

Diagnosis of Bacteria *In Vitro* by Mass Spectrometric Fingerprinting: A Pilot Study

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Abstract. The identification of bacteria by using conventional microbiological techniques can be very time-consuming and circumstantial. In contrast, the headspace screening of bacterial cultures by analyzing their emitted volatile compounds using mass spectrometry might provide a novel approach in diagnostic microbiology. In the present study different strains of *Escherichia coli*, *Klebsiella*, *Citrobacter*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Helicobacter pylori* were investigated. The volatile compounds emitted by these bacteria *in vitro* were analyzed using proton-transfer-reaction mass spectrometry, which allows rapid and sensitive measurement. The detected patterns of volatile compounds produced by the investigated bacteria were compared and substantial differences regarding both quantity and quality were observed. In conclusion, the present study is the first to describe headspace screening of bacterial cultures as a potential diagnostic approach in medical microbiology.

The rapid identification of infectious agents is of paramount importance in order to guide further antimicrobial treatment and patient management. However, most bacteria can be identified only on the basis of time-consuming laboratory techniques, whereas others can only be empirically treated on the basis of clinical manifestation. Thus, any strategy or method that would shorten time to diagnosis could be of great clinical benefit. Recently, mass spectrometry has been shown to possibly meet this claim. Probert et al. investigated the composition of volatile organic compounds (VOC) in the headspace of stool samples obtained from patients suffering from infectious diarrhea. Depending on the causative organisms different specific VOC-patterns could be described [8]. Using this novel approach rapid diagnosis of diarrhea seems to be within reach. In contrast, conventional microbiological techniques may take up to several days.

The fact that different bacterial species emit large amounts of VOC, such as isoprene [6], hydrogen cy-

nide (particularly synthesized by *Pseudomonas aeruginosa*) [4], and volatile sulfur compounds [1], is well known. However, the potential diagnostic value of these compounds (e.g., headspace screening of bacteria cultured in Petri dishes) has not been investigated. Therefore, the aim of our study was to describe spectra of such compounds emitted by various bacterial species *in vitro* using proton-transfer-reaction mass spectrometry (PTR-MS), in order to obtain chemical “fingerprints” indicative for certain bacteria.

Materials and Methods

Materials. Different strains of *Escherichia coli* ($n = 4$), *Klebsiella* ($n = 4$), *Citrobacter* ($n = 3$), *Pseudomonas aeruginosa* ($n = 4$), *Staphylococcus aureus* ($n = 4$), and *Helicobacter pylori* ($n = 2$) were examined. These pathogens were obtained from routine samples. Bacteria were cultivated in Petri dishes and the following types of growth media were used: MacConkey agar (Oxoid, Basingstoke, UK) for *Escherichia coli*, *Klebsiella*, *Citrobacter*, and *Pseudomonas aeruginosa*; Mannitol-salt agar for *Staphylococcus aureus*; Pylori agar (Biomerieux) for the cultivation of *Helicobacter pylori*. Blank agars of each type without bacteria were used as background controls. The headspace of the bacterial cultures (Petri dishes) was screened

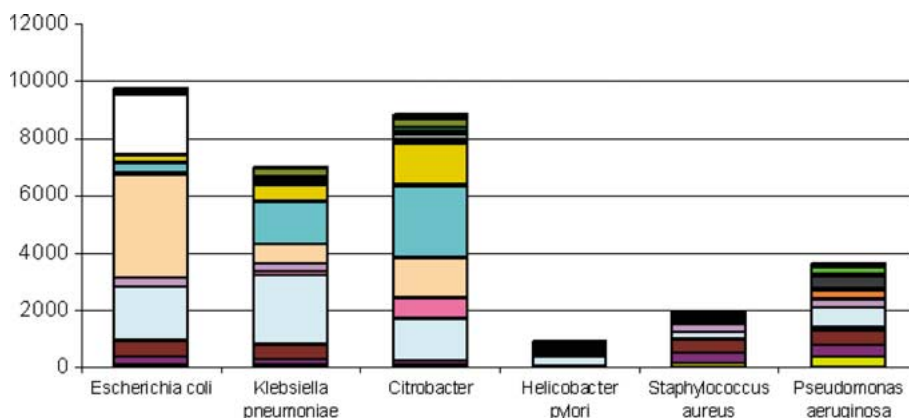


Fig. 1. Mass spectra of the examined bacteria. The concentration is given in parts per billion by volume (ppbv) and plotted on the y-coordinate. All measured mass concentrations (ppbv) of one bacterium are plotted on top of each other and the additive columns of all the measured bacteria are compared.

after 24 h (*E. coli*, *Klebsiella*, *Citrobacter*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) or 72 h (*Helicobacter pylori*; a longer period of time in order to obtain a comparable size of the colonies) after bacteria had been plated onto the Petri dishes. Furthermore, the size and the condition of the colonies were carefully documented in order to explain differences in the concentration of the measured masses. However, due to the defined time schedule no large variations regarding the size (especially between single samples of one bacterium) were observed.

Method. Before and during measurement, all samples were stored at 37°C. For analysis, the Petri dishes were closed with prepared top covers (with two holes). Two Teflon tubes were fixed in the holes. The gas in the headspace of the bacterial cultures and the equivalent control agars (blank) was siphoned off by the first Teflon tube (heated; 37°C) and fed into the PTR-MS apparatus. The other Teflon tube was used to equalize the negative pressure in the airtight Petri dish.

The analysis of the samples was performed using PTR-MS. This technique uses protonated water, H_3O^+ , as a chemical ionization reagent to measure volatile compounds in the parts per billion by volume (ppbv) to parts per trillion by volume (pptv) range. The H_3O^+ reacts with neutral molecules (M) according to $\text{H}_3\text{O}^+ + \text{M} \rightarrow \text{MH}^+ + \text{H}_2\text{O}$. This reaction only occurs if these neutral molecules have larger proton affinities than H_2O . Almost all compounds have larger affinities and therefore proton transfer occurs on every collision with a rate constant k , typical values of which are $1.5 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1} < k < 4 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$. The count rate of ions is determined in the ion detection system. There is a linear relationship between the recorded normalized count rate of ions at different m/z values (according to our settings: 21–229) and the concentration of M in the original trace gas, so that the latter can be calculated. The formula includes the normalized count rate of ions and of the primary ion H_3O^+ , the temperature in the drift tube, the pressure in the drift tube, the mass-dependent transmission efficiency, the rate constant, and the reaction time [7].

After baseline conditions were established each sample was measured at least five times. The average was used for the further analysis of the data. The detected masses in the range of 21–229 atomic mass units (amu) were used for the creation of mass spectra. Descriptive statistics were used to evaluate the findings and summarized data are given as parts per billion volume (ppbv).

Results

A mass spectrum of 209 different masses was detected in the headspace of the samples. For the most part, these

masses represent hydrocarbons, in particular alkanes, alkenes, alcohols, ketones, and organic acids.

The measured concentrations were used for the creation of primary patterns (mass spectra). In order to avoid falsification of the data due to the different agars that had been used, the masses in the headspace above blank agars were also measured and the detected mass concentrations were subtracted from the measured masses of the equivalent bacteria. In this way the final mass spectra were created.

The final spectra of the investigated bacterial cultures differed explicitly from each other. Certain masses were emitted only by certain bacteria. Comparing the profiles of *Klebsiella pneumoniae* and *Helicobacter pylori* it could be shown that *K. pneumoniae* produces larger amounts of masses of low atomic mass (30–90 amu) whereas *H. pylori* produces larger amounts of masses of high atomic mass (more than 160 amu). The latter masses are not emitted by *K. pneumoniae* or only at low concentrations. Moreover, regarding the quantity of single measured mass concentrations significant differences between the investigated bacteria were observed. For example, Mass 38 was consistently found in the headspace of all *E. coli* samples at similar concentrations of 11–14 ppbv whereas all other bacteria emitted lower concentrations. By comparing total concentrations of all measured masses, the Gram-negative bacteria examined (with the exception of *H. pylori*) could be shown to produce larger amounts of volatile compounds than the Gram-positive *Staphylococcus aureus* (Fig. 1).

Discussion

This study is the first describing the headspace screening of bacterial cultures as a potential diagnostic tool in microbiology. Proton-transfer-reaction mass spectrometry was used for analysis. The results show that this

approach seems to be able to differentiate the studied bacterial strains *in vitro* by using the detected volatile compounds, or rather their mass spectra, as chemical “fingerprints.”

The bacteria examined produced different total concentrations of volatile compounds. In particular, *Staphylococcus aureus* produced smaller amounts than most of the Gram-negative species investigated. The reason for this finding cannot be stated at the moment because, due to the methodological limitation, a detailed discussion of the results is not possible. Most questions can only be answered by using a combination of gas chromatography and mass spectrometry (GC-MS), which allows a detailed identification and evaluation of the substances. This method will also help us to gain new insights into both the production of volatile compounds by different bacterial species and their metabolism.

Using conventional methods, the diagnosis of the investigated bacteria might take between 24 and 72 h. Due to the high sensitivity of the method, which measures concentrations of volatile compounds at pptv levels [7], and the availability of online (real-time) measurement, it is conceivable that this time could be reduced by detecting the characteristic mass spectra (or rather VOC patterns) earlier. It must be pointed out that the rapid and accurate identification of the causal pathogen is fundamental for the adequate clinical management of a disease. A temporally delayed diagnosis increases the rate of morbidity and mortality and also leads to an increased economic burden on the health care system [2].

A search of PubMed and other libraries identified the following similar studies regarding VOC analysis of microorganisms. Buzzini et al. studied the production of VOC in 98 tropical ascomycetous yeast strains. Alcohol, aldehydes, and esters were predominantly emitted by these fungi. Differences in the VOC profiles between the different species were used to cluster the yeast strains into 25 VOC phenotypes [3]. Korpi et al. described the analysis of VOC produced by mixed microbial cultures in building materials [5]. However, no study except that by Probert et al. [8] describes the evaluation of volatile compounds emitted by bacterial species as a potential laboratory technique in medical microbiology.

Finally, some potential limitations of our study should be briefly addressed. The number of each experimental setup (*n*) is rather small. But at least this

pilot study points out that the mass spectrometric headspace screening of bacterial cultures *in vitro* might lead to a novel diagnostic tool. One limitation of the PTR-MS method is the fact that each measured mass with its corresponding atomic mass units (range 21–229 amu) can represent a combination of various substances with the same amu. The use of GC-MS would be of great benefit to identify the emitted substances exactly. However, PTR-MS has been shown to be an excellent screening tool, featuring rapid real-time measurement and a highly sensitive analysis.

The main outcome of the present study is the first description of headspace screening of bacterial cultures as a potential diagnostic approach in medical microbiology. In the future it might become possible to correlate specific patterns of volatile compounds with causative agents *in vivo*. The headspace screening of urine in patients with urinary tract infection or the analysis of exhaled air in patients suffering from bacterial pneumonia are conceivable. Nevertheless, much further research is required and the reliability of this approach has to be proven.

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