E. faecium* strains. Similarly for the clustering of the

Figure 4



Classification scheme based on the Raman spectra of six different Enterococcus species. Cluster analysis of four repetitive measurements was performed using the first derivatives of the spectra, considering the spectral ranges between 400 to 980, 1020 to 1140, 1190 to 1500, and 1500 to 1800 cm^{-1}, with the aim to exclude the spectral features that are caused by the carotenoid pigmentation of E. casseliflavus strain 16 and E. hirae strain 6. All spectral ranges were equally weighted. Ward's algorithm was applied.

Raman data, again the *E. faecium* strains cluster into two groups, however in this case, it is strain 19 that clusters apart. To further investigate the findings, isolates 15 and 19 were retyped by 16S RNA sequencing. The sequencing revealed high sequence identities for *E. faecium*, *E. hirae* and *E. durans*, indicating a high relatedness within this group.

In conclusion we have shown the potential usefulness of vibrational spectroscopic techniques in the differentiation of enterococci from various sources, including isolates from food, patient material, and strain collections. The results of our study reflect the high discriminatory power of the IR and the Raman technique that allows accurate differentiation of closely related bacterial species such as enterococci. Comparison of FT-IR and Raman clustering showed that there was considerable consistency between both methods, since very similar classification schemes were obtained. This is most encouraging considering that IR and Raman "see" the total cell composition and structure on the basis of different molecular vibrational modes. In addition, both spectroscopic techniques proved to be capable of discriminating accurately at the strain level, which opens the door for using these physicochemical techniques as tools for epidemiological studies. In comparison to conventional automated identification systems which have been confirmed to be only reliable for the more common clinical isolates such as *E. faecalis* and *E. faecium*, the FT-IR and Raman methods proved to be also applicable for less frequently encountered *Enterococcus* species, for instance *E. hirae* and *E. durans*. Moreover our study indicates that vibrational spectroscopic techniques might not only be superior to conventional phenotypic methods but also turned out to be more broadly applicable than genotypic identification by means of one or even a few very specific PCR analyses which are limited to the identification of only four *Enterococcus* species. Finally, the species differentiation based on the spectroscopic data is consistent only with the analysis results obtained by the 16S RNA sequencing. Though regarded as the "gold standard", 16S RNA sequencing is not appropriate for routine analysis due to its complexity and high costs. Because of this vibrational spectroscopy is not only advantageous as a tool for taxonomic studies but also proves to be very rapid and reliable as a potential routine classification method.

Acknowledgments

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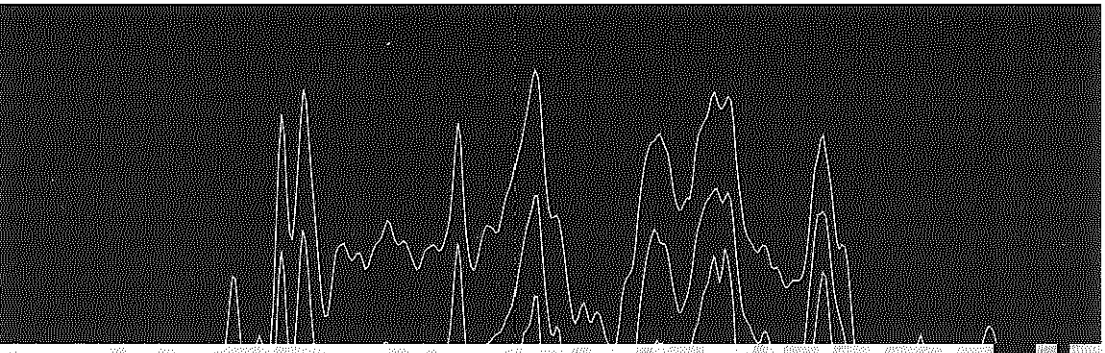

3. Raman spectroscopic method for identification of clinically relevant microorganisms growing on solid culture medium

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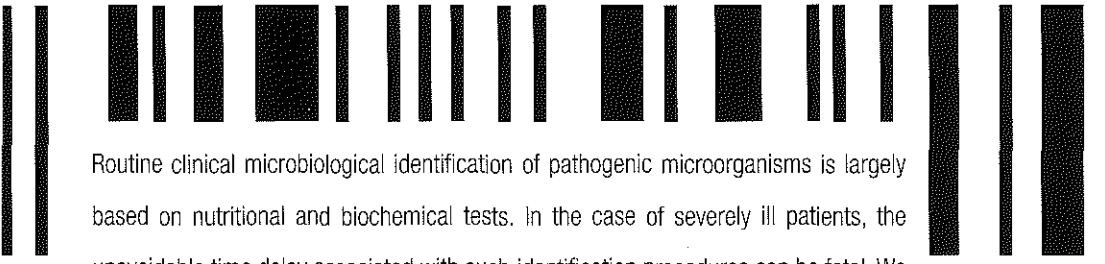


Routine clinical microbiological identification of pathogenic microorganisms is largely based on nutritional and biochemical tests. In the case of severely ill patients, the unavoidable time delay associated with such identification procedures can be fatal. We present a novel identification method based on confocal Raman microspectroscopy. With this approach it is possible to obtain Raman spectra directly from microbial microcolonies on the solid culture medium, which have developed after only 6 hours of culturing for the most commonly encountered organisms. Due to the limited thickness of microcolonies, some of the underlying culture medium is sampled together with the bacteria. Spectra measured at different depths in a microcolony contain different amounts of the medium signal. A mathematical routine, involving vector algebra, is described for the non-subjective correction of spectra for variable signal contributions of the medium. To illustrate the possibilities of our approach for the identification of microorganisms, Raman spectra were collected from 6 hour microcolonies of 5 bacterial strains on solid culture medium. The classification results show that confocal Raman microspectroscopy has much potential as a powerful new tool in clinical diagnostic microbiology.

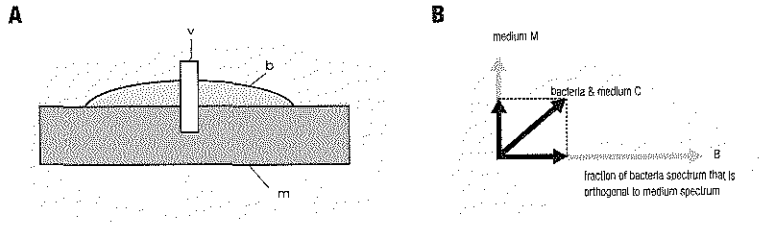
Introduction

Routine microbiological identification of clinical samples is largely based on nutritional and biochemical characteristics of microorganisms. Following receipt of patient material, microbes are usually first cultured for 16 to 24 hours on solid culture medium. A biomass of 10^6 to 10^8 cells is then used for biochemical assays and after or in parallel with the identification assays, an antibiotic susceptibility profile of the microorganism is generated. Consequently, it is usually not until 2 to 3 days later that the clinician is presented with the full results of this labor-intensive procedure. In critical life-threatening infections, such a delay can be fatal for the patient. Therefore, common clinical practice is to start broad-spectrum empiric antimicrobial therapy based on experience with similar cases, before the test results from the microbiology laboratory are known. Early identification of a causative microorganism will enable the clinician to modify and target the initial therapy to the microorganism that causes the specific infection. This practice of streamlining and drug targeting is important to optimize the efficiency of antimicrobial therapy, to reduce the development of drug resistance and other undesired side effects on the microflora. Furthermore, possible toxic effects of broad-spectrum empiric therapy, which sometimes includes a combination of two different antimicrobial agents, can be reduced.

Molecular biological techniques are now being evaluated and used as methods for the identification of microorganisms and the detection of specific antibiotic resistance genes[9, 30, 37-39]. Although these techniques are potentially rapid, they are relatively expensive and require highly skilled personnel. Problems with false-positive reactions due to DNA contamination and false-negative reactions due to inhibitors introduced while preparing or collecting a sample, are complicating factors in DNA amplification-based molecular diagnostics[14, 21, 31, 41]. At present, molecular diagnostics are usually second lines of investigation and are seldom the sole basis for microbial identification. Therefore, there is a need for new techniques that can rapidly identify pathogenic microorganisms and provide information on drug susceptibility.

An alternative approach in microbial characterization is the use of spectroscopic methods. Pyrolysis mass spectrometry has been evaluated as a method for bacterial characterization,[3, 12, 16, 17, 40] however, thus far the high instrument costs have hindered the widespread use of this method[6]. The use of Fourier transform infrared (FT-IR) spectroscopy for microbial identification and characterization is gaining acceptance since Naumann and co-workers published their pioneering work in this field[19].

Figure 1



[A] *Diagrammatic representation of the measurement volume (v) while sampling a bacterial microcolony (b) on a solid culture medium (m).* [B] *Vector diagram of the background subtraction routine depicting the orthogonal vectors of the medium* (M) *and bacteria* (B) *and the vector of the combined bacteria and medium signal* (C)*, see text for details.*

[20,27]. Manfait et al.[5,36,44] have used FT-IR spectroscopy to identify drug resistance in bacteria, indicating the high information content of this technique.

The application of Raman spectroscopy in microbiology has also been explored previously. Studies have been reported in which FT- Raman[13,42] or ultraviolet (UV) resonance Raman[7,15,26] spectroscopy were used to study suspensions and dried films of microorganisms as well as hydrated microbial smears taken from a solid culture medium[23]. Using UV resonance Raman spectroscopy to study bacterial suspensions and bacterial cell constituents, Nelson and Sperry were able to identify microorganisms based on their Raman spectra[29]. They also reported measurements with only very small numbers of cells (1 to 50)[8,11]. However, the application of UV-resonance Raman spectroscopy requires the cells to be suspended in liquid medium in order to avoid damage due to heating[28] and photochemical effects as a result of the strong absorption of UV radiation by nucleic acids and proteins. For the clinical application of Raman spectroscopy targeted at rapid identification of pathogenic microorganisms, this is not a practical solution.

For our studies, we have chosen to use near-infrared- (NIR) multichannel confocal Raman microspectroscopy. The use of NIR laser light minimizes the excitation of sample autofluorescence, which tends to mask the much weaker Raman signal when using visible light excitation. Moreover, the use of a confocal signal detection scheme enables Raman spectroscopic measurements of very small sample volumes (even down to about 1 μm^3)[33,35]. Bacteria are therefore required to be cultured only until microcolonies are formed; microcolonies here being defined as

colonies that develop in 6 hours of growth after plating and having average colony diameters of 10-110 μm (depending on the type of microorganism). Raman spectra can be directly acquired from the microcolonies on solid culture media. When performing measurements on microorganisms still growing on the solid culture medium, there are minimal sample preparation steps prior to spectral acquisition. Since spectra with good signal-to-noise ratios can be obtained of microcolonies within a few minutes of signal collection time, this Raman spectroscopic approach offers the potential for rapid identification of microorganisms. However, a major obstacle of this approach is the presence of signal contributions from the underlying culture medium in the bacteria Raman spectrum due to the limited thickness of the microcolonies (figure 1A). Because the medium signal contribution is neither negligible nor constant, it will interfere with strain identification. We have developed a new approach to deal with this problem. The method does not aim to subtract the exact amount of signal that is contributed by the culture medium. Instead, we subtract all signal contained in the combined microorganism and culture medium spectrum that is indistinguishable from the culture medium spectrum.

In this paper, the methodological aspects of obtaining and analyzing Raman spectra of microorganisms directly on solid culture medium are discussed. In so doing, confocal Raman microspectroscopy can be developed for the rapid, routine identification and characterization of microorganisms.

Materials & Methods

Sample preparations The various bacterial strains used in the studies were derived either from the American Type Culture Collection (ATCC) or from the collection of the Department of Medical Microbiology and Infectious Diseases from the University Hospital Rotterdam (UHR and BM labeled strains). Strains were stored at −80°C in a brain heart infusion broth (Becton Dickinson, Franklin Lakes, NJ) containing 10% glycerol, until use. Following an overnight passage (37°C) on Mueller Hinton (MH) medium (Merck, Darmstadt, Germany), the strains were re-cultured on MH medium for 6 hours at 37°C prior to Raman measurement of microcolonies.

For studies involving bacterial smears on CaF_2 substrate, a second overnight (16 hours) culturing step was performed on MH medium. From these overnight colonies, a biomass from several well-isolated colonies was picked up using an inoculating loop and smeared onto a CaF_2 substrate. The samples were allowed to dry in air prior to the Raman measurements.

Ribonucleic acid (RNA) from baker's yeast (Sigma, St. Louis, MO) was dissolved in water to a concentration of 80 mg/ml prior to measurement.

Raman measurements Raman spectra were acquired using a Renishaw System 1000 Raman microspectrometer (Renishaw plc, Gloucestershire, UK). The accompanying Leica DM-LM microscope was fitted with an 80x near-infrared objective (MIR Plan 80x/0.75, Olympus). The spatial resolution of the setup was determined to be approximately 1.5 μm in the lateral direction and 7-8 μm along the optical axis. This depth resolution is to a larger degree dictated by the entrance slit width of the spectrometer. The spectrometer was equipped with a 300 lines/mm grating. Raman signal was collected in the spectral interval from 250 cm^{-1} to 2150 cm^{-1}, with a spectral resolution of 8 cm^{-1}. Raman measurements were performed using 830 nm excitation from a titanium sapphire laser (model 3900, Spectra Physics, Mountain View, CA) pumped by an argon-ion laser (series 2000, Spectra Physics), delivering 100 mW of laser power on the sample.

The constant background signal contribution originating from optical elements in the laser light delivery pathway was subtracted from all spectra. The reference spectrum of a tungsten band lamp of known temperature was used to correct for the wavelength dependent signal detection efficiency of the Raman setup[34-43].

Correction for background medium signal contribution A non-subjective method was developed to subtract Raman signal contributions of the culture medium from spectra obtained from bacterial microcolonies growing on the culture medium. This procedure involves the use of vector algebra. In mathematical terms, a Raman spectrum of bacteria on culture medium consisting of n data points can be thought of as a vector in a n-dimensional space. Similarly, the spectrum of the culture medium alone can be thought of as another vector in this n-dimensional space. The combined bacteria and medium vector can now be decomposed into a vector parallel to and a vector orthogonal to the medium vector (M) (figure 1B). (Throughout the text, uppercase boldface type is used to denote vectors). The projection of the "combined signal vector" (C) of bacterial and medium signal on M (proj_M) gives the amount of signal in C, that cannot be distinguished from M. Subsequent subtraction of this projection from C results in the desired non-medium related bacteria spectrum (B), (i.e. the vector component of C orthogonal to M, see equation 1). When equation 1 is elaborated in terms of the dot product of C and M, we obtain equation 2. A similar approach for spectral subtraction was described earlier by Berger et al.[4] for the subtraction of a pure component spectrum from the spectrum of a mixture, containing that component, as a first step in a linear multivariate calibration algorithm.

$$\mathbf{B} = \mathbf{C} - \text{proj}_{\mathbf{M}}\mathbf{C} \qquad (1)$$

$$\mathbf{B} = \mathbf{C} - \frac{\mathbf{C} \cdot \mathbf{M}}{\|\mathbf{M}\|^2}\,\mathbf{M} \qquad (2)$$

In the case of measurements on fully hydrated microcolonies, a second variable that can interfere with microorganism identification is the water concentration in the measuring volume. Since fluctuations in ambient temperature and humidity levels can influence the water content of the culture medium, and hence the water signal contribution in the Raman spectra, it is necessary to eliminate this variable as well. The vector correction routine we have developed therefore involves a "double correction" approach. This procedure starts with independently correcting both the combination spectrum (i.e. the spectrum of bacteria and medium) and the medium spectrum for water signal contributions by subtracting their respective projections on the water vector (equations 3 and 4, proj_W is the vector projection on the water vector, W). The two resulting spectra are then used in the next vector correction step to actually correct the combination spectrum for the medium signal (equation 5).

$$C' = C - \text{proj}_W C \quad (3)$$

$$M' = M - \text{proj}_W M \quad (4)$$

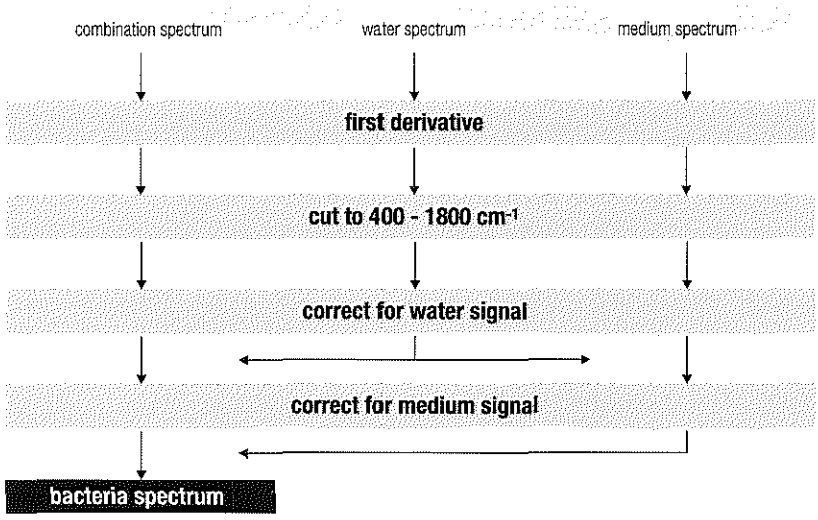
$$B' = C' - \text{proj}_{M'} C' \quad (5)$$

In the work presented here, this method was applied to first derivative spectra instead of the actual measured spectra. In most cases Raman spectra of biological molecules, cells, or tissues contain a broad, relatively featureless background signal, usually ascribed to fluorescence. Its intensity and shape may vary somewhat (and sometimes quite considerably) from measurement to measurement and from strain to strain. When the method described above is applied to spectra that only differ in their Raman to fluorescence background signal ratio, the resulting "corrected" spectra would differ. Therefore the fluorescence background signal contribution needs to be eliminated before application of the vector correction method. This is achieved by making use of first derivative spectra.

It is important to note that the vector correction procedure is not the same as subtraction of the exact amount of signal contributed by the medium. When a pure bacteria spectrum (i.e. without medium signal contribution) is decomposed into vectors parallel and perpendicular to the medium vector, the parallel component would normally not be (exactly) zero. This implies that the result of the vector correction method described is not the exact "pure" bacteria spectrum, but the component of the bacteria spectrum that is orthogonal to the medium vector. Therefore this subtraction method may affect the possibilities of a precise biochemical interpretation of the spectra. However, since the components of the microorganism spectrum that are parallel to the medium and/or water vector cannot be distinguished from the actual signal contributions of water and medium (whose intensity varies from measurement to measurement), they cannot be considered as useful information when it comes to microorganism identification.

A schematic of the whole vector correction procedure is given in figure 2. The code for the calculations in this scheme was developed under the Matlab software package (The Mathworks Inc, Natick, MA).

Figure 2



Schematic representation of the protocol used for correcting data. First derivative spectra are cut to the spectral region of 400 to 1800 cm^{-1}. Cut spectra are then corrected for water and culture medium signal contributions using the vector correction routine described in the materials and methods section.

Validation of the medium subtraction method The application of the vector correction method to non-subjectively subtract background signal contributions is expected to result in reproducible spectra regardless of the amount of medium and water signal initially present in the raw data. This method was tested in several ways.

First, a simulation was performed, in which the measurement of spectra at different depths within a microcolony on solid culture medium (and therefore with varying medium signal contributions) was mimicked. The resulting spectra were subsequently subjected to vector correction in order to remove water and culture medium signal contributions. Secondly, actual measurements at different depths in a microcolony growing on a solid culture medium served as an illustration of the practical situation. Finally, the effect of separately correcting for water signal contributions in addition to medium signal correction with the "double correction" procedure was investigated.

1 Simulation

To simulate measurements at different depths in a bacterial colony, a pure bacteria spectrum (i.e. without culture medium signal contributions) and a spectrum of the culture medium were added in different ratios. Raman spectra of dried bacterial smears on CaF_2 substrate served as the pure bacteria spectra, since in the spectral region of interest CaF_2 has no Raman features. Raman spectra were collected of a dried bacterial smear (*Escherichia coli* ATCC 25922) on CaF_2, of Mueller Hinton culture medium and of water. Raman spectra of the bacterial smear and of the culture medium were obtained at random locations in the sample, comprising 60 minutes total signal collection time. The same signal collection time was used to obtain a spectrum of water. The Raman spectrum of the culture medium used for subtraction in the vector correction routine was also obtained with a high signal-to-noise level. The spectra were scaled to standard normal variance (SNV; i.e. zero mean and unit variance)[2]. The bacterium and medium spectra were then added in the ratios of 1:1, 1:5 and 1:10. First derivative spectra were calculated for the combination spectra, the second MH spectrum and the water spectrum, followed by cutting all the spectra to the region of interest, 400-1800 cm^{-1} (schematic representation in figure 2). These combination spectra were corrected for the water and medium signal contributions by an independently measured culture medium spectrum, as described above. Having a second independent spectrum parallels the scenario of correcting acquired microcolony spectra with a reference culture medium spectrum.

2 Depth measurements

To illustrate the validity of the vector correction method on spectra of bacterial samples growing on culture media, Raman spectra were acquired directly from a 6 hour microcolony (*E. coli* ATCC 25922) with the laser focused at 3 depths within the microcolony. Spectra were acquired from the top (2 μm below surface), middle (4 μm below surface) and bottom (10 μm below the surface) of the microcolony, each in 5 minutes signal collection time. Water and medium Raman spectra were obtained in 60 minutes signal collection time as described above. A Raman spectrum of RNA was obtained in 30 minutes signal integration time. Data treatment was performed as described in figure 2.

3 "Double correction"

The effect of correcting for both water and medium signals was illustrated by culturing *E. coli* ATCC 25922 on three media that only differed in their water content. Separately, 2.25 g, 3.8 g and 5.6 g of dehydrated MH medium (Difco laboratories, Detroit, MI) were dissolved in 100 ml dis-

tilled water to prepare the solid culture media. Raman spectra of water and the three media were collected as described above. Spectra were treated as outlined in figure 2 and for illustration purposes, the water correction step was initially omitted.

Identification/classification of bacterial strains Spectra were obtained of 6 hour microcolonies of 5 bacterial strains (*Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* UHR 28624, *Staphylococcus epidermidis* UHR 29489, *Escherichia coli* ATCC 25922 and *Enterococcus faecium* BM 4147). These measurements were performed in triplicate on separate days. Measurements were carried out on 5 microcolonies per bacterial strain, with 5 spectra obtained from various positions within each microcolony. For each measurement Raman signal was collected for 30 seconds. Raman spectra of the Mueller Hinton culture medium were obtained at random locations in the medium, comprising 60 minutes signal collection time. A water spectrum was also obtained in 60 minutes total signal collection time.

Vector corrections for water and medium signal contributions were performed as described above. Per strain, the 5 spectra collected from each microcolonies were averaged. The complete spectral range (400-1800 cm^{-1}) of the microcolony spectra was then used in the subsequent multivariate analysis. Data from all the three days were combined and analyzed together as one data set.

For multivariate analyses, the amount of data was first reduced using principal component analysis (PCA)[22] performed using the Matlab PLS toolbox (Eigenvector Research Inc, Manson, WA). A total of n-1 PCA scores were calculated (n being the number of spectra in the analysis), typically accounting for 99-100% of the variation in the data set. These PCA scores were used in a cluster analysis (SPSS, Chicago, IL). Ward's clustering algorithm method and squared Euclidean distance measure were used in generating the dendrogram of the hierarchical cluster analysis.

Linear discriminant analysis (LDA) was performed on principal component scores using SPSS. Two thirds of the data was used as a training set and one third as a test set. Selection of principal components that were included in the LDA model was based upon the Wilk's lambda[32] method and 95% F-test inclusion criterion[32], thus maximizing group separation. The strength of the model based on the training set was evaluated using the leave-one-out method[32].

Results and Discussion

The Raman spectrum obtained directly from a bacterial microcolony on solid culture medium contains signal contributions of both bacteria and the culture medium. The relative signal contribution of the culture medium will vary as it critically depends on the exact depth at which the laser light is focused as well as on the thickness of the microcolony. Since the medium signal contribution is not negligible and variable, it would interfere with multivariate analysis intended for strain identification.

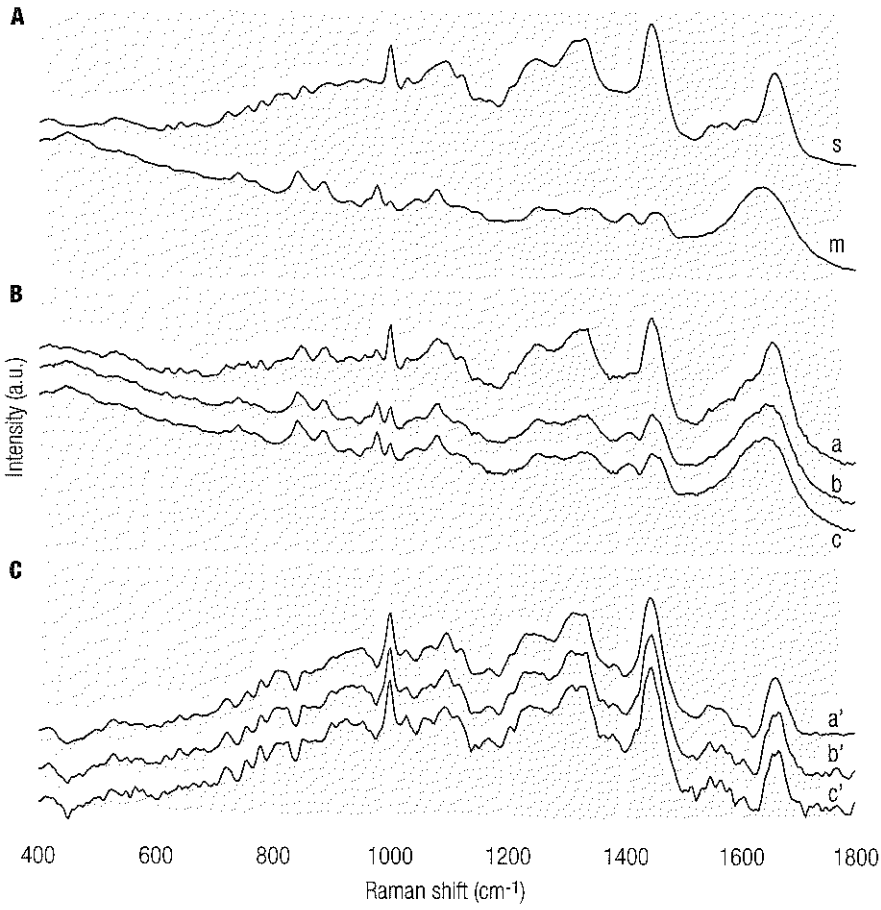
Correction for signal contributions of the culture medium In figure 3A, a spectrum of a dried smear of bacteria and a spectrum of medium from a culture plate are shown. The spectrum of the medium does not contain any clear marker bands that could be used to determine the exact intensity of the medium signal contribution. Therefore, it is not possible to accurately and non-subjectively subtract the medium signal contribution based on the intensity of such a band. For this reason we have developed the vector correction method described in the materials and methods section, which was evaluated in 3 experiments.

As explained in the materials and methods section, the result of the vector correction method is a spectrum that, when viewed as a vector, is perpendicular to the medium spectrum (i.e. not only medium signal is subtracted, but also the component of the bacteria spectrum that is parallel to the medium spectrum). However, for the purpose of readability we will, from here on, refer to the action of the vector correction method as "subtraction of medium signal".

Simulated experiment

The two spectra of figure 3A were used to simulate an experiment in which measurements are carried out at different depths within a microcolony on solid culture medium. To achieve this, the bacteria spectrum and a medium spectrum were co-added in various ratios (figure 3B) (see materials and methods section for details). Spectral features belonging to the bacteria become less obvious when the fraction of the medium is increased in the combination spectrum. For example, there was an increasing sloping background contribution due to the greater water content of the medium. In the spectra with higher proportions of medium added in, the water contribution was also more noticeable by a marked broadening of the 1550-1700 cm^{-1} region. This spectral region overlaps with Raman features predominantly arising from C=O amide backbone

Figure 3



Validation of the vector correction routine. Simulation of measurements at different focusing depths within a bacterial microcolony.
[A] *Raman spectrum of E. coli* ATCC 25922 *bacterial smear on* CaF_2 *substrate (s) and Raman spectrum of Mueller Hinton medium (m) to be used for the simulation.* [B] *Spectra obtained by adding the bacteria spectrum and a medium spectrum in the ratios: (a)* 1:1, *(b)* 1:5 *and (c)* 1:10. [C] *Resulting bacteria spectra (a',b',c') after vector correction for water and medium signal contributions. (a.u. = arbitrary units)*

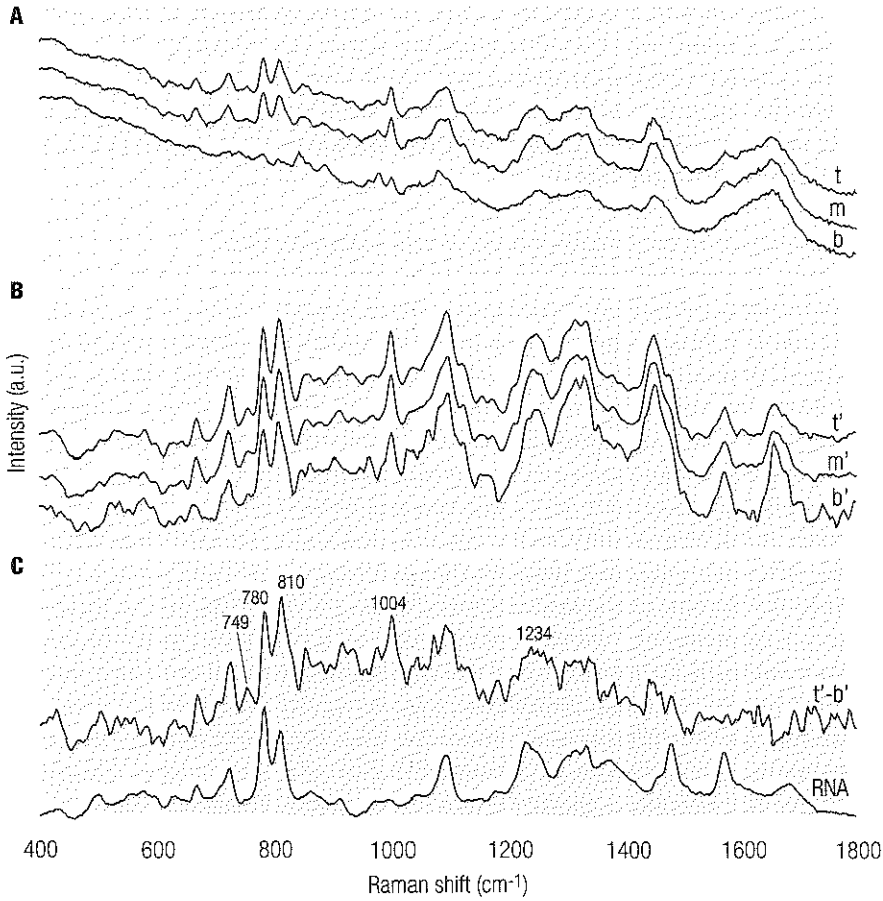
groups within proteins. In addition, various other spectral changes are observed around 1300 cm^{-1}, 1000 cm^{-1} and between 800-900 cm^{-1}.

Using a second medium spectrum, the vector correction method was used to subtract out the corresponding signal contributions. This routine resulted in bacteria spectra with high similarity, illustrating that with the correction scheme, the background signal could be reproducibly subtracted (figure 3c). There were no residual features of the medium following vector correction.

Measurement on microcolony growing on solid culture medium

Having demonstrated that the vector correction procedure is able to non-subjectively subtract medium signal contributions to yield similar bacteria spectra, Raman spectra were acquired at different depths within an actual bacterial microcolony (*E. coli* ATCC 25922). We observed that, at the various focusing depths, there are clear differences in the untreated spectra (figure 4A). Following subtraction of water and medium signal contributions, the spectra were very similar (figure 4B). However, it was noticed that there were still some spectral differences between spectra taken at different depths within the microcolony, as became clear when the difference was taken between the spectra acquired from the top and from the bottom of the microcolony (figure 4c). Also depicted in figure 4c is a spectrum of a solution of RNA from which a water spectrum has been subtracted. Comparison of the difference spectrum with this RNA spectrum reveals many similarities, suggesting that different RNA levels account for the differences observed. However, not all the features in the difference spectrum can be accounted for by RNA bands alone. Among others, the difference spectrum also shows peaks of thymine (749 cm^{-1}) and phenylalanine (1004 cm^{-1}) (Table 1), indicating the presence of more biological differences. Hence, these spectral differences observed are not artefacts of the correction routine. They arise from the naturally occurring biochemical heterogeneity of the bacterial colonies. One explanation could be that even in microcolonies with a thickness of 6 to 8 μm the various layers contain bacteria in different growth stages (older versus younger cells) and that these differences are reflected in changes in the Raman spectrum. Manoharan et al. reported increased RNA levels in bacteria entering the logarithmic phase[25]. We speculate that the cells in the higher layers of the microcolony are more actively dividing than cells in the deeper layers. Further investigations into microcolony heterogeneity are currently underway.

Figure 4



Raman spectra of E. coli ATCC 25922 *measured at various focusing depths, (t) top = 2 µm, (m) middle = 4 µm and (b) bottom = 10 µm, below the surface of the microcolony.*

[A] *Spectra obtained at the various depths before vector correction.* [B] *Spectra (t', m', b') following vector correction for water and the underlying Mueller Hinton culture medium signals.* [C] *Difference spectrum between the vector corrected spectra acquired from the top and the bottom of the microcolony (t'-b') and the Raman spectrum of a solution of ribonucleic acid* (RNA) *after a water spectrum has been subtracted. (a.u. = arbitrary units)*

Table 1

Tentative wavenumber assignments of some of the Raman features in the spectra presented[a]

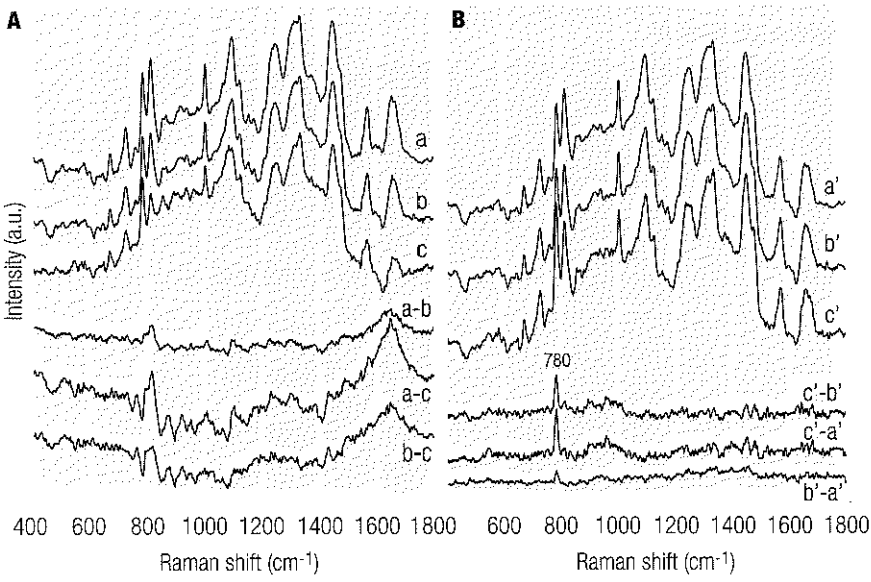
wavenumber (cm^{-1})	tentative assignment
533	δ (COC) glycosidic ring
668	ν (CS)
725	ρ (CH_2)
749	thymine (T) ring
780	uracil (U) ring
810	C–O–P–O–C in A-RNA backbone
856	ν (CC)
	ν (COC) 1,4-glycosidic link
1004	ν (CC) aromatic ring (Phe)
1095	ν (CC) skeletal
	ν (COC) 1,4-glycosidic
1220-1290	amide III
1334	δ (CH)
1452	δ (CH_2)
1573	δ (NH) and ν (CN), amide II
1630-1680	amide I

[a] δ, *Deformation;* ν, *stretching;* ρ, *rocking; Phe, phenylalanine (refs 13 and 42).*

Correction for varying water contribution

In our subtraction method, it is necessary to use a double correction approach involving water and the culture medium. The validity of this approach was demonstrated in a study in which spectra were measured from bacteria grown on various culture media that only differed in water content. Figure 5A shows bacteria spectra after vector correction for the medium alone. Although the spectra look remarkably similar, difference spectra revealed residual variation in the spectra especially in the region around 1650 cm^{-1}. This broad band is characteristic of the Raman spectrum of water. When the extra correction step for the water signal was performed, very similar spectra were obtained although the intensity of one peak at 780 cm^{-1} was higher in the medium richer in nutrients (i.e. lower water content)(figure 5B). This peak could be attributed to uracil (RNA) (Table 1), but one would expect to observe other RNA peaks in the difference spectrum as well. Further research should give additional information and is currently being performed.

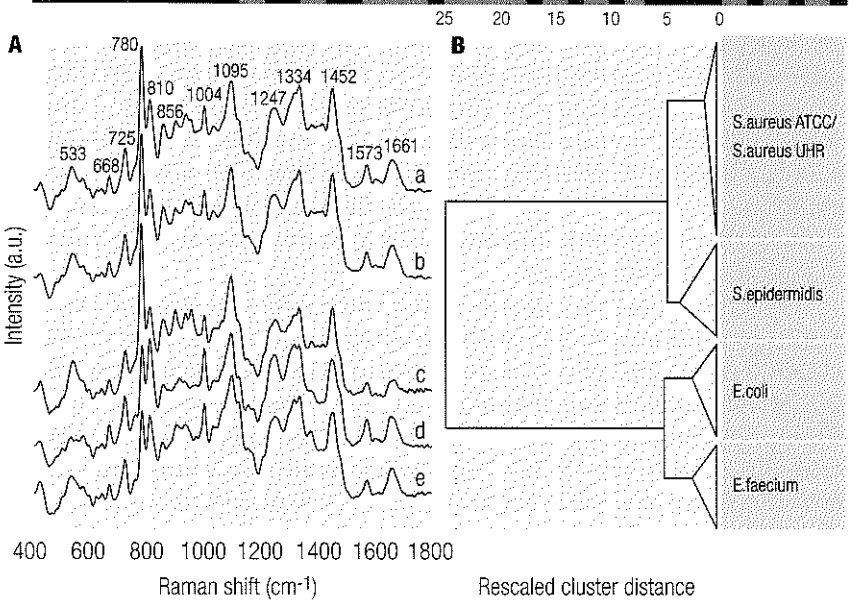
Figure 5



Raman spectra of E. coli ATCC 25922 *cultured on* MH *media with different water content.* [A] *Spectra after vector correction for only the corresponding medium (in order of decreasing water content: a, b, c) and their difference spectra.* [B] *Bacteria spectra (in order of decreasing water content: a', b', c') after vector correction for both the water and corresponding medium signals and their difference spectra.*
(a.u. = arbitrary units)

Limitations in the comparison of organisms cultured on different media therefore do not originate from the correction method presented here, but rather from intrinsic physiological cell differences. Minimization of these influences can be achieved by thorough standardization in the preparation of culture media and in the culture conditions. Commercially available, ready-to-use media have the advantage that major manufacturers use standard protocols and quality controls in their medium preparations. To what extent minor fluctuations from batch to batch can influence the identification process is not yet clear and needs further investigation. The presence of heterogeneity within microcolonies suggests that the Raman signal from several locations in the colony needs to be averaged in order to optimise reproducibility.

Figure 6



[A] *Representative Raman spectra of* 5 *bacterial strains obtained from* 6 *hour microcolonies growing on* MH *medium: (a) Staphylococcus aureus* ATCC 29213 *(b) Staphylococcus aureus* UHR 28624 *(c) Staphylococcus epidermidis* UHR 29489 *(d) Escherichia coli* ATCC 25922 *and (e) Enterococcus faecium* BM 4147. [B] *Dendrogram of hierarchical cluster analysis (squared Euclidean distance measure and Ward's cluster algorithm) performed on the principal component scores of the vector corrected Raman spectra. Repeated measurements of the same strain on* 3 *separate days were included in the analysis. Bacteria spectra were collected in* 2.5 *min. signal integration time.*
(a.u. = arbitrary units)

Identification of bacteria Now that complicating factors such as the variability in the water and medium signal contributions are eliminated, the Raman spectra can be treated by multivariate analysis for (non-subjective) classification of bacterial strains. Representative spectra acquired from 5 bacterial strains are shown in figure 6A. Closer inspection of the spectra reveal that there are indeed spectral differences characteristic of the various strains. For example, the *Staphylococcus* strains are characterized by markedly high intensities at 780 cm^{-1}. To the naked eye, the two *S. aureus* stains resemble one another very closely. The spectra of *E. coli* have a characteristically intense band at around 1004 cm^{-1} arising

from the ring breathing vibration found in the amino acid phenylalanine. The biological relevance of the increased intensity of this peak in *E. coli* Raman spectra is presently unknown. Raman spectra arising from *S. epidermidis* and *E. faecium* have slightly poorer signal-to-noise levels when compared to the other strains. This is due to the fact that 6 hour microcolonies of these strains are very thin. The colony thickness was approximately 1-3 μm for *S. epidermidis* and *E. faecium* as opposed to between 6-8 μm for the strains of *S. aureus* and *E. coli*. These differences lie in the intrinsic biological growth difference of the strains and are reflected in the resulting Raman spectra.

Hierarchical cluster analysis of the microcolony spectra of the 5 strains over 3 days resulted in the dendrogram shown in figure 6B. We observed that there are two major clustering branches consisting of the *Staphylococcus* strains in one group and *E. faecium* and *E. coli* in the other. Within this latter group, a clear division is observed between the *E. faecium* and *E. coli* strains. In the *Staphylococcus* branch subclusters are formed of *S. epidermidis* and *S. aureus*. Therefore, it is possible to distinguish these bacterial genera and the *Staphylococcus* species based on their Raman spectra.

Within the clusters containing spectra of one strain, spectra measured on the same day tended to cluster together indicating the presence of some day-to-day variation. This variation however did not interfere with identification down to the species level of the *Staphylococcus* strains, suggesting that spectral differences between the various species are greater than any subtle day-to-day variation in the spectra of the strain.

When data of all 3 days were analyzed together, the two *S. aureus* strains could not be clearly separated with the unsupervised classification approach. PCA followed by the supervised linear discriminant analysis on only the *S. aureus* spectra resulted in a 100% correct classification of the training set and 83% of the test set. When hierarchical cluster analysis was performed on the PCA scores of each day separately, 100% separation of the two *S. aureus* strains was obtained. This observation suggests that when data of the three days were combined, the complete discrimination of the two *S. aureus* strains was hindered by day-to-day variations in the spectra. The effect of small deviations in wavenumber calibration of the spectra as well as fluctuations in the biochemical composition of the cells are currently being investigated as possible sources of this day-to-day variation. The issue of repeatable wavenumber calibration of multichannel Raman instruments and instrument-to-instrument calibration transfer is an area of active research. A thorough analysis of the problems involved as well as potential solutions was recently published by Mann and Vickers[24].

Other studies using FT-IR spectroscopy combined with multivariate analysis, have also reported the successful separation of different bacterial species such as *Eubacterium* spp.[9], *Lactobacillus* spp.[10], *Streptococcus* spp. and *Enterococcus* spp.[18]. Hence, despite the fact that the various Raman spectra of microorganisms look similar upon first inspection, as with FT-IR spectra, there is a high information content in these spectra which can be used in multivariate analysis for the discrimination of microorganisms.

Overall then, the classification results presented indicate that it is possible to identify bacteria from their Raman spectra acquired directly from microcolonies growing on solid culture medium.

Conclusion

We have presented a novel approach of the use of Raman microspectroscopy for the identification and characterization of clinically relevant microorganisms. An approach for measuring Raman spectra of 6 hour microcolonies directly on the solid culture medium, could in principle enable identification results to be obtained within the same day of receipt of patient material. In contrast, conventional microbiological approaches require significantly more time to arrive at the same result. With respect to other spectroscopic methods mentioned in the introduction, Raman spectroscopy has the advantage of minimizing culturing time, sample handling and the use of chemicals and disposables. Using online data processing routines, the Raman spectra can be analyzed and clustered rapidly. However, key features to the successful application of this technique are the careful correction for variable parameters, such as water signal contributions of the culture medium and the amount of culture medium signal collected with the Raman signal of bacteria. Averaging of signal over several positions in the microcolony is fundamental as well, in order to compensate for colony heterogeneity. The methods must be rigorously standardized, such that reproducible spectra of good signal-to-noise levels are obtained. Furthermore, the use of a standard growth medium with carefully controlled composition is essential for accurate classification of bacteria. The different signal contributions of different media can, in principle, be compensated for, through the application of double vector correction schemes. However, it is more than likely that differences in medium composition also have an influence on the overall biochemical composition of the bacteria. This is, therefore, a point of concern for all spectroscopic methods.

To our knowledge this is the first report on spectroscopic measurements of bacteria directly on solid culture media. Good signal-to-noise levels could be obtained after an incubation period of 6 hours followed by 2.5 minutes of Raman signal collection. From the limited data set presented here it appears that bacteria may be distinguished at the genus and species level on the basis of unsupervised analysis of their Raman spectrum. Supervised methods, such as wavenumber region selection methods[43] and minimisation of day-to-day variation in instrumental and/or biological parameters, should enable discrimination down to the strain level. Therefore we conclude that when thoroughly standardized and optimized, confocal Raman microspectroscopy has potential as a powerful new tool in diagnostic microbiology.

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4. Investigating microbial (micro)colony heterogeneity by vibrational spectroscopy

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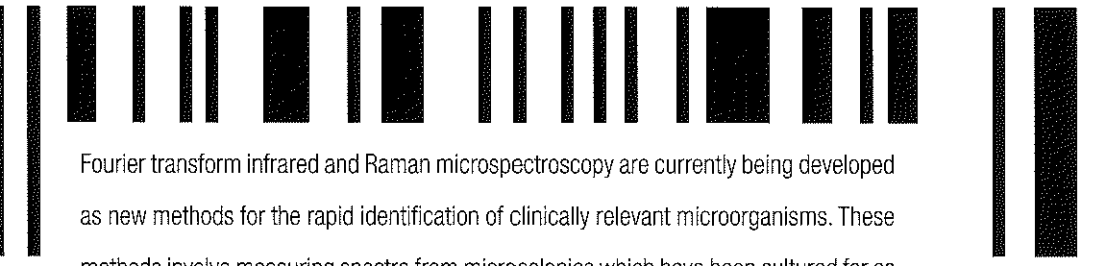
4. Investigating microbial (micro)colony heterogeneity by vibrational spectroscopy



Fourier transform infrared and Raman microspectroscopy are currently being developed as new methods for the rapid identification of clinically relevant microorganisms. These methods involve measuring spectra from microcolonies which have been cultured for as little as 6 hours, followed by the non-subjective identification of microorganisms through the use of multivariate statistical analyses. To examine the biological heterogeneity of microorganism growth which is reflected in the spectra, measurements were acquired from various positions within (micro)colonies cultured for 6, 12 and 24 hours. The studies reveal that there is little spectral variance in 6 hour microcolonies. In contrast, the 12 and 24 hour cultures exhibited a significant amount of heterogeneity. Hierarchical cluster analysis of the spectra from the various positions and depths reveals the presence of different layers in the colonies. Further analysis indicates that spectra acquired from the surface of the colonies exhibit higher levels of glycogen when compared to the deeper layers of the colony. Additionally, the spectra from the deeper layers present with higher RNA levels than the surface layers. Therefore, the 6 hour colonies with their limited heterogeneity are more suitable for inclusion in a spectral database to be used for classification purposes. These results also demonstrate that vibrational spectroscopic techniques can be useful tools for studying the nature of colony development and biofilm formation.

Introduction

In recent years, there has been much effort invested into the development of new techniques for the identification of microorganisms. Many of these methods are aimed at providing the clinician with more rapid identification of the microorganism responsible for infection in order to begin the appropriate course of antimicrobial treatment therapy[1, 9, 15, 21, 27, 31, 44, 51]. The emergence of these novel methods reflect the rise in drug-resistant microorganisms, which requires that antimicrobial treatment be more effectively managed[2, 12, 28, 52]. Among the new methods are those based on vibrational spectroscopic techniques, namely Fourier transform infrared (FT-IR) and Raman spectroscopies. Vibrational spectroscopic methods are reagentless procedures in which there is no need to add dyes or labels for spectral measurement. These non-destructive techniques are based on the absorption (FT-IR) or scattering (Raman) of light directed onto a sample. The amount of light absorbed or scattered depends on the molecules found within the sample and the environment in which these molecules are found. With these highly sensitive techniques, the frequency of light in the resulting spectrum provides biochemical information regarding the molecular composition and molecular structure of, and molecular interaction in cells and tissues[24, 55]. Raman and infrared spectroscopies are complementary techniques which together can provide a more complete impression of the biochemical information within a sample. Furthermore, these two methods differ such that each is capable of providing information not easily obtainable by the other. For instance, with FT-IR spectroscopy, hydrated samples are difficult to measure since water absorbs so strongly that its signal masks other interesting peaks in the spectrum. On the other hand, water is less problematic in Raman spectroscopy enabling measurement of hydrated samples. However, the signal-to-noise ratio of the resulting Raman spectrum is overall poorer than that obtained by FT-IR spectroscopy when spectra are measured for the same amount of time. When these sensitive techniques are coupled to a microscope, spectra can be acquired from microorganisms cultured for short periods of time (~ 6 hours) on or from solid culture media since large biomasses are not required for spectral measurement.

The application of various spectroscopic techniques to identify and characterize microorganisms has been explored previously[3, 9, 14-16, 20-23, 25-27, 29-35, 37, 42, 49, 54, 56]. These studies have shown that it was possible to discriminate among various microorganisms at the genus, species and strain level[14, 21-22, 25, 27, 29-32, 34, 49], and studies report the ability to differentiate microorgan-

isms from various serogroups[21-23, 37]. Furthermore, the use of FT-IR spectroscopy to identify drug resistance has also been investigated[3, 42]. However, many of these previous studies are based on microorganisms cultured for 16 to 24 hours or longer prior to spectral measurement. Our research is aimed at developing new rapid methods for the identification and characterization of clinically relevant microorganisms through the use of confocal Raman and Fourier transform infrared microspectroscopies. Current microbiological diagnostic methods require 2-3 days and involve culturing of microorganisms until a suitable biomass is obtained for subsequent tests. Such methods are inherently slow especially in life-threatening situations such as cases of meningitis, sepsis and critically ill patients in the intensive care units of hospitals[52]. With microspectroscopic methods, microorganisms can be cultured for as little as 6 hours prior to spectral measurement. It is intended that these novel diagnostic methods are able to quickly provide the clinician with results within the same day that patient samples are obtained.

A critical aspect of these rapid identification methods based on vibrational spectroscopy, is the development of spectral reference databases against which clinical results can be compared in order to arrive at non-subjective identification and classification schemes. Not only must the spectra contained within the database be derived from rigidly standardized protocols regarding the culturing and measurement conditions, the database needs to be comprehensive such that it reflects any kind of intrinsic biological diversity and heterogeneity found within microorganisms. Our interest in the development of microcolonies is in regards to the need to understand the heterogeneity of microorganism growth from the point of view of spectral variance. We have previously described a Raman method of characterizing cultures after 6 hours of growth[27]. In this paper, we present the investigation of spatial colony heterogeneity and its biochemical basis in 6 hour and older cultures by Raman and FT-IR microspectroscopies. The findings based on 5 well-characterized microorganisms after growing on solid culture medium for various time periods are reported. For comparison, similar studies were performed using a conventional approach involving intrinsic fluorescence spectroscopy. From this investigation, the nature of the variance of spectra derived from these microorganisms provides insight on the development of microorganisms grown on solid culture medium.

Materials and Methods

Strains and sample preparation A group of 5 well-characterized reference strains were obtained from the collection of the Pasteur Institute (Paris, France): *Staphyloccocus aureus* CIP 4.83, *Staphylococcus aureus* CIP 53.154, *Escherichia coli* CIP 53.126. *Escherichia coli* CIP 54.8T, and the American Type Culture Collection: *Candida albicans* ATCC 90028. These strains were stored in brain heart infusion broth (Becton Dickinson, Paramus, N.J.) containing 10% glycerol at −80°C until ready for use. Sample preparation involved one overnight passage on solid culture medium (to acclimatize the strain), followed by re-culturing the strain for various incubation times; typically for 6 hours, 12 hours and 24 hours to yield (micro)colonies ranging from less than 50 μm in the short incubation times to as large as 2000 μm after the longer culture times. Typical culture conditions for the bacterial strains involved growing at 37°C on Mueller-Hinton medium (Merck, Darmstadt, Germany). The yeast strain was cultured at 37°C on Sabouraud-glucose 2% medium (Merck).

For the Raman studies, a biomass from the overnight passage was used to streak out the strains in 4 segments. Following incubation, spectra were typically acquired directly from microorganisms still growing on the culture plate of well isolated (micro)colonies found in the 3rd or 4th segment.

For the infrared studies, following incubation, microcolonies were transferred from the agar plate to an infrared transparent zinc selenide optical plate using a specially designed stamping device[30-32]. Imprints were allowed to air dry (approx. 15 min.) prior to spectral measurement. Spectra were acquired from the imprinted microcolonies on this substrate.

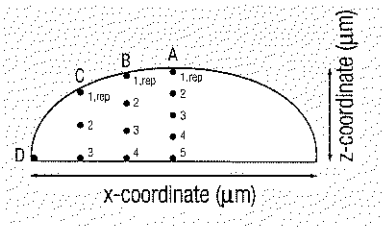
For the fluorescence studies, from the incubated plates, colony imprints were done manually onto glass slides.

Spectral acquisition and data treatment:

Raman spectroscopy

Raman spectra were acquired using a Renishaw System 1000 Raman microspectrometer (Renishaw plc, Gloucestershire, United Kingdom) equipped with a 300 lines/mm grating as described previously[27]. The accompanying Leica microscope was fitted with an 80x near-infrared objective (MIR Plan 80x/0.75, Olympus). Raman signal was collected in the spectral interval from 250 cm^{-1} to 2150 cm^{-1}, with a spectral resolution of 8 cm^{-1}. Measurements were performed using 830 nm excitation from a

Figure 1



Schematic representation of the various measuring positions within a colony for spectra acquired by Raman microspectroscopy. Letters refer to the lateral measuring positions, while numbers refer to the depth measuring positions; "rep" refers to a measurement of the surface position as described in Materials and Methods.

titanium:sapphire laser (model 3900, Spectra Physics, Mountain View, Calif.) pumped by an argon-ion laser (series 2000, Spectra Physics), delivering 100 mW of laser power on the sample.

The cultured plates were taken from the incubator and placed directly under the microscope objective for measurements. Firstly, from each (micro)colony, the diameter and depth were estimated. Thereafter, various measurement positions were determined to give 4 equally-spaced lateral positions from the centre to the edge of the colony. At the thicker lateral positions, various depths within the colony were also selected (figure 1). At each measurement position, 5 spectra at 30 seconds were acquired and averaged. At the very edge and at the bottom of the colony closest to the culture medium, 10 spectra each at 30 seconds were acquired and averaged in order to improve the spectral signal-to-noise ratio. The depth spectra were acquired beginning from the surface and working towards the bottom of the colony. Following the deepest measurement, a repeat measurement of the surface was taken as a duplicate check (labelled "rep" in figure 1). With the use of a computer-controlled xyz-stage, it was possible to determine the lateral and focusing positions reproducibly.

Following spectral acquisition, the constant background signal contribution originating from optical elements in the laser light delivery pathway was subtracted from all spectra. The reference spectrum of a tungsten band lamp of known temperature was used to correct for the wavelength dependent signal detection efficiency of the Raman set-up[36, 55]. Spectral treatment also involved taking the first derivative of the Raman spectra, scaling to standard normal variance (i.e. zero mean and unit variance) and using the spectral region between 400-1800 cm^{-1} for further analysis. Despite the use of a confocal arrangement in which typical measuring volumes were approximately 1.5 µm in the lateral direction and 7-8 µm along the optical axis, there exists the possibility of sam-

pling the underlying culture medium especially for measurements toward the bottom of the colony. Therefore, it was necessary to correct the acquired spectra for the variable underlying signal of the culture medium using a vector correction approach [details are provided in ref. 27. As stated therein, it should be stressed once again that care must be taken in making biochemical interpretation of spectra having been treated with the vector correction method]. Data reduction was then performed through principal component analysis using the Matlab PLS Toolbox (Eigenvector Research Inc., Manson, Wash.). The principal component analysis scores accounting for 99.9% of the total variance captured were used in a hierarchical cluster analysis (SPSS, Chicago, Ill.) using Ward's clustering method and squared Euclidean distance measure.

Infrared spectroscopy

FT-IR spectra were recorded on a FT-IR microscope, model IR Scope II, which was interfaced to an IFS 28/B spectrometer (Bruker Optics, Karlsruhe, Germany) and was equipped with a motor-driven xy-stage. For each spectrum, 256 interferrograms were co-added and averaged. Fourier transformation was done using a Blackmann-Harris 3 term apodization function and a zerofilling factor of 4, resulting in a nominal resolution of 6 cm^{-1}. Spatial heterogeneity of the microcolonies was examined by linearly mapping the microcolony in 10 µm steps in the x and y directions and using an aperture size of 30 µm. The first derivative spectra between the range 820-1780 cm^{-1} were vector normalized as described previously[4]. Hierarchical cluster analysis was performed using Pearson's product moment correlation coefficient and Ward's algorithm.

The infrared measurements were done independently at two different laboratories. Alternatively, FT-IR absorption spectra were collected using a UMA 500 infrared microscope coupled to a FTS-40A spectrometer (Bio-Rad Laboratories, Spectroscopy Division, Hemel-Hampstead, United Kindom), equipped with an mercury-cadmium telluride narrow-band detector and a microscope diaphragm varying from 10 x 10 to 500 x 500 μm^2. Measurements on single microcolonies were performed by setting the microscope aperture from 60 x 60 μm^2 up to 100 x 100 μm^2. Absorption spectra were obtained in the microscope transmission mode using the following parameters: 4 cm^{-1} resolution, 5 kHz scan speed, 32-64 scan coaddition, triangular apodization and spectral range of 800-4000 cm^{-1}. No baseline correction or smoothing was applied to the data. The data was first normalized using the z-score function (i.e. zero mean and unit variance) in Matlab (The Math Works, Inc., Natick, Mass.) and then subjected to cluster analysis using Matlab's Statistics Toolbox employing Ward's algorithm and Euclidean distance measure.

Intrinsic fluorescence

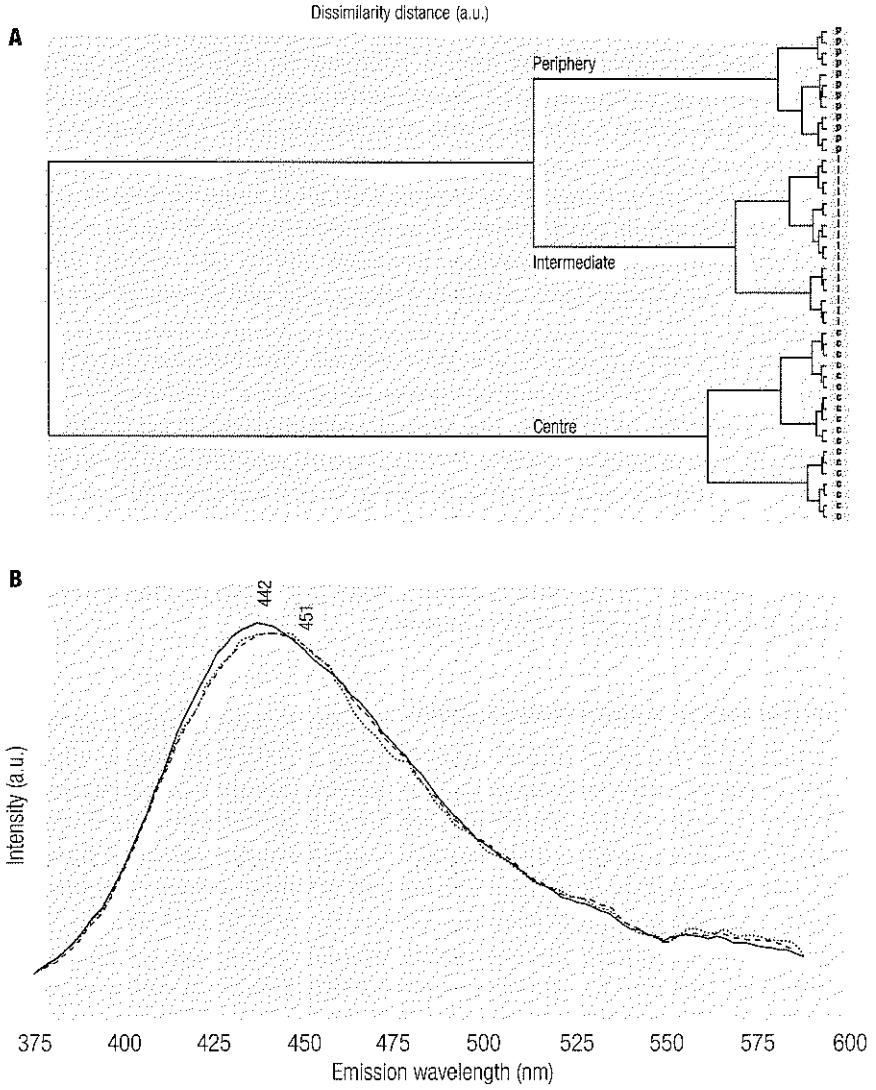
Fluorescence emission signals from (micro)colony imprints on glass slide were measured by exciting in the UV (360 nm) with laser power of 1μW, using an argon ion laser (model 2065A, Spectra Physics). Spectra were recorded using a UV confocal laser microspectrofluorometer (Dilor, Lille, France). For the UV measurements, an Olympus BH2 microscope containing a 100x objective was employed. Point-by-point analysis was done using the spectral imaging acquisition mode, which consisted of scanning the laser over the (micro)colony by moving the computer-controlled xy-stage. For each (micro)colony, one hundred points were collected. The data gave an emission spectral profile, which in turn produced a spectral image that can be compared to the conventional optical image. All spectral manipulations were done with the LabSpec software (Dilor). The spectral profiles obtained were used in a hierarchical cluster analysis constructed with Ward's method and Euclidean distance measure (Statistica, Statsoft, Tulsa, Okla.).

Results and Discussion

In the development of new routine methods based on vibrational spectroscopic techniques for the rapid identification of microorganisms, spectra derived from rigidly standardized protocol are used to establish a spectral database for the non-subjective classification and identification of clinically relevant microorganisms. Thus, it is imperative that the database be comprehensive so that the natural variance of the microorganism is captured within the spectral database. A potential problem is that in recent years, it is becoming more widely accepted that microorganisms are not necessarily unicellular organisms but rather, multicellular organisms able to form complex communities with specific division of tasks and population differentiation[39-41]. Furthermore, it is known that biofilms are elaborate structures composed of microcolonies attached to a surface and within these microcolonies, the bacteria are organized into communities with functional heterogeneity[6]. Given that colonies of microorganisms are complex multicellular communities, it is necessary to establish at what growth stage infrared and Raman spectra should be acquired from such (micro)colonies. In doing so, any heterogeneity which can interfere with the discrimination of microorganisms can be minimized. As the aim of these new methods is to be able to provide the clinician with laboratory results on the same day patient material is acquired, the culture time should be kept short; for example approximately 6 hours growth time. To gain an understanding of the spectral heterogeneity, the development of (micro)colonies was monitored over several culture times and at various positions within the (micro)colonies.

In the discussion to follow, a large part of the analysis is based on the results of subjecting the data to hierarchical clustering analysis. This is a procedure that non-subjectively groups the input cases (i.e. the spectra) based on similarities of their properties (the spectral characteristics). When graphically displayed, the result of the analysis forms a dendrogram: the relationship between the input cases is represented by the distance at which they connect on a dissimilarity scale (e.g. figure 2A). The more similar the cases are, the smaller their connecting distance on the dissimilarity scale. Dendrograms resemble the phylogenetic trees that arise from taxonomical classification. Groups of similar members can be readily visualized. Since the spectral information reflects the biochemistry of the sample measured, the distance in the dendrograms can be interpreted as a measure of how biochemically different the various spectra are and, hence, the measurement positions within a colony.

Figure 2

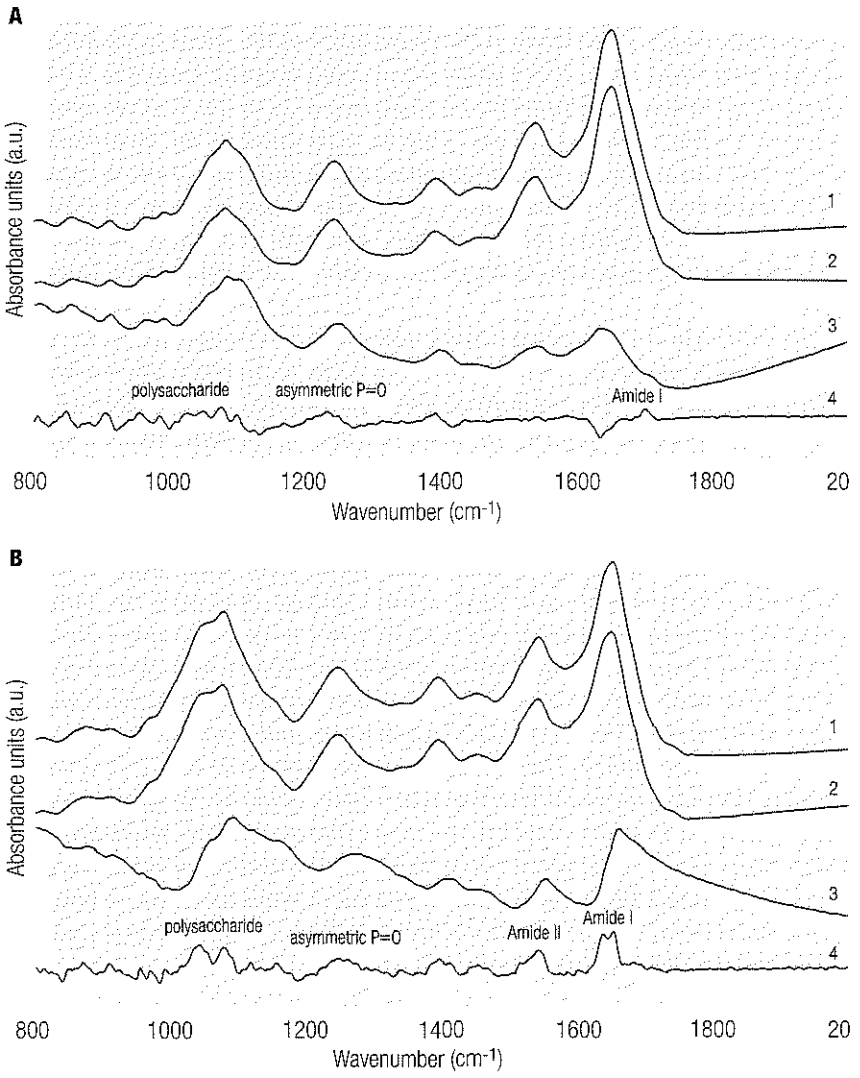


[A] *Dendrogram of hierarchical cluster analysis of uv (360 nm)-excited intrinsic fluorescence spectra (derived from the spectral image after filtering) of E. coli* CIP 54.8T *microcolony imprint. Spectra acquired from various positions within the P: peripheral, I: intermediate, and C: central regions of the microcolony imprint.* [B] *Averaged intrinsic fluorescence spectra corresponding to spectra acquired from the central (——), intermediate (- - - -), and peripheral (·····) regions of the microcolony imprint. a.u., arbitrary units.*

Fluorescence of colony imprints As a starting point in the investigation of (micro)colony heterogeneity, an approach involving fluorescence microspectrometry was first employed. A series of UV-excited intrinsic fluorescence spectra were acquired from a microcolony imprint of *E. coli* CIP 54.8T cultured for 6 hours. The spectra were normalized to account for possible differences in intensity due to variation in the thickness of the microcolony imprint. Subjecting the data to hierarchical cluster analysis revealed that the intrinsic fluorescence was not homogeneously distributed over the microcolony. As shown in the dendrogram in figure 2A, the spectral profiles from the various positions within the imprint form their own sub-clusters (central, intermediate, and peripheral). When the averaged fluorescence spectrum from each sub-cluster is examined (figure 2B), the various spectra are quite similar. The averaged spectrum from the centre of the microcolony has an emission wavelength maximum of 442 nm. In contrast, the spectra corresponding from the intermediate and periphery regions have a broader spectral profile with a flatter maximum possibly corresponding to the superposition of two maxima at 442 nm and 451 nm. Despite these differences, further information regarding the compositional heterogeneity of the various regions could not be directly obtained from the intrinsic fluorescence data. Hence other methods were required.

Infrared spectra of (micro)colony imprints In order to gain a further understanding of the biochemical heterogeneity of (micro)colonies, vibrational spectroscopic techniques were employed. Hierarchical cluster analysis of FT-IR spectra acquired from colonies of *E. coli* CIP 54.8T which were 100 μm or larger, produced a dendrogram similar to that shown in figure 2A, with the spectra tending to cluster into different groups depending upon the measurement position within the colony (not shown for brevity). By examining the individual infrared spectra and calculating difference spectra, it is possible to gain a better understanding of the source of the clustering scheme observed. In figure 3A, the FT-IR spectra acquired from the centre and the edge of an *E. coli* CIP 54.8T colony are shown. Although to the untrained eye these two spectra look remarkably similar, any differences that exists can be highlighted by taking difference spectra. The difference spectrum which results from subtracting the spectrum of the edge from that of the centre is shown as well as the difference obtained from subtracting the first derivative spectra from the two measuring positions. Since infrared bands tend to be quite broad thereby potentially masking peak differences, the differences are more apparent in the derivative spectra. Comparison of the peak positions with those from empirical studies in the literature[24] reveals differences

Figure 3



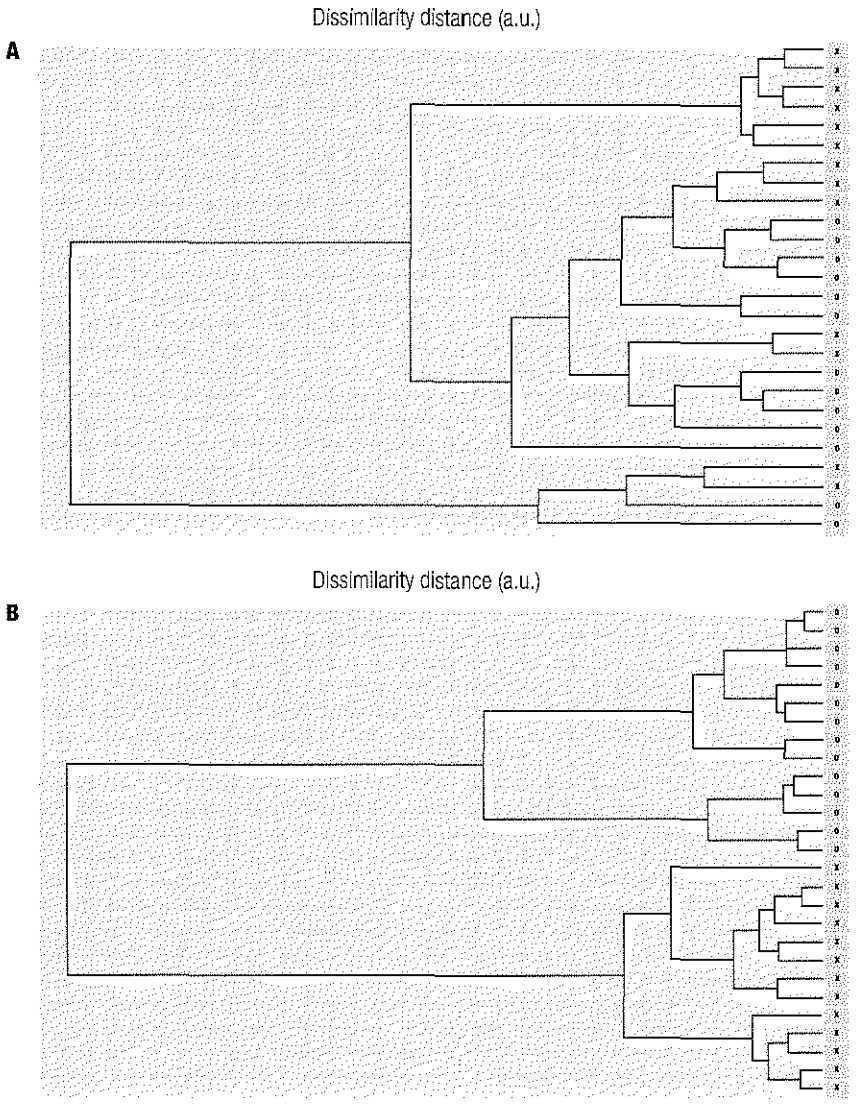
Original FT-IR *spectra from a* 100 µm *E. coli* CIP 54.8T *colony* [A] *and a* 100 µm *C. albicans* ATCC 90028 *colony* [B] *measured at the centre (1) and edge (2) positions of the colony. The corresponding difference spectrum (magnified* 5*x) (3) between centre and edge is shown, as well as the difference spectrum (magnified* 2*x) (4) obtained from the 1st derivative spectra. The difference spectra were obtained by* 1*-to-*1 *subtraction of vector normalized spectra (normalization between* 820 *and* 1780 cm^{-1}*). a.u., arbitrary units.*

in the spectral region around 1230 cm^{-1} which can be assigned to the phosphate double bond asymmetric stretching vibration of phosphodiester, free phosphate and monoester phosphate functional groups. Smaller alterations are also observed in the protein amide I regions (approximately 1620-1670 cm^{-1}). This band arises predominantly from the C=O stretching vibration of the amide C=O groups of proteins. Furthermore, changes visible around 1400 cm^{-1} may be attributed to the symmetric stretching vibrations of COO^- functional groups and very weak changes were observed in the carbohydrate region around 900-1200 cm^{-1}. Similar changes were observed for the other bacterial and yeast strains[35], although the changes were more pronounced in the yeast strains (figure 3B).

Interestingly, in a separate study measuring FT-IR spectra from 12 hour colonies of the two *E. coli* strains (CIP 54.8T and CIP 53.126), heterogeneity between spectra acquired from the centre and the edge of a colony was large enough to influence the discrimination between the different strains. As shown in figure 4A, the spectra arising from the 2 strains formed mixed clusters. However, when spectra acquired from younger colonies (approximately 7 hours culture time) were subjected to cluster analysis, two major clusters were formed corresponding to the different strains (figure 4B). These results suggest that with the older colonies, there is significant heterogeneity in the spectra from various positions within the colony. Similar studies performed with 50 μm size colonies or growth time of about 6 to 7 hours revealed that there was very little variance in the infrared spectra sampled from the centre or periphery of the colony. Therefore, it appears that until 6 to 7 hours of growth, there is very little heterogeneity observed in the composition of microcolonies. However, beyond this time frame, marked biochemical differences such as changes in the protein amide I bands, phosphate moieties likely arising from nucleic acids, protein constitution, and carbohydrate moieties, are noted. These differences likely influence the classification results observed (figures 4A and 4B).

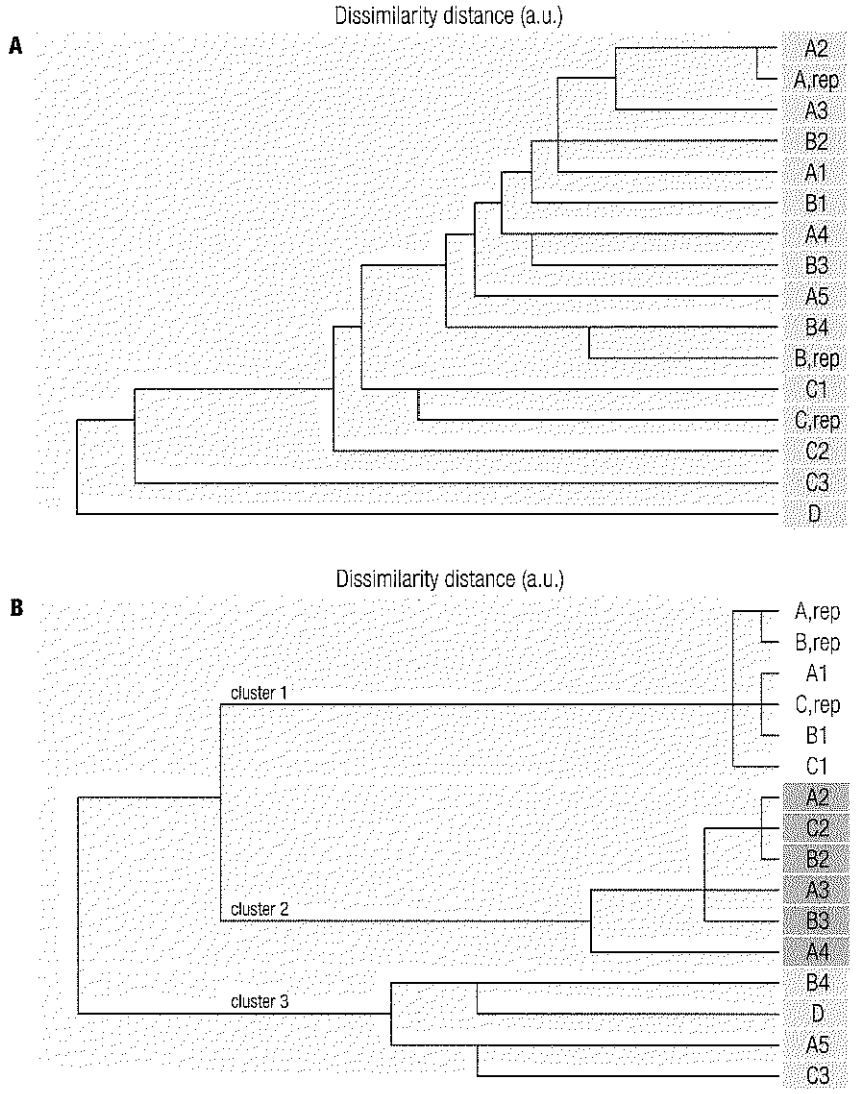
Raman spectra directly from (micro)colonies With infrared microspectroscopy, spectra were acquired through the entire depth of the colony at the central, intermediate, and peripheral regions. However, with this approach, any heterogeneity arising from various depths within the colony would not be readily revealed. Insight into the heterogeneity of microcolonies can also be obtained from confocal Raman microspectroscopy, in which spectra can be acquired from the various lateral positions throughout the colony as well as at various depths within the colony. The Raman spectra acquired in this manner were subjected to

Figure 4



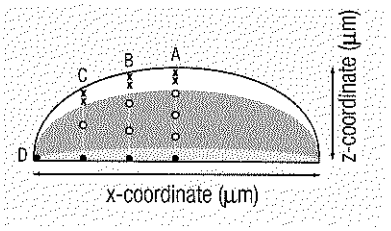
Dendrogram from hierarchical clustering analysis of FT-IR *spectra of* 12 *hour cultures* [A] *and* 7 *hour cultures* [B] *of E. coli* CIP 54.8T *(denoted by x) and E. coli* CIP 53.126 *(denoted by o). a.u., arbitrary units.*

Figure 5



Dendrograms from hierarchical cluster analysis of Raman spectra from various measurement positions within a 6 hour E. coli CIP 53.126 *microcolony* [A] *and a 24 hour E. coli* CIP 53.126 *colony* [B]. *Shading highlights the various clusters and corresponds to the shading in figure 6. The labels correspond to the measuring positions in the schematic diagram of figure 1 and 6. a.u., arbitrary units.*

Figure 6

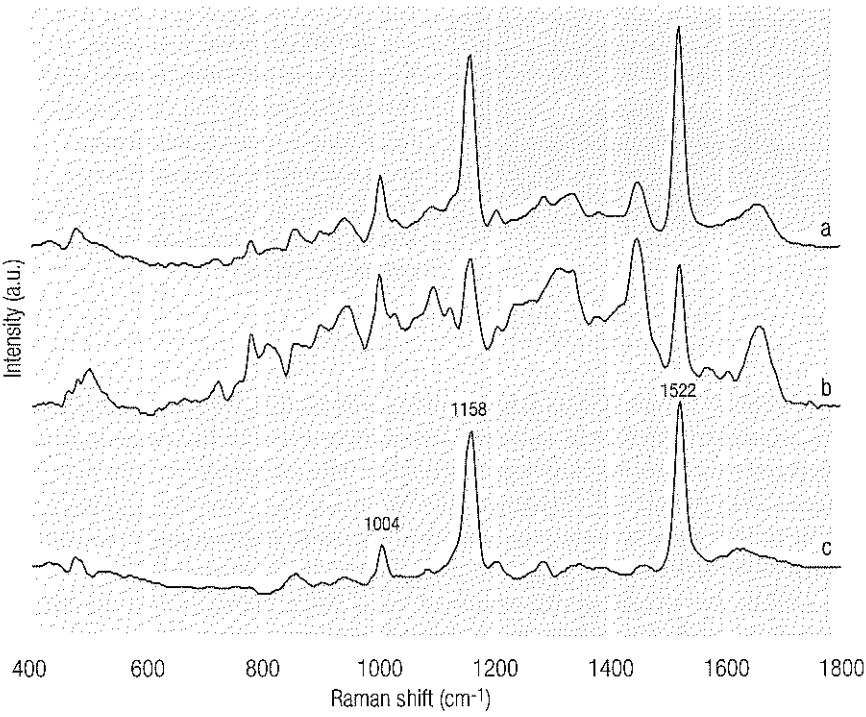


Diagrammatic projection of the various clusters (X, cluster 1; O, cluster 2; •, cluster 3 as determined from figure 5) from the hierarchical cluster analysis of Raman spectra from various measurement positions within a 24 hour E. coli cip 53.126 colony. Shading is used to highlight the various layers within the colony.

hierarchical cluster analysis. In figure 5A, the results are shown for spectra acquired from 6 hour cultures. Visual inspection of the dendrogram revealed no obvious groupings or clusters. This observation is in accordance with the infrared findings for 6 hour colonies, thereby suggesting that at this growth stage, the cultures are quite homogeneous overall. Such observations were apparent irrespective of the strain studied.

Unlike the dendrograms obtained for 6 hour cultures, the dendrograms of spectra acquired from microorganisms grown for 12 and 24 hours showed distinct sub-clusters (figure 5B). When the members of the same cluster were assigned to a group and the various groups projected onto a schematic diagram depicting the measurement location of each spectrum, it appears that there are different layers in the 12 and 24 hour colonies (figure 6). Similar findings were observed for the various strains studied for 12 and 24 hour cultures. Further examination of the spectra indicate that for *S. aureus* CIP 4.83, the clustering differences arise from distinct spectral peaks at 1004, 1158, and 1522 cm^{-1} which can be assigned to the various C–C vibrations found in carotenoids (figure 7)[29, 30, 33, 54]. The clustering reveals that the carotenoid concentration is higher within the upper layers of the colony and less prominent in the deeper layers. Carotenoids are responsible for the yellow-orange pigmentation observed in the 12 and 24 hour colonies and are one of the classical characteristics of this species. Studies have shown that *S. aureus* is very sensitive to the bactericidal effects of fatty acids such as oleic acid. The incorporation of such lipophilic agents into the membranes results in increased membrane fluidity and thus a decrease in membrane-associated functions[5]. It is believed that the production of carotenoids might help *S. aureus* stabilize its cell membrane, thereby preventing potentially lethal fatty acid-induced changes in the fluidity of its membrane[5]. Other studies have also shown that pigmented *S. aureus* strains are far more resis-

Figure 7

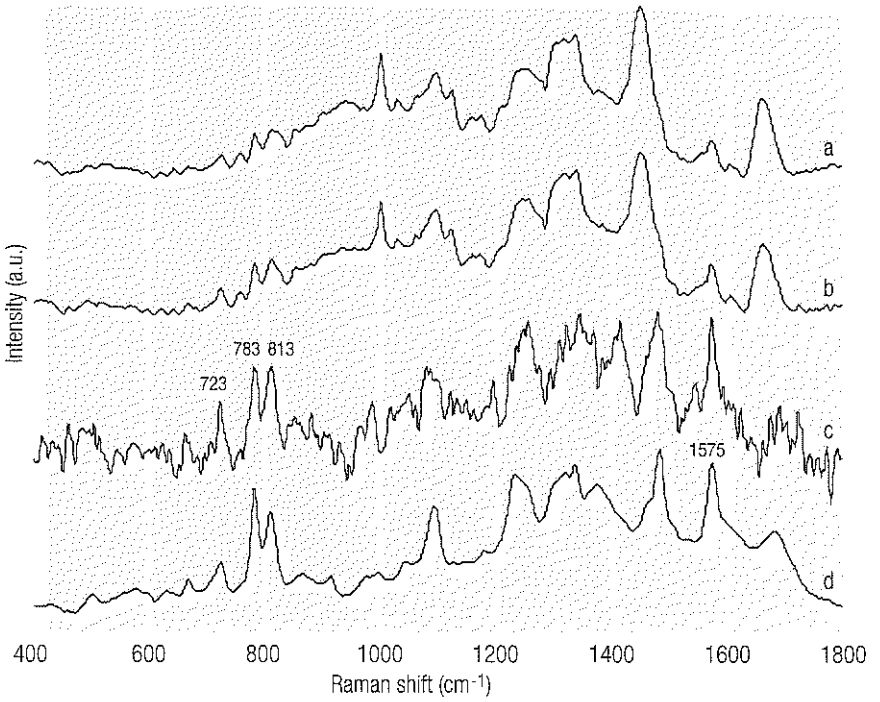


Averaged Raman spectra of the members of cluster 1 (surface layer) (a) and cluster 2 (layer beneath surface) (b) and the corresponding difference spectra (a-b) from a 24 hour colony of S. aureus CIP 4.83 *(c) are shown. a.u., arbitrary units.*

tant to singlet oxygen lethality than are carotenoidless *S. aureus* mutants[8]. Hence, the bacterium might use the carotenoid pigmentation as a mechanism to resist killing by fatty acids and to quench singlet oxygen, thus protecting against lethal effects of photosensitization. Previous studies[18] have shown that the carotenoid production is mainly correlated with the time of growth, and this finding has also been observed by FT-IR spectroscopy[29]. Therefore, it might signify that older cells which produce significant pigmentation are found towards the surface layers of the colony. Alternatively, our finding of higher carotenoid concentration in the upper layers of older colonies might suggest a means by which the colony protects itself from its environment.

Interestingly, the Raman spectra of the other *S. aureus* strain, CIP 53.154, showed that this strain does not produce the characteristic pig-

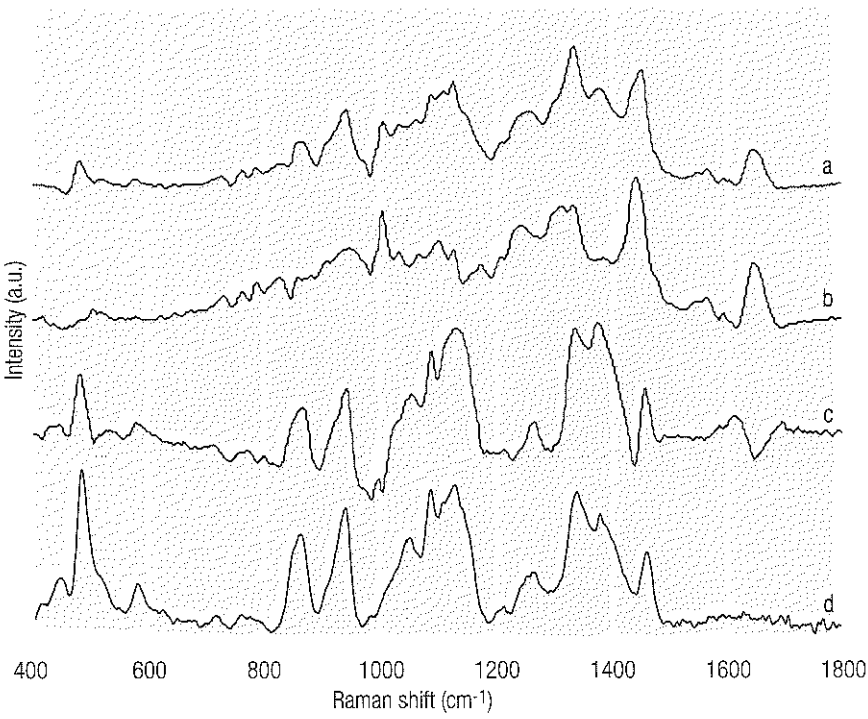
Figure 8



Averaged Raman spectra of the members of cluster 2 (layer beneath surface) (a) and cluster 3 (still deeper layer) (b) and the corresponding difference spectra (b-a) from a 24 hour colony of E. coli CIP 54.8T *(c) are shown. The Raman spectrum of* RNA *(d) is also shown. a.u., arbitrary units.*

mentation. Non-pigmented derivatives of *S. aureus* are known to exist and are often found in subcultures of stored organisms[53]. The cluster analysis shows a similar sort of distinction, with spectra acquired from the surface layers clustering together and those within deeper layers clustering as a group. However, the lack of pigmentation suggests that another spectral feature is responsible for the formation of distinct clusters. This clustering trend was found for *S. aureus* CIP 53.154, *E. coli* CIP 53.126, *E. coli* CIP 54.8T and *C. albicans* ATCC 90028. Closer examination of the Raman difference spectra showed that in the deeper layers of 12 and 24 hour colonies, there are characteristic spectral peaks at 723, 783, 813, and 1575 cm^{-1} (figure 8). These features all arise from the nucleotide and phosphate backbone vibration found in RNA[19]. It appears that the RNA

Figure 9

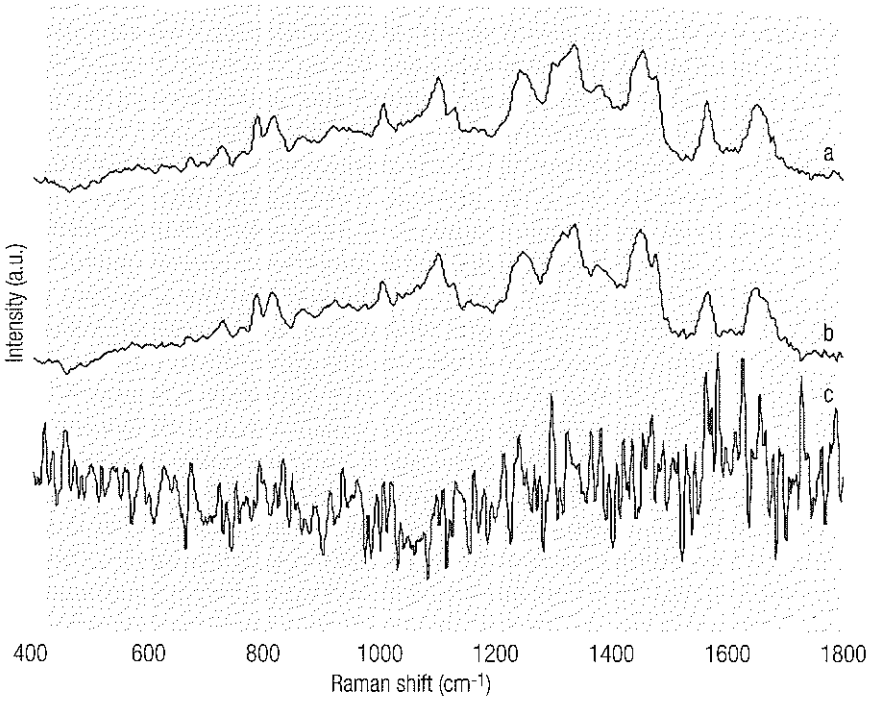


Averaged Raman spectra of the members of cluster 1 (surface layer) (a) and cluster 2 (layer beneath surface) (b) and the corresponding difference spectrum (a-b) from a 24 hour colony of E. coli CIP 53.126 *(c) are shown. The Raman spectrum of glycogen (d) is also shown. a.u., arbitrary units.*

concentration is higher in the deeper layers of the colony. Observations of the decrease in RNA content in older cells which have transitioned to the stationary phase from the logarithmic stage has been reported with FT-IR spectra of *Bacillus subtilis*[29]. Hence, this finding again suggests that the colony is composed of older cells in the surface layers and younger cells in the deeper layers which are more actively dividing thus, reflecting a higher RNA content.

Aside from RNA differences, it was noted that 12 and 24 hour colonies from the bacterial strains contain a relative higher glycogen concentration in the surface layers (figure 9). This glycogen difference is not observed with younger 6 hour bacterial microcolonies (figure 10) or with the yeast strain. At present it is unknown whether the glycogen is contained

Figure 10



Raman spectra are shown from different depths corresponding to the measuring positions in the schematic diagram of figure 1, *with* A1 *from the surface (a) and* A3 *from deeper within the colony (b) and the corresponding difference spectrum (a-b) from a* 6 *hour microcolony of E. coli* CIP 53.126 *(c). a.u., arbitrary units.*

within the cells of the surface layers or found extracellularly in the form of a film. Previous FT-IR studies have also found increases in the carbohydrate C–O stretching mode of 24 hour cultures of *Bradyrhyzobium japonicum* strains which have been transferred from liquid to solid culture medium. From transmission electron micrographs, the authors ascribed such changes to alteration of the bacterial wall component, possibly the formation of glycocalyx[56]. The organization of colonies into distinct layers has also been observed with *E. coli* strains (cultured for 2 weeks) in which vertical sections through colonies revealed a stratification of different cell types, as could be seen with standard microscopic reagents such as staining with toluidine blue[42]. Previous reports in the literature using scanning electron microscopy to study the surface structure of *E.*

coli colonies growing for over 24 hours on agar medium in normal petri dishes have revealed that each colony secretes extracellular materials, some of which form a skin or framework over its surface[38-39]. Other studies have shown that at later stages of colony development (20-24 hours), the surface film of *E. coli* colonies became thicker. On the other hand, the film was not observed for colonies cultured for 6 to 16 hours of growth[45-48]. Therefore, it is possible that the glycogen-rich surface layer observed with Raman microspectroscopy is the polysaccharide-rich extracellular coat, commonly known as the glycocalyx, of bacterial cells. These exopolysaccharides are mainly composed of homopolysaccharides (cellulose, levans, dextrans and glucans) and heteropolysaccharides (monosaccharides including a uronic acid)[43]. It is thought that the formation of the glycocalyx serves as an integral matrix for a biofilm and that following the adhesion of bacteria to a substrate, the glycocalyx forms a protective milieu for cell division and microcolony formation and growth[7, 11]. Some studies propose that the glycocalyx either acts as a diffusion barrier or, by complexing antibacterial agent, excludes and/or influencing the penetration of antimicrobial agents to the underlying cells[10, 13]. Modern medicine increasingly relies on the use of indwelling medical devices such as catheters and prosthetic joints for multiple purposes. These so-called foreign bodies are implanted for a short period, intermittently or permanently. One of the most frequently encountered complications of these devices is the development of infections. The ability of bacteria to adhere to the surface of these indwelling devices by binding to biofilm layers is still not completely understood [Endtz, personal communication]. Hence, there is much interest in the development of biofilms, associated with disease in humans due to the increasing use of medical devices and the difficulty, resulting from resistance to antimicrobial agents, of effectively controlling infection[6, 13, 17].

Overall, these infrared and Raman studies of the development of microorganisms cultured for various growth times reveal that there is significant colony heterogeneity in the strains cultured for 12 and 24 hours. These differences can be attributed to higher glycogen content in the surface layers and to increased levels of carotenoid pigmentation in certain *S. aureus* strains. Furthermore, a relative higher RNA content was observed in the deeper layers of the colony. Therefore spectra derived from these older colonies are quite variable, indicating the need to sample spectra from a multitude of positions within these colonies in order to capture the biological variance of the various cell types. The lack of group clusters and absence of obvious spectral differences in the various spectra obtained from 6 hour cultures suggest that the microcolonies at this growth stage are very homogenous in terms of molecular composition. Thus, these spectra are suitable for inclusion in and building of

spectral libraries of microorganisms. With the development of a comprehensive spectral database, it should be possible to use Raman and FT-IR microspectroscopies to provide a rapid identification and classification of clinically relevant microorganisms. Moreover, the present study demonstrates that vibrational microspectroscopy can be applied to further understand the heterogeneity of microorganism growth. For example, the attachment and microcolony formation of biofilms as well as the actual mechanisms of biofilm resistance to antimicrobial agents still remain unclear[13]. FT-IR spectroscopy, including attenuated total reflectance spectroscopy, has been used previously to study bacterial growth and biofilm formation[56]. The use of Raman microspectroscopy to probe various layers within a colony, can be extended to study the formation of sessile communities found at the base of the biofilm. These sessile cells are believed to be the root of many persistent and chronic bacterial infections since they can withstand host immune responses, unlike their non-attached planktonic counterparts which are killed by antibiotic therapy[6]. The knowledge gained from such studies can be used to develop new strategies for the treatment of infection, especially those associated with indwelling medical devices.

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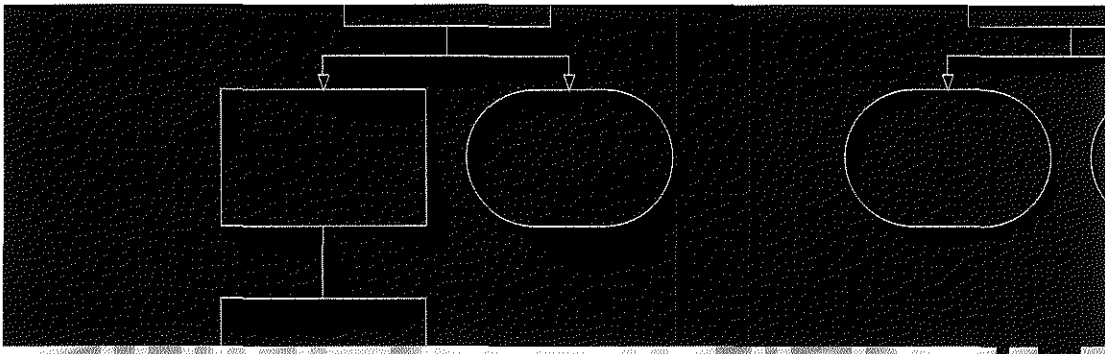

5. Rapid identification of *Candida* species by confocal Raman microspectroscopy

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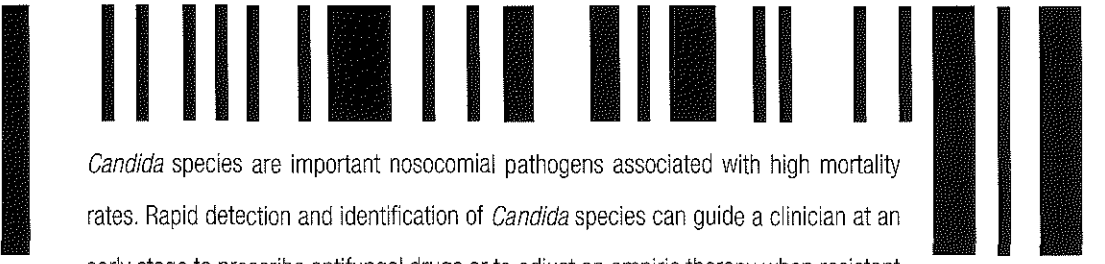


Candida species are important nosocomial pathogens associated with high mortality rates. Rapid detection and identification of *Candida* species can guide a clinician at an early stage to prescribe antifungal drugs or to adjust an empiric therapy when resistant species are isolated. Confocal Raman microspectroscopy is highly suitable for the rapid identification of *Candida* species, since Raman spectra can be directly obtained from microcolonies on a solid culture medium after only 6 hours of culturing. In this study, we have used a set of 42 *Candida* strains comprising 5 species that are frequently encountered in clinical microbiology, to test the feasibility of the technique for the rapid identification of *Candida* species. The procedure started either from a culture on Sabouraud medium or from a positive vial of an automated blood culture system. Prior to Raman measurements, strains were sub-cultured on Sabouraud medium for 6 hours to form microcolonies. Using multivariate statistical analyses, high prediction accuracy (97% to 100%) was obtained employing the Raman method. Identification with Raman microspectroscopy may therefore be significantly faster than identification using commercial identification systems that allow various species to be identified and which often require 24 to 48 hours before a reliable identification is obtained. We conclude that confocal Raman microspectroscopy offers a rapid, accurate, and easy-to-use alternative for the identification of clinically relevant *Candida* species.

Introduction

Yeasts of the genus *Candida* are increasingly encountered as the cause of nosocomial infections. These opportunistic pathogens are often isolated from critically ill patients on intensive care units (ICUs), e.g. patients receiving broad spectrum antimicrobial therapy or patients with intravascular devices[28, 42]. *Candida* species are the fourth most commonly encountered nosocomial pathogens in bloodstream infections in the US, and candidosis is associated with high mortality rates[5, 14, 17, 31, 43]. Of the *Candida* species encountered in clinical practice *Candida albicans* is the most prevalent. *C.albicans* is often susceptible to the azole group of antifungal agents. However, there is a shift towards the more azole-tolerant species, such as *C.glabrata*, *C.tropicalis* and *C.krusei*, possibly related to the increasing use of itraconazole and fluconazole, the antifungal drugs of first choice in candidiasis[3, 30, 31, 40, 45]. Rapid identification of these species is therefore relevant for the clinician in determining the correct antifungal agent.

Conventional identification of *Candida* species is based on an extensive series of tests, e.g. carbohydrate fermentation and assimilation, growth at 37°C and 42°C, colony and cell morphology, and the ability to form germ tubes[27, 45]. Available commercial yeast identification systems are derived from this conventional approach, e.g. Vitek2 (bioMerieux, Lyon, France), API 20C (bioMerieux, Basingstoke, UK), the Rapid Yeast Plus system (Innovative Diagnostic Systems, Norcross, GA) and the Minitek system (Becton Dickinson Microbiology Systems, Cockeysville, MD). The performance of commercial identification systems has been extensively evaluated for most clinically relevant *Candida* species. Once enough biomass was obtained from the initial culture (16 to 24 hours for most commonly encountered species), results were obtained after 4 hours to several days of incubation, depending on the system. Identification accuracy was reported between 59% and 99%, and seemed to improve with the increase in number of tests included in the system[10, 11, 19, 27, 33, 41, 44-46]. Most of the rapid (same-day) identification systems are designed to discriminate between two species or to confirm a presumptive identification. Rapid systems enabling identification of various species are at best limited to the more common species seen in the clinical laboratory[45]. Therefore, the need for rapid multi-species tests still exists.

Rapid identification of microorganisms in general has been shown to have a major impact on the morbidity, mortality and duration of hospitalization[2, 7, 16]. Doern and co-workers showed that when an empirically started antimicrobial therapy had to be changed based on laboratory re-

sults, this change could be made ca. 15 hours earlier when rapid techniques were applied[7]. For *Candida* species involved in bloodstream infections on the ICU it was shown by Ibrahim et al. that initial therapy was inadequate in 95% of the cases, because no antifungal agent was included[16]. Due to the inadequacy of the initial therapy a mortality rate of about 60% was observed in the patient group with *Candida* infections. Hence, early recognition of a *Candida* infection would help the clinician to select a proper treatment. Combined with a rapid identification of the causative organism, this treatment could be optimized, if required, in an early stage of the infection.

Vibrational spectroscopic techniques are highly suitable as a basis for the development of rapid identification methods. Fourier transform infrared (FT-IR) spectroscopy and Raman spectroscopy provide information about the molecular composition of a sample. The overall molecular composition of microbial species and strains is sufficiently different to lead to reproducible differences in FT-IR and Raman spectra, to an extent that the spectra can be used as highly specific spectroscopic fingerprints that enable identification of microorganisms[6, 8, 9, 12, 13, 15, 22, 25, 26]. Recently we reported a new and rapid method "to record" Raman spectra of microbial microcolonies, directly on solid culture media[23]. Reproducible Raman spectra can be obtained from microcolonies of 10-100 μm in diameter, such as will develop for most commonly encountered microorganisms after about 6 hours of culturing[4, 23]. A good impression of the potential identification accuracy of Raman-based methods was obtained from a comparison of vibrational spectroscopic methods with genotypic identification methods. Raman spectra were obtained from dried smears on glass slides of overnight cultures of *Enterococcus* species. A cluster analysis carried out on the Raman database thus established, showed that clustering of strains occurred in accordance with genotypic species identification, whereas routine phenotypic methods failed in a number of cases[20].

Here we present results from a study aimed at the development of a rapid and accurate identification method for clinically relevant *Candida* species. An identification algorithm is described and tested which carries out *Candida* species identification based on Raman spectra obtained of 6 hour microcolonies on solid a culture medium, with or without prior passage through a blood culture system.

Materials and Methods

Yeast strains and identification A collection of 42 *Candida* strains was used (table 1). Strains were either obtained from culture collections (American Type Culture Collection ATCC, VA: *C. albicans* ATCC 90028, *C. glabrata* ATCC 66032, *C. kefyr* ATCC 66028, *C. tropicalis* ATCC 750; Centraal Bureau voor Schimmelcultures CBS, Utrecht, The Netherlands: *C. krusei* CBS 573) or were clinical isolates identified to the species level by the conventional identification method mentioned in the introduction.

Sample preparation Samples were stored at −80 °C in a brain-heart infusion broth (Becton Dickinson, Franklin Lakes, New Jersey) containing 10% glycerol until use. Thirty-two strains were sub-cultured on Sabouraud+2% glucose (SAB) medium for 6 hours at 30 °C prior to Raman measurement of microcolonies, following an overnight passage (30 °C) on SAB medium (Merck, Darmstadt, Germany). The dataset of spectra obtained from microcolonies prepared in this way will be referred to as the '*SAB-dataset*'.

For 34 strains (table 1), microcolonies were prepared after a passage through a blood culture system, in order to determine if this would affect the identification ability of the Raman method. The strains were seeded at 10^3 CFU/ml in 10 ml blood from healthy volunteers. The seeded blood samples were used to inoculate Mycosis culture vials of the automated BACTEC blood culture system (Becton Dickinson). When the culture vials were flagged as positive by the system (within 24 hours for most strains), several drops of the liquid culture medium were plated on SAB medium and cultured for 6 hours at 30 °C prior to Raman measurement of microcolonies. The data obtained from samples pre-cultured in the BACTEC system will be referred to as the '*blood-dataset*'.

Confocal Raman microspectroscopy Raman spectroscopic measurements were performed as described previously[23]. Briefly, the solid culture medium containing the microcolonies was placed directly under the microscope of a Renishaw System 1000 Raman microspectrometer (Renishaw plc, Gloucestershire, UK). The microscope was fitted with an 80x near-infrared objective (MIR Plan 80x/0.75, Olympus). Samples were excited using 100-150 mW of 830 nm laser light from a titanium-sapphire laser (model 3900, Spectra Physics, Mountain View, CA) pumped by an argon ion laser (series 2000, Spectra Physics). The constant background signal contribution originating from optical elements in the laser light deliv-

Table 1

Composition of the strain collection used in this study.

species	strain[a]	SAB-dataset[b]	blood-dataset[b]	origin	identification method[c]	reference
C. albicans	2	x		blood	CA	41
	3	x	x	blood	CA	41
	11	x		blood	CA	41
	12	x	x	blood	CA	41
	25	x		blood	CA	41
	42	x	x	tissue	CA	41
	6319	x	x	oral cavity	GT/GS	39
	ATCC 28367	x	x			
	ATCC 38696	x	x			
	ATCC 90028	x	x			
C. glabrata	32	x		blood	CA	41
	37	x	x	blood	CA	41
	46	x	x	blood	CA	41
	326 I/95		x	stomach biopsy	API ID 32C	d
	33371 I/94		x	oral swab	API ID 32C	d
	ATCC 66032	x				
	ATCC 90030		x			
C. kefyr	13	x	x	blood	CA	41
	29	x	x	tissue	CA	41
	52	x	x	faeces	CA	41
	53	x	x	oral rinse	CA	41
	146 I/96		x	BAL*	API ID 32C	d
	430 II/96		x	BAL*	API ID 32C	d
	ATCC 66028	x	x			
C.krusei	4	x		tissue	CA	41
	7	x	x	blood	CA	41
	8	x	x	sputum	CA	41
	10	x	x	tissue	CA	41
	14	x		BAL*	CA	41
	15	x	x	tissue	CA	41
	28	x		oral rinse	CA	41
	40	x	x	tissue	CA	41
	47	x	x	tissue	CA	41
	CBS 573	x	x			

(continued on page 114)

species	strain[a]	'SAB-dataset'[b]	'blood-dataset'[b]	origin	identification method[c]	reference
C. tropicalis	19	x	x	blood	CA	41
	45	x	x	ascites	CA	41
	48	x	x	tissue	CA	41
	326 II/95		x	stomach biopsy	API ID 32C	d
	ATCC 750		x			
	M38 I/96		x	oral rinse	API ID 32C	d
	M56 I/93		x	sputum	API ID 32C	d
	M675 I/93		x	blood	API ID 32C	d
Total	**42**	**32**	**34**			

[a] *strain identification;* ATCC: *American Type Culture Collection,* CBS: *Centraal Bureau Schimmelcultures* [b] *strains in the* 'SAB-*dataset*' *were only cultured on Sabouraud medium, strains in the 'blood-dataset' were obtained from spiked blood cultures (see Materials and Method section for details);* 'x' *indicates that a Raman spectrum of the strain is included* [c] CA: *assimilation of carbohydrates,* GT: *germtube formation in human serum,* CS: *chlamydospore formation on cornmeal agar,* API ID 32C *from bioMerieux (Lyon, France)* [d] *strains kindly provided by the laboratory of Prof. Dr. D. Naumann of the Robert Koch Institute, Berlin, Germany* *BAL: *broncho-alveolar lavage*

ery pathway was subtracted from all spectra. The reference spectrum of a tungsten band lamp of known temperature was used to correct for the wavelength dependent signal detection efficiency of the Raman set-up[39-47]. Calibration of the wavenumber-axis was performed using the known wavelengths of the atomic lines from neon and argon.

Per yeast sample 5 microcolonies were selected. Within each microcolony, spectra were obtained from 10 randomly chosen locations, using a signal collection time of 30 seconds per measurement. For each sample measured, the 50 spectra thus obtained were averaged. The yeast Raman spectra used for this study were obtained over a 3-month period.

Sixty Raman spectra of the SAB medium were obtained at random locations in the medium, comprising 30 minutes signal collection time. Sixty water spectra were also obtained in 30 minutes total signal collection time.

Spectrum treatment All spectrum analyses were performed on first derivatives of the measured spectra. This was done in order to minimize the influence of the broad, relatively featureless signal background usually ascribed to fluorescence, on which the Raman spectra are superimposed and which may vary from sample to sample[23].

In a previous paper we described a method for orthogonalizing microbial signal contributions to the background signal contribution of the solid culture medium[23]. This is necessary because the actual signal contribution of the culture medium critically depends on the exact position of the laser focus in the colony, and therefore unavoidably varies from one measurement to the next. After the orthogonalizing procedure, Raman spectra obtained from a particular microcolony look the same, irrespective of the intensity of culture medium signal contributions initially present. Collection of a database of spectra over an extended period of time necessitates the use of culture plates from different batches, which will show slight variations in composition and water concentration. Moreover inhomogeneities within 1 culture plate can be encountered at the microscopic scale at which the Raman experiments take place. This means that in order to be able to compare all spectra of yeast microcolonies, they must be orthogonalized with respect to all culture medium spectra. In the previous paper this was accomplished by sequentially orthogonalizing a spectrum to all medium spectra[23]. Here we have applied a more efficient method. The spectra of all the culture plates and of water were subjected to a principal component analysis. The first principal components, accounting for 99 % of all signal variance within this dataset of spectra were used to construct a principal component subspace (PC-subspace). Microcolony spectra were projected onto this PC subspace and only the spectrum component orthogonal to this PC-subspace was retained for further analysis. After this procedure, microcolony spectra are obtained that are both independent of the amount of medium signal contribution originally present, and independent of batch to batch variations in medium composition (unless these affect the biochemical composition of the cells, e.g. due to effects on growth rate[23]).

All procedures used for spectrum treatment and data analysis were developed using the Matlab 5.3 software package (The Mathworks Inc., Natick, MA) and the multivariate statistical analysis toolbox PLS-toolbox 2.0.0c (Eigenvector Research Inc., Manson, WA) unless otherwise stated.

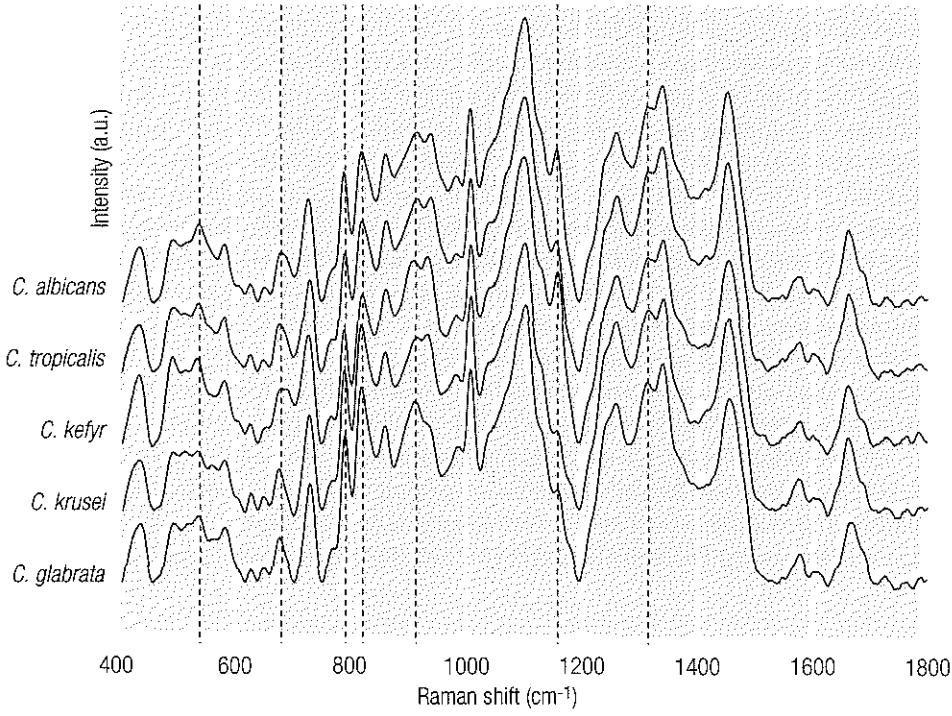
Data analysis *Principal component analysis (PCA).* Before multivariate statistical analyses, a data reduction was performed using PCA, this is a well-known method to reduce the dimensionality in a dataset[18, 35]. The maximum number of n-1 principal components was calculated (n being the number of spectra in the analysis), typically accounting for 99-100% of the variation in the data set.

Hierarchical cluster analysis (HCA) was performed on the n-1 principal component (PC) scores, obtained for each spectrum, using Ward's clustering algorithm and the squared Euclidean distance measure to generate a

dendrogram. For HCA the SPSS statistical software package (SPSS, Chicago, IL) was used.

Linear discriminant analysis (LDA). For LDA only PC scores accounting for more than 1% of the variance in the data set were retained. A two-sided t-test was used to individually select those PC scores that showed the highest significance in discriminating the different microbial groups presented. The number of PC scores that was used as input for an LDA model was kept at least two times smaller than the number of spectra in the smallest model group to prevent overfitting in the LDA model[1].

Figure 1



Representative Raman spectra of 5 Candida species used in this study. Spectra are averages of those obtained from the 'SAB-dataset'. Dotted lines indicate spectral features of which the relative intensities differ between the spectra of different species
(a.u. = arbitrary units).

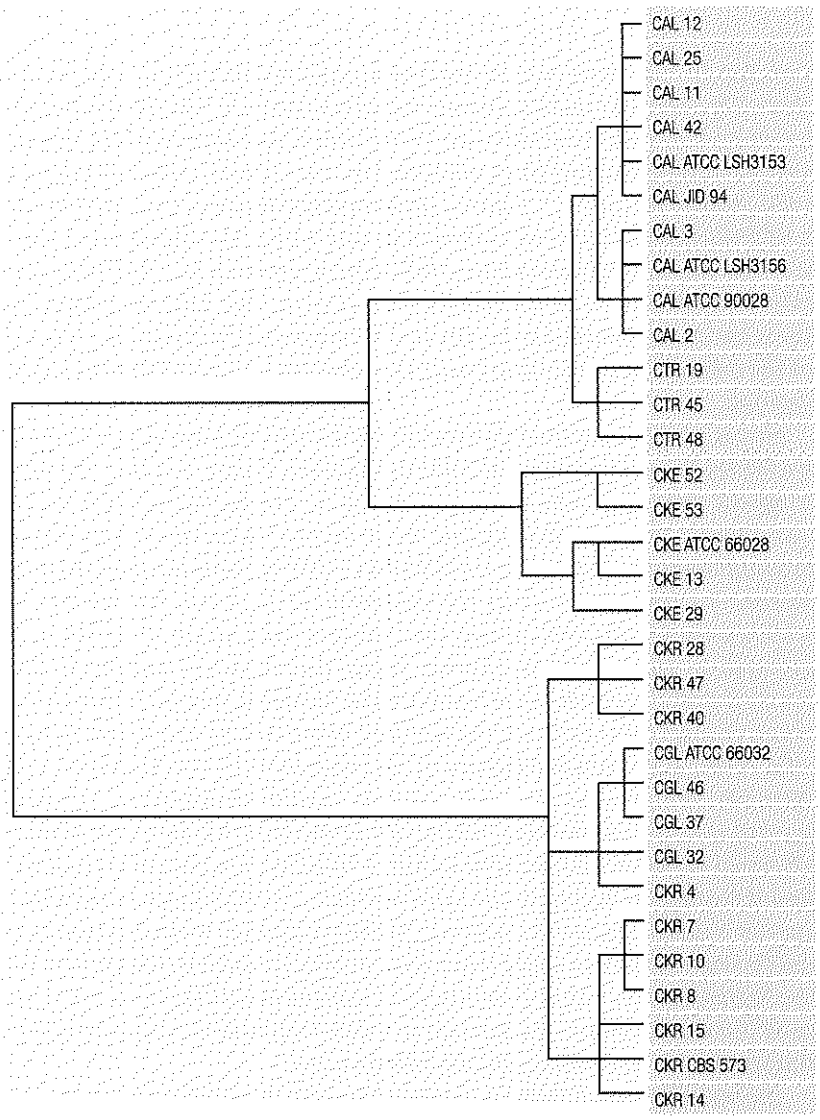
Results & Discussion

Our aim in this study was to develop a rapid identification scheme for clinically relevant *Candida* species, based on confocal Raman microspectroscopy. From earlier studies we have learned that reproducible Raman spectra can be obtained from microbial microcolonies, still growing on a solid culture medium[4,23]. Figure 1 shows typical Raman spectra from the 5 different *Candida* species included in this study. The highlighted spectral features show characteristic differences between the different species. The differences, from species to species, in the relative heights of these bands are believed to be due to differences in biochemical make-up of the cell-wall. A precise band assignment is the subject of further investigation.

HCA is a non-supervised method to obtain information about the dissimilarity between spectra of different species. Figure 2 shows the dendrogram resulting from a HCA performed on the Raman spectra in the 'SAB-dataset' (i.e. spectra obtained from strains cultured on Sabouraud medium only; see Materials and Methods section). Separate clusters were formed for the species with the exception of *C. krusei*, the spectra of which were distributed among two clusters. One strain, *C. krusei* 4, is grouped in the *C. glabrata* cluster, using this objective approach. The spectral differences observed between the averaged spectra of the two *C. krusei* clusters were only very minor and could not be attributed to specific molecular fractions (figure 3). As explained in the Materials and Methods section, the HCA uses squared Euclidean distances between spectra as input parameters. This result shows that this measure of overall signal variance encountered within a set of spectra obtained from different strains belonging to the same species, can be as large as the interspecies signal variance. In order to facilitate species identification it is necessary therefore to apply supervised analysis methods, which look for the signal variance that is relevant to species discrimination. Here we used the results of the HCA method as a first step in developing a sequential species identification scheme based on LDA.

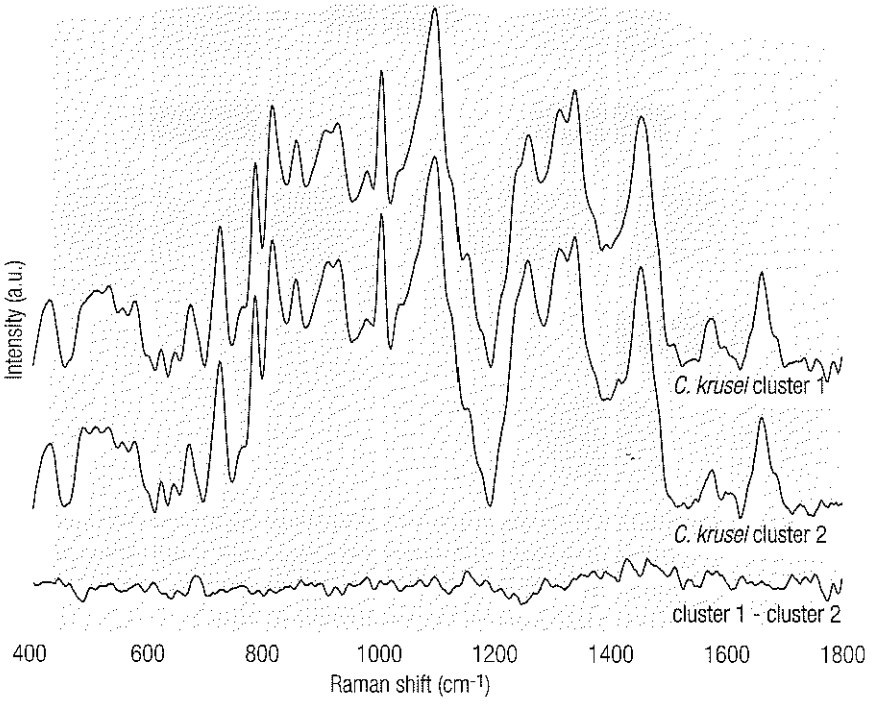
LDA was applied on those PC scores that were most informative for the separation of the different species involved. In order to reduce the complexity of the LDA model used, a method was chosen in which the *Candida* species were separated at different levels. The use of different models in a sequential approach to separate microorganisms based on FT-IR spectra was earlier described by Udelhoven et al.[38]. For preparing the models in this study, the similarity between the species, as observed in the HCA, was used to distinguish the different levels (figure 4). Model 1 separates *C.*

Figure 2



Dendrogram resulting from HCA *of Raman spectra of Candida strains in the* 'SAB-*dataset' (see Materials and Methods section). Squared Euclidean distance measure and Ward's clustering algorithm were used in the analysis.* CAL: *C. albicans,* CGL: *C. glabrata,* CKE: *C. kefyr,* CKR: *C. krusei,* CTR: *C. tropicalis. (a.u. = arbitrary units).*

Figure 3

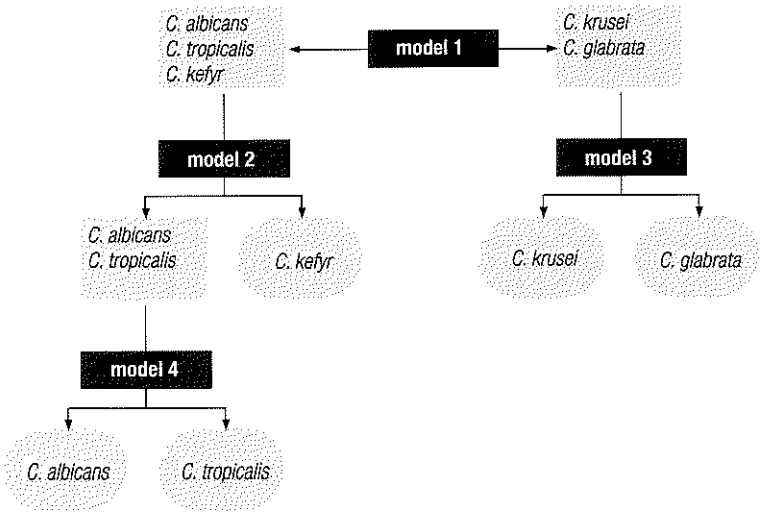


Raman spectra arising from the averages of C. krusei cluster1 (strains 28, 40 and 47) and C. krusei cluster 2 (strains 7, 8, 10, 14, 15 and CBS 573) as shown in figure 2. The difference spectrum at the bottom shows the spectral differences between cluster 1 and cluster 2 (a.u. = arbitrary units).

albicans, *C. tropicalis* and *C. kefyr* from *C. krusei* and *C. glabrata*. This distinction is based on the largest dissimilarity observed. Model 2 discriminates between *C. albicans*/*C. tropicalis* and *C. kefyr*. Model 3 is designed to discriminate between *C. krusei* and *C. glabrata*. Finally, model 4 further separates *C. albicans* and *C. tropicalis*. Based on the outcome of model 1, an unknown spectrum is either projected on model 2 or model 3. When model 2 predicts the Raman spectrum as belonging to either *C. albicans* or *C. tropicalis* the spectrum is finally projected on model 4 to distinguish between these species.

The prediction accuracy of the identification model was determined using a 'leave-one-out' evaluation[34]. The spectra of all but one strain were used to generate the LDA models 1 to 4. For the strains that were included

Figure 4



Schematic representation of the sequential identification procedure based on LDA *models 1 to 4. Spectra to be identified are predicted using model 1, depending on the result the next projection will be on model 2 or 3 etc.. Models are produced based on the major groupings found in the dendrogram of figure 2.*

in both the '*SAB-dataset*' and the *'blood-dataset'*, both spectra were left out. The spectrum or spectra from the strain that was left out was used to test the accuracy of the identification models. By repeating this procedure, and leaving the spectrum or spectra of each strain out in turn, information is obtained on the reproducibility of the identification procedure, i.e. if there was enough discriminating information in the Raman spectra to identify unknown spectra correctly. When the 'leave-one-strain-out' evaluation was performed on the '*SAB-dataset*' all 32 strains (100%) were correctly identified (table 2). This indicates that although the HCA showed two separate *C. krusei* clusters and one misclassification, a supervised method is able to identify characteristic spectral differences between *C. krusei* and *C. glabrata*, which otherwise remain hidden in non-species-specific signal variance. Furthermore, performing the 'leave-one-strain-out' evaluation with both the *'SAB-dataset'* and the *'blood-dataset'* included again yielded a high prediction accuracy of 97.0% (tabel 3). Two strains from the *'blood-dataset'* were misidentified, *C. tropicalis* 40 was predicted as *C. albicans* and *C. krusei* M38 1/96 was identified as *C. glabrata*. Taking the results of the combined dataset into account, there were no significant differences in identification accuracy between the '*SAB-*

Table 2

	Raman identification					
	C. albicans	*C. tropicalis*	*C. kefyr*	*C. krusei*	*C. glabrata*	total
C. albicans	10	-	-	-	-	10
C. tropicalis	-	3	-	-	-	3
C. kefyr	-	-	5	-	-	5
C. krusei	-	-	-	10	-	10
C. glabrata	-	-	-	-	4	4
						32

Results of the 'leave-one-strain-out' evaluation using the 'SAB-dataset'.

Table 3

	Raman identification					
	C. albicans	*C. tropicalis*	*C. kefyr*	*C. krusei*	*C. glabrata*	total
C. albicans	17	-	-	-	-	17
C. tropicalis	1	10	-	-	-	11
C. kefyr	-	-	12	-	-	12
C. krusei	-	-	-	16	1	17
C. glabrata	-	-	-	-	9	9
						66

Results of the 'leave-one-strain-out' evaluation using the 'SAB-dataset' and the 'blood-dataset' combined.

dataset' and the *'blood-dataset'*. This indicates that the pre-treatment or culturing of the *Candida* strains prior to Raman measurements did not significantly influence the accuracy of the LDA models used. Therefore, considering the speed and ease at which various species can be identified, confocal Raman microspectroscopy has the potential to develop into a more powerful and faster technique than the rapid identification systems available today.

Vibrational spectroscopies have been used by several authors to study *Candida* species[21, 22, 32, 35-38]. To our knowledge however, this is the first time that confocal Raman microspectroscopy has been used for identification purposes. Performing measurement directly on the solid culture medium from microcolonies has several advantages in a clinical diagnostic setting. The most obvious advantage is the short time required for obtaining microcolonies on which measurements can be performed. Furthermore, besides the minimal sample handling required, there is no

need to use labels or dyes and there is only a limited need for disposables. A known problem with *Candida* infections on the ICU is that therapy is started relatively late, because of the lack of early clinical manifestations and the delay in laboratory detection procedures. Consequently, *Candida* infections are associated with high mortality rates[24]. The results presented here show that high prediction accuracy could be achieved within 6 hours after a sample was cultured on SAB medium or when blood cultures became positive. In our routine microbiological laboratory, positive BACTEC cultures due to a *Candida* infection are analyzed using the Vitek2 system (bioMerieux, Lyon, France). After the BACTEC culture vials are flagged as positive by the system an additional 24 to 48 hours are required for identification. Rapid recognition of a species with a possible tolerance towards azole agents (*C. glabrata*, *C. tropicalis* and *C. krusei*), present the clinician with an option to monitor very closely the effect of treatment with such an antifungal agent, or choose a different agent, at an early stage of the infection. Our current aim is to use the Raman identification in a prospective study of blood cultures in our tertiary care hospital. We conclude that confocal Raman microspectroscopy may offer a rapid, accurate, and easy to use alternative for the identification of clinically relevant *Candida* species.

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6. Rapid identification of pathogens by vibrational spectroscopy

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6. Rapid identification of pathogens by vibrational spectroscopy

Background: Rapid identification of microbial pathogens can reduce infection-related morbidity and mortality of hospitalized patients. Vibrational spectroscopies are highly suitable for this purpose. Raman spectra and Fourier-transform infrared spectra constitute detailed representations of the overall molecular composition of microbial cells. Because this composition is species (and even strain) specific, the vibrational spectra are too, as was previously shown for *Enterococcus* and *Candida* species [*Kirschner et al, J Clin Microbiol 39, 1763-1770 (2001), Maquelin et al, J Clin Microbiol 40, 594-600 (2002)*]. Moreover only little biomass is required. Raman spectra can be obtained directly of microbial microcolonies, of 10-100 microns in diameter, growing on the solid culture medium. Infrared spectra are obtained of microcolony imprints made on an infrared-transparent window. For most clinically relevant microorganisms such microcolonies will develop in about 6 hours after a solid culture medium is inoculated.

Methods: A prospective clinical study was carried out in which the causative pathogens of blood stream infections in hospitalized patients were identified. Reference libraries were created of Raman and infrared spectra of microorganisms, with a high prevalence in blood stream infections. They were used to develop identification models, based on linear discriminant analysis and artificial neural networks. These models were tested by carrying out vibrational spectroscopic identification in parallel with routine diagnostic phenotypic identification, which has a typical turnaround time of 1 to 2 days.

Findings: Raman and infrared spectra were collected of microcolonies, 6 to 8 hours after microbial growth was detected by an automated blood culture system. High identification accuracy was achieved in both the Raman (92.2%, 106/115) and infrared (98.3%, 119/121) studies.

Interpretation: Vibrational spectroscopies enable simple, rapid and accurate microbial identification. These advantages can be easily transferred to other applications in diagnostic microbiology, e.g. to accelerate identification of fastidious microorganisms.

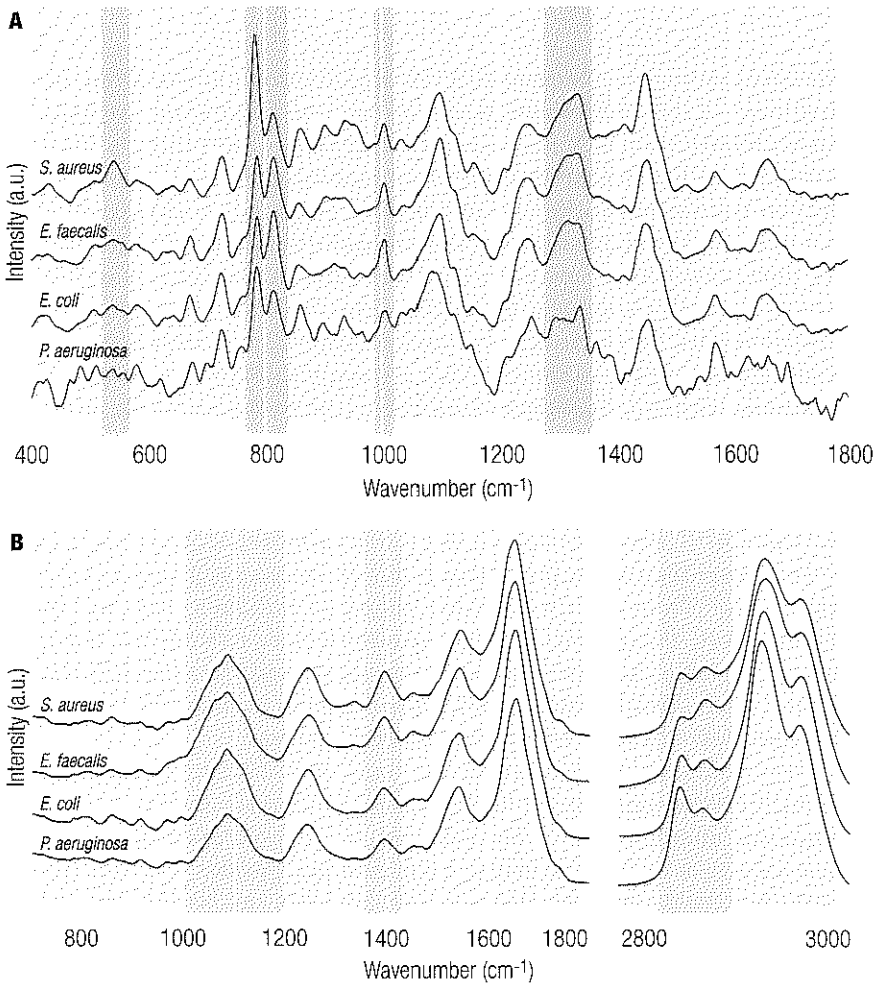
Introduction

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[3]. Most commercially available identification systems in routine use in hospitals are based on physiological and nutritional characteristics of microorganisms. These systems require a pure microbial culture and a high inoculum[16]. Consequently, a turnaround time of 24 hours (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. It has been reported that, as a result of this, 10 to 30% of patients suffering from bloodstream infections in intensive care units do not initially receive correct anti-microbial therapy[12, 28]. Mortality rates in this group have been reported to be 30 to 60% higher than in the group that promptly receives appropriate therapy[7, 12]. Apart from the risk that the empirical treatment may not be effective at all, this practice may lead to adverse toxic side effects[26] and is known to aggravate problems with resistance against anti-microbial agents[9]. Early identification enables the clinician to precisely target a pathogen with the most effective antimicrobial agent.

Novel genotypic approaches for the rapid identification of clinically relevant microorganisms are finding their way into the field of clinical diagnostic microbiology[22, 25]. For example, the amplification of specific gene sequences by the polymerase chain reaction, enables very sensitive methods to be developed[22]. Furthermore, microbes can be detected and identified in complex matrices using fluorescence *in situ* hybridisation, targeting the conserved 16s rRNA[1].

A radically different approach towards the development of identification methods is based on spectroscopic techniques[4, 17, 18]. These techniques are characterized by a minimum of sample handling: no extractions, amplifications, labelling or staining steps of any kind are required. We have developed Raman and Fourier transform infrared (FT-IR) spectroscopic techniques for the rapid and accurate identification of clinically relevant microorganisms. Vibrational spectra reflect the overall molecular composition of a sample. Since different organisms differ in their overall molecular composition, their Raman and FT-IR spectra (figure 1) will also be different. The spectra can serve as spectroscopic fingerprints

Figure 1



Typical Raman [A] *and* FT-IR [B] *spectra of 4 microorganisms as included in the reference database used for the identification of pathogens isolated from blood. Some markedly spectral differences between the species have been highlighted. In* [A]*:* 540 cm^{-1}*, glycosidic ring of carbohydrates;* 780 *and* 810 cm^{-1}*, uracil and phosphate backbone of nucleic acids resp.;* 1004 cm^{-1}*, phenylalanine;* 1250-1350 cm^{-1}*, mixed region with information on proteins, lipids, carbohydrates and nucleic acids. In* [B]*:* 900-1200 cm^{-1}*, carbohydrate region;* 1400 cm^{-1}*, C=O and* COO^- *vibrations in carbohydrates and proteins;* 2850-2900 cm^{-1}*, protein and lipid vibrations (e.g.* 2850 cm^{-1}*,* CH_2 *in fatty acids;* 2870 cm^{-1}*,* CH_3 *in lipids and proteins).*

that enable highly accurate identification of microorganisms[17]. *Enterococcus* species, such as *E. hirae, E. durans,* and the vancomycin-resistant species *E. casseliflavus* and *E. gallinarum,* were correctly identified by both FT-IR and Raman spectroscopy, while most routinely used identification systems perform poorly[11]. Moreover, only a very low inoculum is required to obtain spectra. Within 6-8 hours after starting a culture on a standard solid culture medium, most commonly encountered pathogens develop microcolonies of 10 to 100 µm in diameter, from which reproducible Raman and FT-IR spectra can be obtained. For Raman spectroscopy, spectra can be collected directly from the microcolonies on the solid culture medium. Confocal signal detection[23] is used to adapt the measurement volume to the thin microcolonies, thereby minimizing signal contributions from the culture medium. Remaining signal contributions from the culture medium are corrected for by a non-subjective background signal subtraction algorithm which orthogonalises the spectra to the medium signal[14]. FT-IR spectra can be collected from imprints of microcolonies on an IR-transparent substrate[17]. Microcolonies are transferred from the solid culture medium to the substrate by employing a special stamping device[19], after which the imprint is allowed to dry.

In order to optimise the spectroscopic contrast between different microorganisms, other sources of variation in molecular composition must be minimised. This requires standardization of culturing conditions, in particular the culture medium, incubation temperature and culturing time. Culturing time is important because microbial colonies growing on a solid culture plate become increasingly heterogeneous with respect to molecular composition. Such heterogeneity is absent in small microcolonies, obtained after 6 to 8 hours of culturing[2].

Standardization of culturing conditions and instrument parameters enables the creation of reference libraries of microorganism spectra. Such libraries are the foundation of microorganism identification algorithms, based for example on linear discriminant analysis[13] or on artificial neural networks[27]. These enable the identification of an unknown microorganism on the basis of its Raman or FT-IR-spectrum. Here we present results of the first prospective clinical study in which the causative pathogens of blood infections were identified by vibrational spectroscopic methods.

Methods

Sample preparation For Raman spectroscopy, the reference strains were seeded at 10^3 colony forming units/ml in 10 ml blood from healthy volunteers. Aerobic culture vials (bacteria) or Mycosis culture vials (yeasts) of the automated BACTEC 9240 blood culture system (Becton Dickinson, Cockeysville, MD, USA) were inoculated with these samples. When the culture vials were flagged as positive by the system, 100µL of the liquid culture medium was plated on Mueller Hinton medium (Merck, Darmstadt, Germany) which supports growth of a wide range of bacteria or Sabouraud + 2% glucose medium (Merck) for yeasts. These cultures were incubated for 6 hours at 37°C or 30°C respectively, prior to Raman measurement of microcolonies. Patient blood samples that were positive according to the BACTEC system were treated in the same manner as described above.

For the infrared measurements of reference strains, three calibrated platinum loops (1 mm in diameter) of biomass from the third quadrant of an overnight culture were suspended in 10 ml pre-warmed liquid medium (Luria broth, Merck). The suspensions were diluted 100 fold for yeasts and 1000 fold for bacteria, and an aliquot of 100 µl of this dilution was spread onto pre-warmed Caso agar plates (Merck) (bacteria) or Sabouraud + 2% glucose agar plates (Merck) (yeasts). Positive patient samples from the BacT/Alert system (Organon Teknika, Eppelheim, Germany) were diluted 100 fold in Luria broth in order to remove the charcoal particles from the culture medium. Two aliquots of 100 µl were than spread on Caso medium and Sabouraud +2% glucose medium respectively. After an incubation period of 6 to 8 hours at 37 °C, the microcolonies were transferred from the agar plate onto a ZnSe substrate, using a specially designed stamping device[19]. After drying in air for 15 minutes, the microbial spots deposited onto the infrared-transparent plate were measured.

Phenotypic identification Microbial identification in the routine clinical diagnostic laboratories was performed using phenotypic identification by API and Vitek systems (both from bioMerieux, Marcy-l'Etoile, France).

Confocal Raman microspectroscopy Raman spectroscopic measurements were performed as previously described[23, 29]. Briefly, the solid culture medium with microcolonies was placed directly under the microscope of a System 1000 Raman microspectrometer (Renishaw plc, Wotton-un-

der-Edge, UK). An 80x near-infrared objective (MIR Plan 80x/0.75, Olympus) focused 100-150 mW laser light (830 nm) on the sample from a titanium-sapphire laser (model 3900, Spectra Physics, Mountain View, CA) pumped by an argon-ion laser (Series 2000, Spectra Physics). Per plate 5 microcolonies were selected. Within each microcolony, spectra were obtained from 10 randomly chosen locations, using a signal collection time of 30 seconds per measurement. For each sample measured, the 50 spectra thus obtained were averaged.

FT-IR microspectroscopy Fourier transform infrared spectra were recorded on an FT-IR microscope, (IR Scope II interfaced to an IFS 28/B spectrometer, Bruker Optics, Karlsruhe, Germany), equipped with a motorized xy-stage, a 15x Cassegrain-objective and a broadband MCT (mercury cadmium telluride) detector. All spectra were acquired over 256 scans. Fourier transformation was done using a Blackmann-Harris 3 term apodization function and a zerofilling factor of 4, resulting in a nominal resolution of 6 cm^{-1}. Per imprint, 10 microcolonies were measured. A background spectrum of the ZnSe substrate was recorded before each sample measurement in order to account for variations in water vapour and CO_2 level, resulting in a measurement time of 18 min. per sample.

Data analysis Raman spectrum analyses were performed using the Matlab 5.3 software package (The Mathworks Inc., Natick, MA) and the multivariate statistical analysis toolbox PLS-toolbox 2.0.0c (Eigenvector Research Inc., Manson, WA). First derivatives of the spectra were used in order to minimise the influence of the broad featureless background due to fluorescence, on which the spectra are superimposed and which tends to vary from sample to sample[14]. Evaluation of infrared spectral data (calculation of derivatives, normalisation, etc.) was performed using the OPUS NT software version 3.1 (Bruker Optics). First derivatives of the original IR spectra were calculated using a 9-point Savitzky-Golay filter to enhance the resolution of superimposed bands and to minimise problems from unavoidable baseline shifts. For spectrum analysis, the first derivative spectra between the range 780-1780 cm^{-1} were vector normalised as described previously (Bruker Optics, Handbook).

Before further analyses of Raman spectra, a data reduction was performed using principal component analysis (PCA)[8]. The maximum number of n-1 principal components was calculated (n being the number of spectra in the analysis), typically accounting for 99-100% of the variation in the data set.

Hierarchical cluster analysis (HCA) of Raman spectra was performed on the n-1 principal component (PC) scores, obtained for each spectrum, using Ward's clustering algorithm and the squared Euclidean distance measure to generate a dendrogram. For HCA of Raman spectra, the SPSS statistical software package (SPSS, Chicago, IL) was used. For infrared data, spectral distances were calculated from Pearson's correlation coefficient and Ward's algorithm was used for hierarchical cluster analysis (OPUS).

For the development of a Raman identification algorithm, linear discriminant analysis (LDA) was performed on the basis of the principal component scores using SPSS and the identification results of the routine identification methods. Selection of principal components that were included in the LDA model was based on the Wilk's lambda method and the 95% F-test inclusion criterion, thus maximising group separation[20]. The model based on the training set was evaluated using the leave-one-out method[20].

For the artificial neural network (ANN) analysis the dimensionality of the FT-IR data was reduced by a manual preselection of wavenumber region(s) containing the most significant spectral information, which was different for different classification levels (figure 2b). A boxcar averaging[5] with two neighbouring data points followed, to further reduce the number of input neurons of the ANN. Finally PCA was performed and the first few scores were retained according to Kaiser's criterion[10].

All multilayer perceptron (MLP) analyses were carried out with the Neurodeveloper software version 2.1 (Synthon, Gusterath, Germany). For each classification level, a fully-connected feed-forward neural network, consisting of 3 neuron layers was trained using the Rprop algorithm[24]. For each neural net, the first layer contained the first few PC scores as input neurons (capturing 99 %-100 % of the variance in the dataset), one hidden layer and an output layer with 2 to 3 output neurons depending on the classification level (figure 2b). Two independent data sets were used for internal calibration of the ANN's, where approximately 80 % of the available reference spectra were employed for the training and the remaining samples for validation purposes. The network that produced the smallest sum of squared errors in the validation pattern set was included in the final hierarchical classification system comprising one toplevel and six subsequent sub-classification levels. The classification results from the trained neural networks were evaluated using the "winner takes all" function[30]. Additionally, the model based on the training set was evaluated using the leave-one-out method.

Results

Reference databases of microorganism spectra and development of microorganism identification models Separate databases of reference Raman and FT-IR spectra were created representing approximately 85% of the microbial species most commonly encountered in blood infections of patients treated in the intensive care units of the University Hospital Rotterdam and the Rudolf Virchow Hospital (Berlin, Germany) (see caption of table 1). The strains included in the reference databases were either well-characterized clinical isolates or were obtained from culture collections.

For the Raman identification of bacteria, a similar approach was used as described previously for the identification of *Candida* species[13]. Briefly, hierarchical cluster analysis of the spectra in the reference library was used as a non-subjective method to determine the major groupings in the data set. These groupings were used as the starting point for the development of an identification tree based on linear discriminant analysis (LDA) (figure 2a). For each division in the tree a LDA-model was developed. Identification of a microorganism occurs by entering its spectrum in this identification tree at model 1. Depending on the outcome, it proceeds to model 2 or 3 etc.. Likewise, a multilayered neural network (figure 2b), consisting of one top-level net and several subsequent subnets, was developed for identification based on infrared spectra.

A first validation of the LDA and artificial neural network (ANN) models was performed using a 'leave-one-out' method in which the spectra of all but one of the reference strains were used to generate the respective models. The strain that was left out, was identified on the basis of its spectrum, in order to test the prediction models. This procedure was repeated for each strain. The results in table 1 show that at the genus-level the models resulted in a near-perfect identification for both databases. In the Raman database the exceptions were 1 *Streptococcus* strain that was identified as *E. faecalis*, and 1 *E. coli*, which is identified as *E. cloacae*. Misidentifications also occurred for 2 *Streptococcus* strains being identified as *E. faecalis* in the infrared database. At the species-level the models performed nearly as good, with 1 CNS strain being misclassified as *S. aureus* in both the Raman and infrared databases. Only the separation of *E. aerogenes* and *E. cloacae* by model 6 of the Raman identification proved more problematic with an 80.0% correct identification in the leave-one-out evaluation. For the yeast strains included in the databases, high identification accuracy was achieved as well (table 1).

Table 1

	Raman		Infrared	
	correct ID	*misidentification*	*correct ID*	*misidentification*
S. aureus	9 (100%)		13 (100%)	
CNS	25 (96.2%)	1x *S. aureus*	17 (94.4%)	1x *S. aureus*
E. coli	16 (97.3%)	1x *E. cloacae*	12 (100%)	
E. cloacae	9 (81.8%)	2x *E. aerogenes*	6 (100%)	
E. aerogenes	7 (77.8%)	2x *E. cloacae*	6 (100%)	
P. aeruginosa	9 (100%)		5 (100%)	
E. faecalis group	8 (100%)		9 (100%)	
E. faecium group	8 (100%)		9 (100%)	
Streptococcus spp	8 (88.9%)	1x *E. faecalis*	9 (82%)	2x *E. faecalis group*
C. albicans	6 (85.7%)	1x *C. kefyr*	7 (87.5%)	1x *C. dubliniensis*
C. dubliniensis	-		5 (100%)	
C. glabrata	5 (100%)		5 (100%)	
C. kefyr	7 (100%)		4 (100%)	
C. krusei	7 (100%)		4 (100%)	
C. tropicalis	8 (100%)		5 (83.3%)	1x *C. albicans*

Results of the leave-one-out evaluation of the Raman and infrared prediction models, based on the strains in the reference databases. For the different species-groups, the following species were included (number of strains in Raman database/infrared database): CNS; *S. epidermidis (10/6), S. schleiferi (3/2), S. saprophyticus (3/2), S. haemolyticus (3/2), S. capitis (3/2), S. lugdunensis (2/-), S. warneri (2/2), S. hominis (-/2); Streptococcus spp; S. agalactiae (1/-), S. oralis (2/3), S. salivarius (2/2), S. pneumoniae (1/2), S. pyogenes (3/3), Streptococcus variant Gr. A (-/1); Enterococcus faecium group; E. faecium (6/5), E. hirae (1/2), E. durans (1/2); Enterococcus faecalis group (identified as such for infrared only); E. faecalis (6), E. casseliflavus (1), E. gallinarum (2).*

Analysis of clinical samples Over a 4-month period, all consecutive positive blood cultures from patients in the intensive care wards (ICUs), and a random selection of positive blood cultures from patients in other wards of the University Hospital Rotterdam were used to test the Raman spectroscopic identification method. A total of 135 blood cultures was collected from 92 patients. Bacteria were isolated from 129 blood cultures and 6 were positive for yeast. Similarly, all positive blood cultures from the ICUs of the Rudolf Virchow Hospital were collected over a 3-month period to test the FT-IR microspectroscopic identification approach. A total of 138 blood cultures were examined from 121 patients, of which 131 contained bacteria and 7 contained yeast.

Figure 2

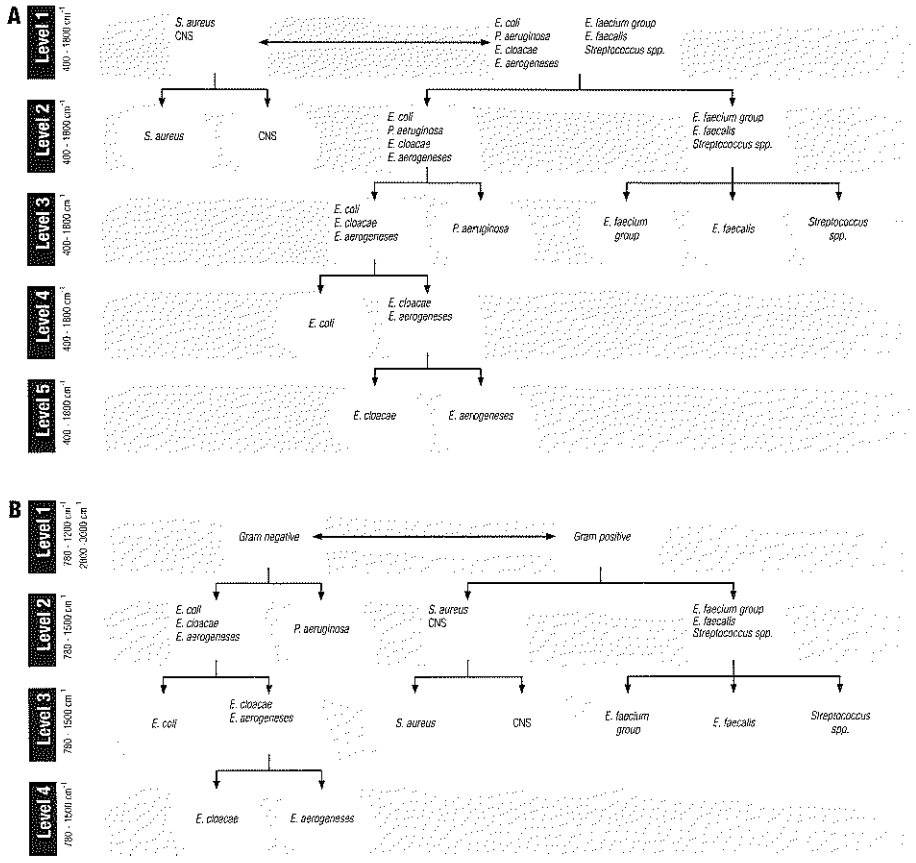


Figure 2
Sequential identification schemes used for the identification of bacteria. (A) Sequential LDA *model used for the identification of microorganisms based on their Raman spectrum, (B) Schematic diagram of the hierarchical network used to identify bacteria on the basis of their infrared spectra. See text for more details.*

A small volume of the liquid culture medium from an automated blood culture system was incubated for 6 to 8 hours on two separate solid culture media. One to support growth of a wide range of bacteria and one to support the growth of yeasts. After the incubation period, cell morphology was inspected by direct microscopy to distinguish bacteria from yeasts. Based on this distinction, vibrational spectra were collected

Table 2

	Raman		Infrared	
	correct ID	*misidentification*	*correct ID*	*misidentification*
S. aureus	19 (100%)		27 (96.4%)	1x *S. aureus*
CNS	37 (97.4%)	1x *Streptococcus*	52 (98.1%)	1x CNS
E. coli	22 (91.7%)	2x *E. aerogenes*	12 (100%)	
E. cloacae	2 (40%)	1x *E. aerogenes*	2 (100%)	
E. aerogenes	3 (100%)		-	
P. aeruginosa	5 (100%)		1 (100%)	
E. faecalis group	3 (100%)		6 (100%)	
E. faecium group	1 (100%)		5 (100%)	
Streptococcus spp	8 (72.7%)	2x *E. faecalis* 1x *E. aerogenes*	7 (100%)	
Candida albicans	6 (100%)		5 (100%)	
Candida glabrata	-		1 (100%)	
Candida tropicalis	-		1 (100%)	

Comparison of phenotypic and vibrational spectroscopic identification of patient samples included in the prospective study. For Raman spectroscopy 115 samples were included and for infrared spectroscopy 121 samples. Indicated are the number of strains included and the percentage correct identification (between brackets), per species or species-group.

from isolates on the culture medium that best supported the growth of that organism, e.g. Mueller Hinton or CASO medium for bacteria and Sabouraud+2% glucose for yeasts (details in Methods section). Raman and infrared spectra thus obtained from patient samples were entered in the respective identification trees for species identification as described above (figure 2).

When a strain was identified by the routine identification methods as a member of a genus, that was not included in the reference spectral databases, it was excluded from the comparison between routine identification methods and vibrational spectroscopic identification. This was the case for 17 strains in both the Raman and the FT-IR tests. In addition, three samples containing mixed cultures of very similar cell morphology, such as *E. coli* and *E. aerogenes*, were excluded from the comparison between Raman spectroscopy and routine identification, as it was not obvious which species was measured in the Raman experiments. However, in all cases the Raman identification corresponded with one of the components in the mixed culture.

Comparison of phenotypic and vibrational spectroscopic identification methods

For 106 out of the remaining 115 samples Raman spectroscopic identification corresponded with the phenotypic identification of the routine diagnostic test (table 2). Four of the 9 misidentifications can be rationalised. The 2 *E. coli* isolates identified as *E. aerogenes* were collected from one patient, only 5 hours apart, making it very likely that the strains were identical. A similar situation occurred for 2 of the 3 *E. cloacae* isolates identified by the Raman method as *E. aerogenes*. Two of the 3 *Streptococcus* species that were misidentified (*S. mitis* and *S. anginosus*) belonged to species not included in the reference database. Infrared spectroscopy correctly identified 119 out of 121 samples (table 2).

Hence, 92.2% (106/115) of the microorganisms included in the comparison were accurately identified by Raman spectroscopy and 98.3% (119/121) by infrared spectroscopy. A near-perfect identification of the main contributors to the bloodstream infections (staphylococci and *E. coli*) was obtained by both methods. The perfect identification of the samples with *Candida* species 6 (Raman) to 8 (infrared) hours after a positive signal from the automated blood culture systems is particularly encouraging, as the routine phenotypic identification required an additional 48 hours.

Discussion

The results of this prospective study show the potential of vibrational spectroscopic methods for rapid and accurate microbial identification. The fundamental strength of this approach is emphasised by the fact that this study was performed at 2 hospitals, employing different spectroscopic methods, different sample handling protocols, different methods for signal analysis, and different prediction models, but yielding equally good results. Correct identification of 92.2% and 98.3% of the samples (for the Raman and infrared methods respectively) was achieved for spectra that were available 6 to 8 hours after signalling of the automated blood culture system.

The signal collection times used in this study (25 minutes for Raman and 18 minutes for FT-IR) currently limit the sample throughput. However, with further optimisation of the instrumentation a reduction in signal collection time to only a few minutes is feasible. This will also facilitate the analysis of mixed cultures by performing the identification on more microcolonies selected on the basis of cell and colony morphology. Apart from the clinical significance a practical reason for our choice to target blood infections first, was that they are nearly always due to a single pathogen. Development of dedicated culture media, which will enhance the rate of microcolony development, is expected to further shorten the necessary cultivation time.

Extension of the reference spectral databases to include a wider range of microorganisms (genera, species and strains) will further increase prediction accuracy, as well as the development of other targeted medical microbiological applications. Intra-abdominal infections with *Candida* species for example, are associated with high mortality rates[6]. Rapid identification is important since some species are intrinsically resistant to antifungal agents of the azole-group, which are usually the agents of first choice in treating this kind of infections. We have previously shown that highly accurate rapid identification (97%) of *Candida* species by vibrational spectroscopic methods is possible[13]. A clinical pilot study in which prospective *Candida*-species identification in intra-abdominal infections by Raman spectroscopy is tested, is currently underway. Another potential application for spectroscopic techniques, which makes use of the fact that very little biomass is needed is the identification of fastidious microorganisms, e.g. Mycobacteria. Routinely used phenotypic identification methods can take between 2 and 8 weeks to be completed for these slow-growing bacteria[21]. However, the rapid identification of *Mycobacterium* spp. is becoming increasingly important, due to the increased

incidence over the last decade. Rapid discrimination between *M. tuberculosis* and *M. avium*, currently the topic of a FT-IR spectroscopic study, is of prime importance for effectively guiding the choice of antibiotic therapy, as the two life-threatening infections in immuno-compromised patients require different types of management and therapy.

Apart from enabling rapid identification, vibrational spectroscopic techniques require virtually no sample handling and no consumables apart from the culture medium, which is in sharp contrast with other rapid identification techniques that are under development, such as molecular genetic approaches. This implies that vibrational spectroscopies lend themselves well for automation, can be used by non-experts, and will be relatively inexpensive to use. Moreover, vibrational spectroscopy also offers possibilities for the development of rapid drug-susceptibility testing[15]. We conclude that Raman and FT-IR spectroscopies provide a novel answer to the need for rapid microbial identification in a clinical diagnostic setting.

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7. Summary

The development of a novel, rapid identification method for microorganisms is presented. The method is based on confocal Raman microspectroscopy, a so-called vibrational spectroscopy, which is based on the inelastic scattering of light. By an interaction with light, molecules can be excited to higher vibrational states. Vibrational spectra are highly cell-type specific and consequently, a Raman spectrum provides a "*spectroscopic fingerprint*" that is unique and corresponds to the biochemical composition of that sample. In the first chapter a general introduction is given to clinical diagnostic microbiology and Raman spectroscopy, the 2 subjects that are combined in this thesis. Subsequent chapters describe the different steps in the development of the Raman identification method: accuracy, reproducibility and speed.

From the results described in chapter 2 it becomes clear that Raman spectroscopy enables high accuracy identification methods to be developed. Raman and infrared spectra were collected from a collection of *Enterococcus* strains. The strains were cultured overnight and some biomass of these cultures was smeared on an optical substrate for measurements. When the results of an objective cluster analysis were compared with an identification according to commercially available phenotypic identification systems, some strains were not classified according to their species. Because it is known that commercially available systems perform poorly with the identification of less common *Enterococcus* species, the strains were also analyzed using the 'gold standard' (16S RNA sequence analysis). From a comparison between the phenotypic and genotypic methods it was shown that the classification based on the infrared and Raman spectra was correct and comparable to the one obtained by the gold standard.

Besides addressing the accuracy of identification, the aim of this study was to make the method as rapid as possible. In order to achieve

this, the amount of pre-treatments to the sample and the culturing time required prior to Raman measurements were minimised. Using a small confocal measurement volume, measurements were performed on very small microbial colonies, without removing them from the solid culture medium. After 6 hours of culturing at 37°C, microcolonies with a diameter of 10 to 100 μm developed for most common, clinically relevant microorganisms. Due to the limited thickness and the irregular shape of such colonies however, it was unavoidable to measure some of the underlying culture medium together with the microbial cells in the microcolonies. The amount of signal contribution from the culture medium is variable and critically depends on the actual focussing depth of the laser and the thickness of a microcolony. Therefore, this signal contribution will interfere with the reproducibility of Raman spectra and needs to be corrected for. No features could be found in the combined bacteria and culture medium spectrum however, that indicated how much signal from the culture medium was initially present. Chapter 3 describes a method that deals with this problem. A mathematical routine, involving vector algebra, was developed and evaluated for the non-subjective correction of spectra for the variable signal contributions of the culture medium. It was shown that reproducible spectra could be obtained independent of the culture medium signal contribution initially present.

In chapter 4 the method to collect "*in situ*" vibrational spectra from microbial colonies was used to study the heterogeneity within colonies of different ages. Microbial cultures of 6, 12 and 24 hours old were used to collect spectra from different locations in the colonies. For the 3 microbial species used (*S. aureus*, *E. coli* and *C. albicans*), the colonies of 12 and 24 hours were most heterogeneous. Reproducible layers were found in these colonies, which were related to differences in growth-phase of the cells in that particular layer. Six hour old microcolonies on the other hand, did not show any heterogeneity. It was concluded that these microcolonies were best suited to build a database of Raman and infrared spectra, which could serve as a reference for identification purposes. Furthermore, with the methodology developed it seems possible to study the composition of biofilms in a similar way as the colonies were studied, i.e. by probing at different depths.

Based on the results of the studies presented in previous chapters, chapter 5 describes how databases can be constructed of Raman spectra from microbial microcolonies. A collection of strains from the yeast genus *Candida* was used. The procedure started either from a culture on a solid culture medium or from a positive vial of an automated blood culture system. Prior to Raman measurements, strains were sub-cultured on a new solid culture medium for 6 hours to form microcolonies. Using

multivariate statistical analyses, high prediction accuracy (97% to 100%) was obtained employing the Raman method. Identification with Raman microspectroscopy may therefore be significantly faster than identification using commercially available identification systems that often require 24 to 48 hours before a reliable identification is obtained.

Finally, a clinical study was performed in which blood cultures were analyzed by Raman- and infrared spectroscopy, parallel to the routine diagnostic microbiological analysis. Chapter 6 describes this study, in which the knowledge obtained from the results of the previous chapters is combined. First, reference libraries were created of vibrational spectra of bacteria and yeasts that are frequently encountered in blood stream infections in hospitalized patients on intensive care units. These libraries were used to develop species identification models, based on linear discriminant analysis and artificial neural networks. The generated models were used for a prospective study of blood cultures from patients on the intensive care and from a random selection of other wards. Six to eight hours after blood samples were identified as positive by an automated blood culture system, Raman and infrared spectra were collected of microcolonies and analyzed by means of the identification models. High identification accuracy was achieved in both the Raman (92.2%, 106/115) and infrared (98.3%, 119/121) studies.

It can be concluded that confocal Raman microspectroscopy is a rapid and accurate technique for the identification of microorganisms. Not only the possibilities of rapid identification are of interest to clinical diagnostic microbiology, also the possibilities to study microorganisms at the strain level (epidemiology), and test for antibiotic susceptibility make the technique a powerful tool for the clinical laboratory.

8. Samenvatting

In dit proefschrift wordt de ontwikkeling van een nieuwe, snelle identificatiemethode voor micro-organismen gepresenteerd. De methode is gebaseerd op confocale Raman-microspectroscopie, een zogenaamde vibratiespectroscopie welke gebaseerd is op de inelastische verstrooiing van licht. Tijdens een interactie met licht kunnen moleculen geëxciteerd worden tot een hoger vibratieniveau. Vibratiespectra zijn uiterst specifiek voor een celtype, met als gevolg dat een Ramanspectrum een unieke *"spectroscopische vingerafdruk"* is, welke correspondeert met de biochemische samenstelling van een monster. In het eerste hoofdstuk wordt een algemene inleiding gegeven in de klinische diagnostische microbiologie en Raman spectroscopie, de 2 onderwerpen welke verenigd zijn in dit proefschrift. De daaropvolgende hoofdstukken beschrijven de verschillende stappen welke hebben geleid tot de ontwikkeling van de Ramanspectroscopische identificatiemethode: nauwkeurigheid, reproduceerbaarheid en snelheid.

Uit de resultaten zoals die beschreven staan in hoofdstuk 2 wordt duidelijk dat Ramanspectroscopie gebruikt kan worden voor de zeer nauwkeurige identificatie van micro-organismen. Raman- en infraroodspectra werden gemeten van een collectie *Enterococcus* stammen. De stammen werden overnacht gekweekt waarna er wat biomassa van de culturen op een optisch substraat werd gesmeerd om daar de metingen op te verrichten. In een vergelijking van de resultaten van een objectieve clusteranalyse met die van een commercieel verkrijgbaar fenotypisch identificatiesysteem, werden sommige stammen niet volgens het juiste species geclassificeerd. Omdat het bekend is dat commercieel verkrijgbare identificatiesystemen de minder vaak voorkomende *Enterococcus* species niet nauwkeurig kunnen identificeren, werden de stammen ook geanalyseerd met de "gouden standaard" (16S RNA sequentie analyse). Uit de vergelijking tussen de fenotypische en genotypische methoden

bleek dat de classificatie gebaseerd op infrarood- en Ramanspectra correct was en vergelijkbaar met de gouden standaard.

Naast de aandacht voor nauwkeurigheid van de identificatie, was het een doel van het hier gepresenteerde onderzoek om de methode zo snel mogelijk te maken. Om dit te bereiken werden het aantal voorbehandelingen en de kweektijd, die nodig zijn voor dat Raman metingen verricht kunnen worden, geminimaliseerd. Door gebruik te maken van een klein, confocaal meetvolume, was het mogelijk om uiterst kleine microbiële kolonies direct op de vaste voedingsbodem te meten. Na 6 uur kweken bij 37°C vormen de meest voorkomende, klinisch relevante, microorganismen microkolonies van 10 tot 100 µm in diameter. Door de beperkte dikte en onregelmatige vorm van dergelijke kolonies, is het echter onvermijdelijk om samen met de cellen in de microkolonie een deel van het onderliggende kweekmedium mee te meten. De signaalbijdrage van het kweekmedium is variabel en is volledig afhankelijk van de focusdiepte van de laser en de dikte van de microkolonie. Deze bijdrage zal daarom de reproduceerbaarheid van de Ramanspectra beperken en dient derhalve gecorrigeerd te worden. In het gecombineerde bacterie en medium spectrum konden geen spectrale kenmerken gevonden worden, welke een indicatie gaven van de initiële signaalbijdrage van het kweekmedium. In hoofdstuk 3 wordt een oplossing voor dit probleem besproken. Een mathematische routine, gebaseerd op vectoralgebra, werd ontwikkeld en geëvalueerd voor de niet-subjectieve correctie van Ramanspectra voor de signaalbijdrage van het kweekmedium. Er werd aangetoond dat het mogelijk is reproduceerbare spectra te verkrijgen, onafhankelijk van de medium-signaalbijdrage die in eerste instantie aanwezig is.

In hoofdstuk 4 werd de methode, om "*in-situ*" vibratiespectra te meten, gebruikt voor het bestuderen van de heterogeniteit in kolonies van verschillende leeftijden. Microbiële culturen van 6, 12 en 24 uur werden gebruikt om spectra te verzamelen van verschillende posities in de verkregen kolonies. Van de 3 species die gebruikt werden (*S. aureus*, *E. coli* en *C. albicans*) waren de kolonies van 12 en 24 uur oud het meest heterogeen. In deze kolonies werden reproduceerbare lagen gevonden, welke gerelateerd werden aan verschillen in groeifase van de cellen in die specifieke lagen. Zes uur oude kolonies daarentegen vertoonde geen enkele heterogeniteit. Er werd geconcludeerd dat deze microkolonies het meest geschikt waren om databases te maken van Raman- en infraroodspectra, welke kunnen dienen als referentie voor identificatie doeleinden. Bovendien lijkt het mogelijk om met de beschreven techniek de samenstelling van biofilms te bestuderen door op verschillende lagen in de biofilm metingen te doen, net als in de kolonies werd gedaan.
Uitgaande van de resultaten van de studies zoals die beschreven werden

in voorgaande hoofdstukken, wordt in hoofdstuk 5 beschreven hoe databases geconstrueerd kunnen worden van Ramanspectra van microkolonies. Een collectie stammen van het gistgenus *Candida* werd hiervoor gebruikt. De procedure werd gestart van een kweek op een vaste voedingbodem of vanuit een positieve kweek van een geautomatiseerd bloedkweeksysteem. Voorafgaand aan de Ramanmetingen werden de stammen op een nieuwe vaste voedingbodem geënt en 6 uur geïncubeerd om microkolonie te verkrijgen. Door gebruik te maken van multivariaat statische analyses, was het mogelijk om met de Ramanmethode een hoge identificatienauwkeurigheid te halen (97 tot 100%). Identificatie met Ramanspectroscopie zou daarom significant sneller kunnen zijn dan de commercieel verkrijgbare identificatiesystemen, welke meestal 24 tot 48 uur nodig hebben om tot een betrouwbare uitslag te komen.

Tenslotte werd er een studie uitgevoerd waarin bloedkweken geanalyseerd werden met Raman- en infraroodspectroscopie, parallel aan de routine diagnostiek van het microbiologisch laboratorium. Hoofdstuk 6 beschrijft deze studie waarin de resultaten uit voorgaande hoofdstukken gecombineerd werden. Eerst werd er een referentiebibliotheek opgebouwd van vibratiespectra van bacteriën en gisten die het meest frequent geïsoleerd worden uit bloedkweken van patiënten op de intensive care afdeling. Deze bibliotheken werden gebruikt om een model te ontwikkelen voor species identificatie, gebaseerd op lineaire discriminant analyse en artificiële neurale netwerken. De ontwikkelde modellen werden vervolgens gebruikt om prospectief de bloedkweken te analyseren van intensive care patiënten en een willekeurige selectie van patiënten van andere afdelingen. Zes tot acht uur nadat bloedkweken als positief werden aangegeven door een geautomatiseerd bloedkweeksysteem, werden Raman- en infraroodspectra metingen verricht aan microkolonies en geanalyseerd met de identificatiemodellen. Hoge identificatienauwkeurigheid werd behaald voor zowel de Raman (92,2%, 106/115) als de infrarood (98,3%, 119/121) methoden.

Er kan geconcludeerd worden dat confocale Raman-microspectroscopie een snelle en nauwkeurige techniek is voor de identificatie van micro-organismen. Niet alleen de mogelijkheid tot snelle identificatie, maar ook de mogelijkheden om micro-organismen op stamniveau te bestuderen (epidemiologie) en antibiotica gevoeligheid te testen, bepalen dat de techniek van belang is voor het klinisch laboratorium.

9. Dankwoord

Nou daar ligt het dan, mijn proefschrift. Alle klassieke symptomen van een promovendus heb ik wel gehad denk ik: enthousiast beginnen, er achterkomen dat je onderbetaald wordt, gekibbel met je begeleider, stress halverwege maar vooral op het eind en een jaar extra nodig hebben om het boekje klaar te krijgen. En als je me nou vraagt of ik er trots op ben? Jaren lang geen besef gehad van de buitenwereld, gespecialiseerd op een heel klein stukje van de wetenschap, duur en relatief oud om de arbeidsmarkt op te gaan, constant maar tijdelijke aanstellingen, moeten soebatten voor onderzoeksgeld... Ja, ik ben er trots op en het is een grote troost dat ik medeplichtigen heb. Want ondanks dat alleen mijn naam op de voorkant van dit boekje staat, is de totstandkoming ervan alles behalve een solo-actie geweest. Er zijn vele mensen die actief of op de achtergrond hebben bijgedragen aan het onderzoek.

Om te beginnen wil ik professor Bruining en Gerwin Puppels bedanken voor de mogelijkheid die ze mij hebben geboden dit promotieonderzoek uit te voeren. Professor Bruining, het was mij tijdens het sollicitatiegesprek al duidelijk dat u een man van daden bent, nog meer dan van woorden. Binnen 2 minuten na het gesprek had u het al besloten: 'Als chirurg op de intensive care moet men snel beslissingen nemen, dus we hebben besloten u de positie aan te bieden'. Tot op de dag van vandaag ben ik blij met uw beslissing. Het heeft mij de mogelijkheid geboden mijn steentje bij te dragen aan dit unieke onderzoek. Gerwin ook jij bent een man van weinig woorden. Op grond van de advertentie in de Volkskrant wilde ik wel eens wat meer weten over de vacature en ik besloot je te bellen. Na dit zeldzaam korte gesprek had ik mijn vragenlijstje afgewerkt en had jij slechts een handje vol woorden verspild. Ondanks die eerste indruk besloot ik toch te solliciteren en al snel ontdekte ik dat die communicatieve spaarzaamheid typisch Twents was. Alhoewel het soms moeilijk communiceren is het iemand die 3 enigszins

samenhangende woorden als volzin beschouwd, denk ik toch dat onze samenwerking mooie vruchten heeft afgeworpen. Zo af en toe hebben we elkaar wel eens tot een uiterste gedreven, waarschijnlijk het gevolg van overeenkomsten in onze karakters. De ongelooflijke lachpartijen (tot de tranen ons over de wangen biggelden) waren er gelukkig meer en die werkten heel relativerend.

Het is het multidisciplinaire karakter van het onderzoeksteam dat het mogelijk maakt een onderzoek uit te voeren zoals wij dat de afgelopen jaren hebben gedaan. Ik ben dan ook veel dank verschuldigd aan de mensen die deel uit maken (hebben gemaakt) van het team. In den beginne waren er Tom Bakker Schut, Peter Caspers en Rolf Wolthuis... en die zijn er nog steeds. Jongens, bedankt voor de samenwerking in de afgelopen jaren. Ik heb ontzettend veel geleerd van jullie drieën, als bijna onuitputtelijke bron van kennis op het gebied van optiek, spectroscopie, computers en analyse technieken. Het was eerst wel even wennen hoor, zo'n laboratorium dat constant op z'n kop staat. Als microbioloog huiverde ik van die chaos en probeerde ik daar orde (lees netheid) in te scheppen. Inmiddels heb ik van jullie beta's de chaostheorie een beetje leren accepteren en schuif ik gewoon de hele kolere zooi opzij als ik m'n metingen wil doen. Het is een kwestie van genoeg alcohol gebruiken (OP DE TAFEL, JONGENS), om het gevaar voor jullie zo veel mogelijk in te perken. Ik hoop dat we nog lang als 'harde kern' op het laboratorium kunnen samenwerken. Tom, onze nestor, jij bent voor mij het prototype van een wetenschapper: filosofisch, anarchistisch, geen carrièrewensen en ongelooflijk slim op vele gebieden. Rolf, ook jij bent thuis op velerlei gebied en het verbaast me wel eens dat je bijna op ieder gespreksonderwerp wel een steekhoudende mening hebt, ook al bestaat die regelmatig maar uit enkele woorden. Net als die Tukkers ben ook jij niet altijd even royaal in je tekst en je telefoongesprekken die voornamelijk uit het woord 'moi' bestaan met hooguit een herhaling daarvan zijn legendarisch. Ik ben blij dat je samen met Peter wilde paranimfen, te meer omdat we de afgelopen 5 jaar met z'n drieën een zelfde doel hebben nagestreefd. Ik heb de eindlijn gehaald en ik weet zeker dat jullie die binnenkort ook gaan halen. Peter, ik denk dat we vaak andere collega's tot wanhoop hebben gedreven met ons specifieke gevoel voor humor. Het was niet ongewoon dat wij al dubbel lagen voordat anderen enig idee hadden waar de aanzet tot een grap nu eigenlijk toe had moeten leiden. Maar ja, snelle jongens als we zijn... en wat die zieke geest betreft: mea culpa, mea maxima culpa.

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we je naar huis moeten sturen want anders was die kleine ook nog op het lab geboren. Ik ben er van overtuigd dat we Ramanspectroscopie voorgoed op de microbiologische kaart hebben gezet. Ook Tamara van Vreeswijk kwam het bacto-team versterken, eerst als stagiaire en daarna als research analist. Tamara, dankzij jouw hulp hebben we een aantal hele grote stappen voorwaarts gemaakt. Onbewust is jouw manier van werken tijdens je stage een maatstaf geworden, waar maar weinig stagiaires aan (hebben) kunnen tippen.

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Op het laboratorium liepen er natuurlijk ook mensen rond die niet direct aan mijn onderzoek hebben bijgedragen. Annemiek Coremans, Karan Kanhai, Sweder van de Poll, Bas de Jong, Maaike Ibelings en de Raman-girls Senada Koljenović en Annieke Nijssen, altijd in voor een geintje of een praatje over het weer, als ik te lang achter mijn computer had gezeten en echt even achter dat ding weg moest. De Raman-girls bleken zich ook fantastisch te ontpoppen als gastvrouwen tijdens de ontvangst van de gasten op 'mijn' minisymposium. Piet Kreeft mag hier niet ontbreken. Piet, ik en de stagiaires konden altijd bij je aankloppen voor hulp, advies en een schuine bak. Enne, nog bedankt dat je als handmodel wilde fungeren voor mijn boekje (wedden dat nu bijna iedereen gaat terugbladeren).

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van Leeuwen was het altijd weer prettig om 'beleefdheden' uit te wisselen. Mannen houdt nou toch op met dat DNA werk, het is nu toch wel pijnlijk duidelijk geworden waar de toekomst ligt!

Dan zijn er natuurlijk nog vele vrienden en bekenden die ik wil bedanken voor de biertjes, etentjes, biertjes, gezellige avonden, biertjes en goede gesprekken. Dat soort dingen zijn goed om alles weer even in perspectief te zien: een bacterie blijft een klein vies beestje dat je niet kan zien, een laser een lampje en een Ramanspectrum een kleuren foto waarop je ziektekiemen kan herkennen. Het is ondoenlijk om al die vrienden te noemen, maar aan Jeffrey Groot kan ik niet voorbij gaan. Je hebt het weer geflikt man, een fantastisch gelikt stukje werk! Onwijs tof dat je bij iedere keer dat het er op aan kwam tijdens mijn hele studie, je grafisch vernuft wilde inzetten om het eindresultaat in de vorm van een verslag, scriptie of proefschrift zo fraai vorm te geven: je bent een kunstenaar!

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10. Over de auteur

De auteur van dit proefschrift werd op 3 maart 1971 geboren in 's-Gravenhage. Nadat hij in 1988 het Hoger Algemeen Voortgezet Onderwijs met succes had afgerond, werd in 1990 het diploma behaald van het Voorbereidend Wetenschappelijk Onderwijs. In dat zelfde jaar is hij begonnen aan de Hogere Laboratorium Opleiding tot medisch microbiologisch analist aan de Haagse Hogeschool. Na een fusie met de Hogeschool Rotterdam en Omstreken werd de studie voortgezet in de bakermat van de Nederlandse microbiologie: Delft. Na afronding van deze opleiding is een extra stage gedaan op het Laboratorium voor Microbiologie aan de Rijksuniversiteit Gent (België). In 1994 is de auteur begonnen aan de studie medische biologie aan de Vrije Universiteit van Amsterdam, alwaar hij zijn kennis van de medische microbiologie verder verbreedde en verdiepte. Kort na het behalen van het doctoraal diploma werd in 1997 met het hier beschreven promotieonderzoek gestart op het Laboratorium voor Intensive Care Onderzoek en Optische Spectroscopie, van de afdeling Algemene Heelkunde van de Erasmus Universiteit Rotterdam en het Academisch Ziekenhuis Rotterdam. Op dit moment (voorjaar 2002) zet de auteur de lijn van onderzoek voort op hetzelfde laboratorium, in samenwerking met de afdeling Medische Microbiologie en Infectieziekten van de Erasmus Universiteit Rotterdam en het Academisch Ziekenhuis Rotterdam.

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