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Research Article

Prospects and possibilities of using Raman spectroscopy for the identification of *Pseudomonas aeruginosa* from turtle *Emys orbicularis* (Linnaeus, 1758) skin

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Abstract

This study describes an express method for identifying microorganisms: *Pseudomonas aeruginosa* by standard Raman spectroscopy, without surface-enhanced Raman spectroscopy (SERS). The short-wavelength 514 nm Ar-Ion laser was used for *P. aeruginosa* spectral identification in the Raman shift range from 3200 cm^{-1} to 200 cm^{-1}. The research results showed a high analytical and diagnostic sensitivity of the technology to the express identification of *P. aeruginosa* and can be used as one of the reliable methods. The proven technology is promising for further research of other microorganisms due to several significant advantages of the method. It does not require long-term cultivation of bacteria and special sample preparation, additional expensive reagents or consumables.

Key words: Bacteria, identification of microorganisms, *Pseudomonas aeruginosa*, Raman spectroscopy, reptiles, turtle

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Introduction

Nowadays, microbiological research is an important and relevant activity in biology and medicine since these studies confirm or deny the presence of certain bacteria with high accuracy and reliability. The classical bacteriological research method and Automated bacteriological diagnostics by different identification systems, VITEK2, Phoenix, MALDI-TOF (matrix-assisted laser description/ionisation time-of-flight mass spectroscopy), Next-generation sequencing, solves the problem of isolating a pure culture of the pathogen with its subsequent identification, but it requires much time and financial investments. Thanks to microbiological research methods, it is possible to establish the causative agents of certain infectious diseases and choose a rational treatment for these diseases (Ramalho et al. 2002). Therefore, developing faster and cheaper routine methods for diagnosing pathogenic microorganisms is a priority area in modern microbiology. *Pseudomonas* spp. is a group of bacteria that can cause several infections. *P. aeruginosa* is the most common disease-causing form of these bacteria, according to the Centers for Disease Control and Prevention (CDC). *P. aeruginosa* belongs to the group of gram-negative bacteria and it is an opportunistic pathogen. Colonies of *P. aeruginosa* consist of rod-shaped bacterium sizes about 1–5 μm long and

0.5–1.0 μm wide (Pauw et al. 2008). They can infect different organs and tissues; the infection is usually severe. Serious infections from *P. aeruginosa* primarily occur in healthcare settings, but people can also become infected from hot tubs and swimming pools or after contact with free-living animals. *P. aeruginosa* is one of the main etiological factors in more than 65% of cases of purulent-septic skin lesions and the fight against these diseases remains an urgent task of modern medicine (Kałużna et al. 2014; Lee et al. 2021; Umbrasko et al. 2022, 2023).

The study of microorganisms that are the causative agents of many infectious diseases is an urgent and important task and progress in solving this can only be achieved if various research methods are used, combining them to obtain the fastest, most reliable and economically justified results (Terrones-Fernandez et al. 2023). The method for detecting microorganisms should not require complex stages of sample preparation while providing rapid identification of the bacteria and be relatively inexpensive and automated. An important criterion for setting up a microbiological study, based on determining the type of bacteria, is its speed. However, the classical bacteriological diagnostic method, based on the isolation and identification of a pure culture of bacteria based on the totality of their specific properties, with all its reliability and information content, is very laborious, financially costly and, most importantly, it is lengthy (it may take several days). In addition to the microbiological method, other laboratory studies allow for obtaining objective information about the microbial composition of the material under study. The use of automated systems with multi-wavelength lasers for the identification of bacteria can speed up the process of microorganism identification by 24–48 hours and obtain reliable information about microorganisms faster (Huang et al. 2004, 2010; Zhu et al. 2014; Pezzotti 2021).

One of the possible methods for the identification of microorganisms is Raman spectroscopy. This method is based on the detection of the molecular structure

Figure 1. Sampling site: Silene Nature Park NATURA2000 (Latvia: 55.690835°N, 26.788760°E).

vibrations of the object and has established itself as a reliable analytical tool in various fields of science (Petjukevičs and Škute 2017; Vaitiekūnaitė and Snitka 2021; Vaitiekūnaitė et al. 2022). It is assumed that bacteria are also characterised by individual spectral lines of Raman scattering (like biomolecule fingerprinting), which make it possible to identify them in a short time (1–2 min) with a very high degree of reliability of the information obtained. In turn, a library of microorganisms is created, based on the spectral information of Raman spectra. Raman spectroscopy makes it possible to apply this method to rapidly identify microorganisms in more comprehensive applications (Patel et al. 2008; Stegelmeier et al. 2019; Kumar et al. 2020; Vaitiekūnaitė et al. 2022). One of the existing problems is the timely delivery of bio-material, as about 30% of bacterial strains do not reach laboratory testing and, thus, cannot be identified. Modern achievements in microbiology using the Raman spectroscopy method and its limitations were considered, as well as the most important world trends in the use of this diagnostic technology for the study and indication of the causative agents of bacterial pathogenic flora and viral infections (Lee et al. 2020; Nakar et al. 2022; Umbrasko et al. 2022).

Based on the preceding, the purpose of this study is to develop an express method for detecting and identifying the bacterium, *P. aeruginosa*, without the use of expensive SERS substrates with metallic gold or silver nanoparticles, based on Raman spectroscopy by analysing and comparing the obtained spectra of analysed samples with the test-control strain: *P. aeruginosa* ATCC 27853 (American Type Culture Collection), as well as optimising the conditions for obtaining spectra and also developing an algorithm for processing primary spectral information.

Materials and methods

Study site, sampling, microbiological identification

For the classic bacteriological method, ten free-living turtles, *Emys orbicularis* (L.) (European pond turtle), were collected and biological material was taken from the skin surface. The sampling site was Silene Nature Park NATURA2000 (Latvia) (55.690835°N, 26.788760°E) (Fig. 1). CliniswabTS Sterile Transport Swabs (Italy) were used to collect samples in wild nature and preserve the microbiological flora for further analysis in the laboratory. Subsequently, *Pseudomonas* spp. was isolated and identified by the classical bacteriological method. Solid nutrient media were used for cultivation and primary microbiological analysis: CHROMagar Orientation and Trypticasein Soy Lab-Agar (BioMaxima S.A., Poland) (Merlino et al. 1996; Garcia and Isenberg 2010). As an objective, daily cultures of the control strain (culture number: *P. aeruginosa* ATCC 27853) and *P. aeruginosa* were taken, which were identified from the skin surface of turtles. Identified colonies from the surface of the skin of turtles were stored in a refrigerator at a temperature of 4 ± 1 °C. Then for the study, the material was warmed up to room temperature 20 ± 1 °C. Bacterial colonies were transferred into 3 ml BHI (Brain Heart Infusion Broth, BioMaxima S.A., Poland) with a disposable sterile loop (COPAN). In the next step, colonies were incubated in a thermostat at 37 ± 1 °C for 24 hours. BHI is a nutrient-rich liquid medium suitable for the inoculation of *P. aeruginosa*. After 24 hours of incubation, the samples were transferred with a sterile disposable viscose swab with a plastic stick (APTACA) to a nutrient medium (Trypticasein Soy Lab-Agar) and incubated in a thermostat at

37 ± 1 °C for 24–48 hours. Isolated colonies were placed in sterile distilled water, the obtained emulsion vortexed ≈ 10 s by Vortex V-1 plus Biosan (Latvia) and visually compared with McFarland 0.5 Standard, 1.5 x 10^8 cells per volume unit (approximate bacterial suspension/ml) and after that, 100 µl of bacterial suspension transferred on sterile microscope 1 – 1.2 mm thick slide (ChannelMED).

Raman spectroscopy of *P. aeruginosa*

Raman scattering spectra were recorded using Renishaw inVia Raman Microscope (United Kingdom), equipped with an optical microscope, Leica DM 2500 (Germany). Raman scattered light from the sample collected through a microscope with a short-distance objective, Leica L 50×/0.50 (eyepiece: HC PLAN 10×/20) and analysed by an inVia Spectrometer. Scattered light focused on a Renishaw air–cooled *Ren Cam* CCD array detector with insertion/retraction speed > 20 mm s^{-1}, repeatability < 0.5 µm, laser spot size ≤ 2 µm FWHM, spatial resolution 2 µm and a field of view > 25µm. During Raman spectroscopy, **>** 50 scans were accumulated for each sample. To improve the noise/signal quality ratio, the laser power was minimised (reduced sample self-fluorescence) and an excitation source Renishaw Stellar-Ren Ar-Ion laser with 514.0 nm wavelength (VIS: 2400 l/mm grating and back-illuminated CCD camera) was used. Lens-focused laser radiation on a quartz glass slide with the sample and Raman spectra were collected by the Raman inVia Reflex microspectroscopy system in the range from 3200 cm^{-1} to 200 cm^{-1} for the full-length spectrum (Fig. 4b) and from 2000 cm^{-1} to 600 cm^{-1} for the short-length spectrum (Fig. 4a). The Ar-Ion laser power was ≤ 50 mW at the laser head and ≤ 0.016 mW at the sample surface (Nova II PD300-3W-V1, Ophir Photonics, Israel). All spectra were read out three times for each bacterial suspension of ten and the results of Raman scattering signals were averaged. All collected data are unsmoothed averages of exposures obtained with an integration time of 2–40 s/exposure. The optimal spectral resolution from the sample surface was reached at 2 cm^{-1}. Data collection was accomplished with Renishaw WiRE 5.5 Raman software, scans, processing (including decomposition of the complex band–shapes analyses) and spectral analysis was controlled by a personal computer. Raman spectral shifts were compared with the Raman spectrum of the control strain (*P. aeruginosa* ATCC 27853) and the S.T. Japan spectral databases, Thermo scientific software, Grams Spectral ID library, Version 9.0.7 and the data from scientific articles.

Results

The results of the research are presented in the form of graphs of the spectral characteristics of *P. aeruginosa* (Fig. 2a) and (Fig. 2b). Ten spectra of *P. aeruginosa* bacterial substances (bacterial colonies suspension, concentration measured according to McFarland 0.5 Standard), systematised and analysed by Raman spectroscopy. In the Raman spectra of control samples, low-intensity lines were detected that were not related to compounds characteristic of bacteria and were taken into account as side noise since the range of detection of Raman shifts and the ratio of line intensities did not correspond to lipids, proteins or nucleic acids. However, it was known that lipids, which are inherent in bacterial cells increasing the reflectivity, also play an important role, probably influencing the intensity level

increase in spectra. It was noted that spontaneous luminescence was absent or was minimal during short-term (up to 60 s) focusing on the sample, which indicates the absence of a noticeable effect of laser radiation on them. Cosmic rays' peaks or unusually high and unstable Raman shifts on the abscissa axis – x was also not considered and was suppressed with cosmic ray removing functions.

The specific Raman spectra observed in this research represent an ensemble of Raman signals that arise from the different molecular vibrations of individual cell components, integrating over nucleic acids, lipids, carbohydrates and proteins. The resulting Raman spectra had a significant number of peaks, for which it was difficult to unambiguously assign to the type of vibrations of groups of atoms in analyte molecules. As can be seen from the graph, the scattered light intensity peaks of this bacterium species coincide in intensity and localisation in the spectral region of Raman scattering in the short and extended spectra for *P. aeruginosa*. The individual Raman shifts were localised at 624 cm^{-1}, 760 cm^{-1}, 808 cm^{-1}, 1002 cm^{-1}, 1032 cm^{-1}, 1145 cm^{-1}, 1150 cm^{-1}, 1178 cm^{-1}, 1207 cm^{-1}, 1330 cm^{-1}, 1359 cm^{-1}, 1445 cm^{-1}, 1580 cm^{-1}, 1600 cm^{-1} and 1620 cm^{-1}; the values and characteristics of the principal obtained components are detailed in table (Table 1).

All obtained spectra from *P. aeruginosa* bacteria had similar characteristics of the values in the studied region 1700–600 cm^{-1} and slightly differ only in the intensity of the peaks. Recorded spectra previously filtered from high-frequency noise (software suppression of cosmic rays) are presented in the paper for further analysis and interpretation. The specific Raman spectra observed here represent an ensemble of signals that arise from the molecular vibrations of individual cell components of gram-negative bacteria, integrating over proteins, lipids and carbohydrates (Fig. 3).

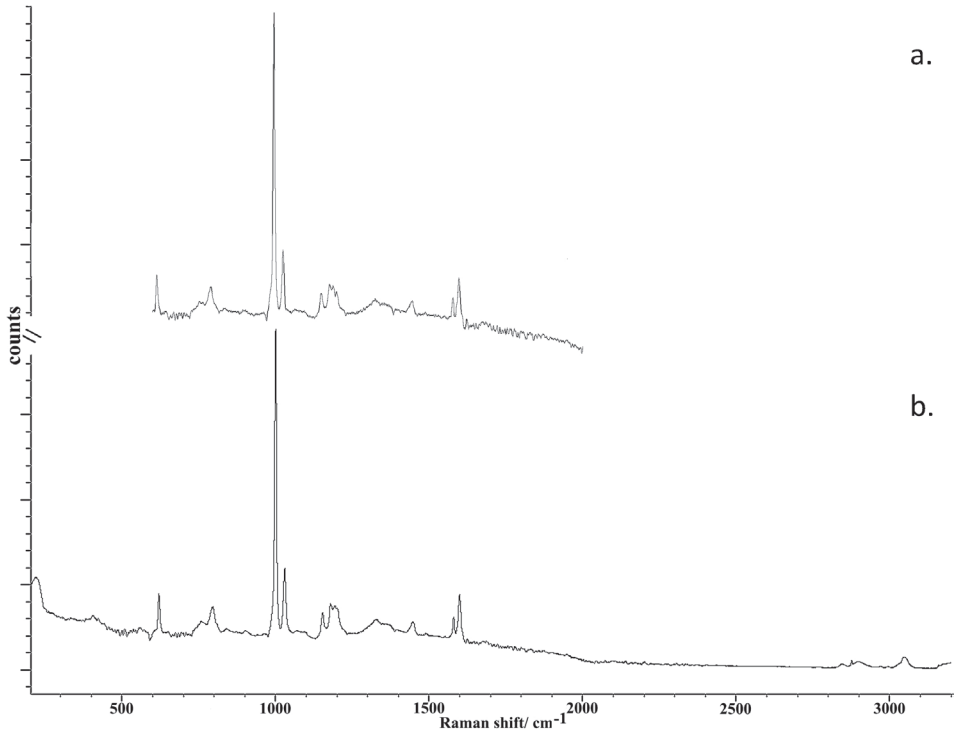

Figure 2. Averaged short-length spectrum: 2000 cm^{-1} – 600 cm^{-1} and extended full-length Raman spectrum: 3200 cm^{-1} – 200 cm^{-1} of *P. aeruginosa* bacteria isolated from turtle skin (**a**) and control strain: *P. aeruginosa* culture number: ATCC 27853 (**b**). On the abscissa axis x– Raman shift (cm^{-1}) along the y-axis is the scattered light intensity (a.u).

Table 1. Raman shifts and tentative assignments of bacteria cells of *P. aeruginosa* isolated from turtle skin.

Raman shifts, (cm^{-1})	Tentative assignment [a]
624	Skeletal vibrations of aromatics rings of amino acid
760	Carbohydrates COO–def, CH_2 rocking
808	Nonpolar amino acids: proline, valine; Polar, uncharged amino acid: υ (CN) tyrosine
1002	amino acid: Phe
1032	C–H in plane, Phe
1145	sulfonic acid residues
1150	Could be associated with the stretching vibration from symmetric glycosidic linkages (C–O–C) and rbr of polysaccharides or C–C str vibrations. ATP
1178	Aromatic amino acids: δ (C–H), Tyr, Phe; Proteins: C–H str Region
1207	Proteins: Amide III, C–C_6H_5 str. Phe, Trp
1330	CH deformations can be assigned to polysaccharides and lipids, as well as to protein
1359	υ(COO–), δ(C–H) proteins
1445	CH deformations can be assigned to polysaccharides and lipids, as well as to protein
1580 (1600–1585)	C=C str, C–C str (in-ring)
1600 (1645–1540)	C–C str (in-ring), Amide II, υ(CN), γ(NH), unsaturated lipids
1620 (1680–1640)	Amide I
650–600	Proteins
1280–1160	B-sheet (proteins)
1333–1313	CH def stretch band
1440–1360	υ(COO–) sym
1460–1440	δ(CH_2) fatty acid molecules without double bonds
1645–1545	Amide II, υ(CN), γ(NH)
2000–1665	Overtones of fundamental or compound vibrations, weak vibrations
3100–2800	C–H str region

[a] Abbreviations: Phe – phenylalanine; Tyr – tyrosine; Trp – tryptophan; rbr – ring breathing; def – deformation; str – stretching; sym – symmetric.

Discussion

The possibility of taking spectra of *P. aeruginosa* directly from the surface of Petri dishes with nutrient agar was initially studied. However, this technology added extra noise to the spectrum due to the agar on which the colonies were grown. The plastic base of the Petri dishes (transparent polystyrene) with nutrient agar on which bacterial colonies were grown (Fig. 4). Raman spectra of *P. aeruginosa* wild type were acquired between 3200 cm^{-1} and 200 cm^{-1}. However, the studies reported here concentrate on vibrational bands found in the fingerprint region 1700 – 600 cm^{-1}. Outside this range, C–H stretching vibrations in the 3050 – 2750 cm^{-1} range dominate spectra. This information is unimportant in this paper, although these Raman bands carry important information about cell membrane fluidity (Huang et al. 2010; Kumar et al. 2020; Pezzotti 2021).

Previously, before Raman scattering, the quality of preparations of the nutrient medium to luminescence capability was checked out. Accordingly, the possibility of the substrate distorting the obtained Raman spectra was neutralised using bacterial suspension drops against direct spectra from the Petri dish surface. The spectra of bacterial suspensions were measured one by one.

Measurements of the bacterial suspension of samples from different groups did not reveal significant differences; however, there was a slight change in the peak height (the intensity of the Raman signal varied depending on the concentration of bacterial suspension in the test sample).

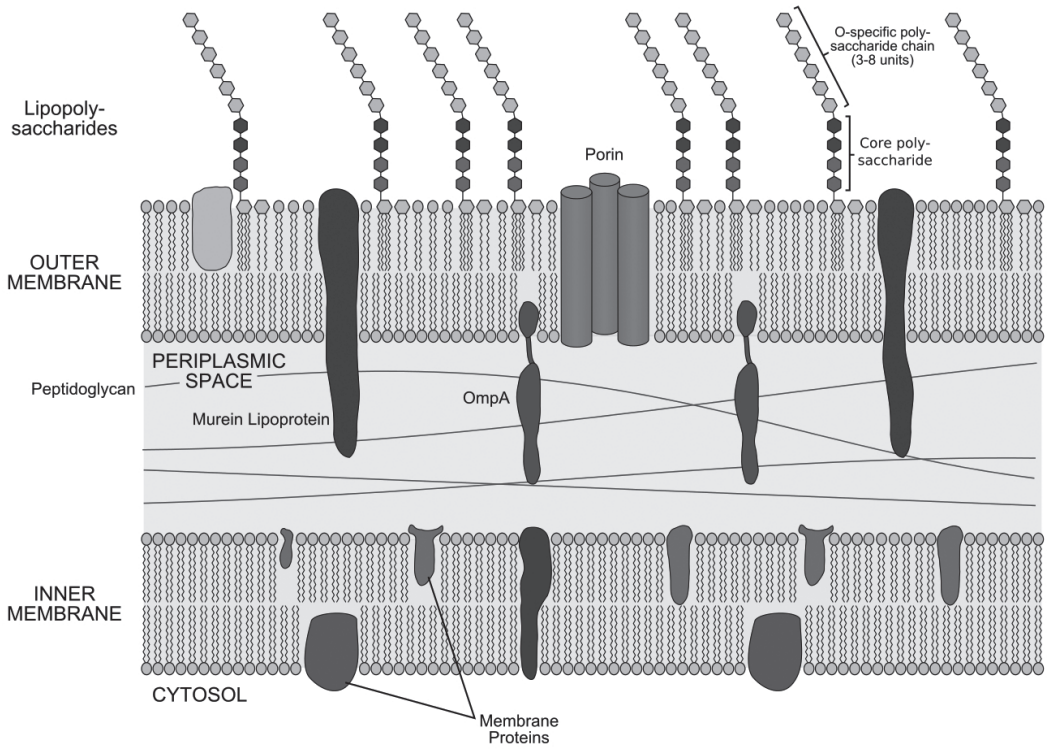

Figure 3. Cell wall components of Gram-negative bacteria (Kagle 2023).

Figure 4. Microscopic image of *Pseudomonas aeruginosa* bacterial cells distribution in agar: (Trypticasein Soy Lab-Agar, BioMaxima).

It was also experimentally confirmed that this bacterial test-suspension by McFarland 0.5 Standard method, 1.5 x 10^8 cells per volume unit, is suitable for Raman spectroscopy during this type of bacterium *P. aeruginosa* analysis and is suitable for Raman spectroscopy without the use of surface signal enhancement technique (SERS).

Each sample of studied bacteria for *P. aeruginosa* is characterised by individual spectral shifts of Raman scattering, which make it possible to identify them in a short time (total time for whole full-range spectrum 1–10 min) and, theoretically, makes it possible to identify a large number of cultures simultaneously. This method is characterised by high sensitivity (100 µl of the prepared suspension according to the McFarland Standard method, 1.5 x 10^8 cells per volume unit) and rapid microorganism identification. The spectra recorded for the same sample remain almost unchanged in the spectrum over a short time, while they could only slightly differ in signal intensity and resolution of the leading bands. It was noted that, to take high-quality, optimally-reproducible spectra, the spectrum from an object should not exceed 60 seconds from one point

Conclusions

The study showed the possibility of obtaining fast and high-quality Raman spectra of *P. aeruginosa* bacterial cells. The used parameters of laser excitation did not cause pronounced destructive changes in bacterial cells. Bacterial cells retained their integrity and cellular organelles with decreased laser beam power. However, the self-luminescence of the samples was reduced to a minimum background effect, which did not significantly affect the quality of the obtained Raman spectra. Laser diagnostics, based on Raman spectroscopy, can be considered an express method for identifying microorganisms and allows detection of the presence of a microorganism even at this concentration (1.5 x 10^8 cells per volume unit). The application of Raman spectroscopy is characterised by high analytical and diagnostic sensitivity and specificity, which is necessary for accurately identifying microorganisms. We can also note the high speed of obtaining results (quantitative and qualitative). The method does not require additional stages of bacterial cultivation or special sample preparation, which are important characteristics for a reliable study and provide analytical reliability and high speed for obtaining results. These advantages of the method give reason to consider it a promising universal express method for microbiological diagnosis of diseases of microbial etiology. The study's results indicate the information content of using Raman spectroscopy to identify microorganisms. However, interpreting these changes and possibly using this technology to study other bacteria requires additional research.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Formal analysis: AP. Investigation: IU. Methodology: IU, AP. Project administration: NŠ. Software: AP. Visualization: AP. Writing – original draft: IU, AP. Writing – review and editing: NŠ.

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Data availability

All of the data that support the findings of this study are available in the main text.

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