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Article Body Template

- **Article title:** Differential mobility spectrometry classification of bacteria

- **Short running title:** DMS classification of bacteria.

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Abstract:

Background: Rapid identification of bacteria would facilitate timely initiation of therapy and improve cost-effectiveness of treatment. Traditional methods (culture, PCR) require reagents, consumables and hours to days to complete the identification. In this study, we examined whether differential mobility spectrometry (DMS) could classify most common bacterial species, genera and between gram-status within minutes. **Materials & Methods**: Cultured bacterial sample gaseous headspaces were measured with DMS and data analysed using k-nearest-neighbor and leave-one-out cross-validation. **Results:** DMS achieved a correct classification rate 70.7% for all bacterial species. For bacterial genera, the rate was 77.6% and between gram status, 89.1%. **Conclusions:** Largest difficulties arose in distinguishing bacteria of the same genus. Future improvement of the sensor characteristics may improve the classification accuracy.

- **Keywords:**
  - Differential mobility spectrometry, DMS, Ion mobility spectrometry, IMS, Bacteria, eNose

Article Body Template

Main body of text

Introduction

Identification of the bacterial species and antibiotic resistance is essential in getting the right diagnosis, to initiate treatment and to prevent worsening of the infection. Early detection also enables quarantine and logistic measures to prevent spreading of the infection. Rapid treatment and identification of the pathogen is especially important in life-threatening situations such as septic infections.

Traditionally, the most common methods of studying bacteria have been the use of gram-staining, culture and biochemical methods. Bacterial staining is characterized by poor sensitivity. [1-3] In bacterial culture, bacteria are grown on petri-dishes with suitable medium and then examined further. The growth medium type can influence bacterial metabolic pathways. [4] The sensitivity of the growing bacteria to antibiotics can also be tested. [5] The major disadvantage of bacterial culture is the time it takes to sufficiently grow the bacteria, usually ranging from one to two days.

Polymerase chain reaction (PCR) is currently considered to be the gold standard of microorganism identification. [6] In PCR, a region of bacterium's DNA is multiplied in a test tube before further assessment. [7] Due to the multiplication, the essential advantage of PCR is its high sensitivity. On the other hand, even a tiny amount of contaminating DNA in the multiplier tube can skew the results, resulting in false positives. Also, genetic polymorphism of pathogens can cause false negatives. [8] PCR also requires expensive equipment, consumables, reagents, distinct conditions and a trained staff. [9] Recently, Real-Time-PCR-methods have surfaced, which are faster in identification than conventional PCR. In a study involving the detection of urine pathogens, Real-Time-PCR achieved results of 82% sensitivity and 60% specificity. [10]

A newer option of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) recognizes the protein profiles of bacteria. In two selected studies, MALDI-TOF MS was able to recognize different species of bacteria with accuracies of 95.2% and 94.4%. [11, 12] While clearly faster than standard culture, MALDI-TOF MS requires a period of bacterial culture to obtain sufficient amount of sample for analysis, resulting in a delay of 1 to 24 hours. The costs and maintenance needs make MALDI-TOF MS feasible only in large-volume laboratories.

An electronic nose, eNose, is a general term used for electronic devices that mimic mammalian olfactory sense by analyzing the Volatile Organic Compounds (VOCs) of the studied sample. A prominent, more accurately defined technology utilized in some eNoses is Ion Mobility Spectrometry (IMS) in which VOCs are first ionized and their movement is then controlled with constant electric fields on their paths to detectors. An extension of IMS is Differential Mobility Spectrometry (DMS), which adds an asymmetric changing electric field to the system, which in turn creates a new element of resolving the ion clusters. DMS is sometimes also called FAIMS, Field Asymmetric Ion Mobility Spectrometry. The main benefit of IMS and DMS technologies is that they operate at atmospheric pressure, are easy to use, rapid and do not require extensive maintenance. To date, studies have shown the ability of IMS to detect urinary tract infection pathogens at an accuracy of over 90% *in vitro*, common wound infection pathogens at 80% accuracy and ultimately methicillin resistant *staphylococcus aureus* (MRSA) from non-resistant form (MSSA) at an overall accuracy of 91% *in vitro*. [13, 14] Recently, it was demonstrated that DMS is capable of discriminating common upper respiratory tract pathogens at an accuracy of 80-85% *in vitro*. [15] However, each of these studies have focused on a single clinical situation, where the number of possible species of bacteria are limited. Our study aimed to test DMS's ability with a larger set of different bacterial

species and a significantly greater number of overall bacterial samples. If the already promising results with *in vitro* studies can be transformed into clinical settings, DMS can offer a feasible, fast and affordable method of microorganism detection.

Materials and methods

Samples

The bacterial samples were obtained from samples of regular patients of Tampere University Hospital, Finland and processed by the hospital's main laboratory services provider, Fimlab Laboratories. The study was approved by Tampere University Hospital Ethics Committee (study code R10066). No personal data was handled in the study. The samples were all grown on blood agar plates and incubated between 1 to 2 days. Measurements were conducted in room temperature. Between each measured plate, plain water samples were measured, in order to clean the system from carry-over of the previous bacterial plate.

Bacteria were measured in random order. Our aim was to measure plates of at least two different bacteria species in each measurement session, in order to minimize the effect of possible sensor drifting and variation in conditions. This was achieved in all but one of the 23 measurement days, when only bacteria of *Staphylococcus aureus* species were measured due to limitations in available bacteria.

In the study, 321 different bacterial plates were used. Each plate was measured twice, thus creating 642 measurements of 41 different bacteria species, in total. A single measurement took approximately 6 minutes. Three different scenarios were examined: one, where DMS tried to distinguish between species of bacteria, one between genera of bacteria and one between gram-positive and gram-negative bacteria. In each scenario, we excluded groups with less than 10 measurements from the study. In the first scenario, therefore, 103 measurements of 26 different species were excluded and the final analysis consisted of 537 measurements of 15 different bacteria species. In the second scenario, 43 measurements of 12 different genera were excluded, leaving 599 measurements of 11 different bacteria genera to the final analysis. The last scenario involved all 642 measurements.

Sensor

The eNose used in our study was Envi-AMC (Environics Ltd, Mikkeli, Finland), which utilizes the aforementioned differential mobility spectrometry (DMS). Within DMS, the molecules of the studied sample are first charged in an ionization chamber with reactant ions. Envi-AMC uses Americium ^{241}Am as an ionization source. The ionized molecules are then directed to a drift tube containing carrier gas at the end of which is a detector plate. In the drift tube, there are two electric fields parallel to the path of ionized molecules: a constant electric field or compensation voltage (Vc) and an asymmetric changing electric field working as a separation voltage (Vrf), which makes the molecules zigzag on their way towards the detector plate. The movement of molecules and how much they are affected by the two electric fields depends on their mass, shape and charge, practically by the cross-sectional area of the molecule. If an ion comes in contact to the wall of the drift tube before reaching the detector plate, it loses its charge and is unaccounted for. Upon reaching the detector plate, an ion donates its charge and generates current, which is then registered by the system. The different portion of the total number of ions that reach the detector plate with each combination of Vc and Vrf values give rise to a distinct dispersion plot of each sample. The process is carried out twice, separately for positive and negative ions, so each measurement produces two dispersion plots: a positive channel and a negative channel (figure 1). [16]

The measurement chamber stood externally to the Envi-AMC device itself. The chamber was supplied by compressed air which was dried and filtered with activated charcoal- and molecular sieve (5Å) before

entering the chamber. From the chamber the air along with VOC's of the sample were drawn into the Envi-AMC DMS sensor.

We used separation voltage (Vrf) values ranging from 440 V to 680 V and compensation voltage (Vc) values from -0.8 V to 9.0 V. Resolution of the positive channel was 80 Vc and 25 Vrf data points. The negative channel also had 80 Vc points but only 15 Vrf points. The difference in Vrf data points between channels was due to a human error and only noticed after the measurements were conducted.

Statistical analysis

Matlab 2016Rb (The Mathworks, Natick, MA, USA) was used to conduct the statistical analysis. In order to streamline the analysis, the number of Vc points in both channels was condensed through averaging to 40, post-measurement. The data matrices of positive and negative channels were then combined and transformed into a 1600-dimensionial data vector for final analysis. For classification, K-nearest neighbour (kNN) method was used. In kNN, the samples and their data vectors are reviewed in Euclidean space. Each sample is then classified to the class which has the most representatives in the sample's immediate vicinity. The number of k denotes how many neighbors are taken into consideration. If, for example, k is 1, then the sample is classified according to the class of its nearest neighbor. If k is 5, the sample is classified to the class which has the majority of the sample's 5 closest neighbors. In order to avoid over-fitting, results were cross-validated using leave-one-out cross-validation.

Results

In the first scenario, of the 537 measurements of 15 different species of bacteria, DMS achieved a correct classification rate of 70.7% while using kNN with a k value of 1 (Figure 2). With k = 3, the rate was 59.9% and with k = 5, 54.9%. Correct classification by chance in this scenario was 17.8%, which is calculated by assuming that every measurement is classified to the class with the most representatives. Examples of the DMS spectra of three different species, *S. aureus, E. coli, Str. agalactiae* are presented in figure 2.

Samples of the following species were also measured but not taken into account in the first scenario: *S. ludgunencis, S. epidermidis, S. simulans, S. maltophilia, S. liquefaciens, S. caprae, S. warneri, S. schleiferi, S. capitis, Str. dysgalactiae, B. cereus, B. fragilis, C. indologenes, C. amycolatum, C. striatum, C. freundii, A. denitrificans, A. baumannii, A. naeslundii, P. anaerobius, P. vulgaris, E. hormaechei, E. aerogenes, K. oxytoca, M. morganii, H. influenzae*.

In the second scenario, of the 599 measurements of 11 different genera of bacteria, a correct classification rate of 77.6% between genera was achieved with k = 1 (Figure 3). With k = 3 and k = 5, the rates were 68.9% and 66.6%, respectively. Correct classification by chance was 28.7%.

The third scenario consisted of all 642 measurements of 41 different species from 23 different genera of bacteria. With k = 1, a correct classification rate of 89.1% between gram-positive and gram-negative bacteria was achieved (Figure 4). With k = 3 and k = 5, the rates were 81.5% and 81.0%, respectively.

Discussion

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Previously, the ability of IMS and DMS to differentiate bacteria has been shown with distinct narrow sets of bacteria of different clinical situations. [13-15] To our knowledge, this study is the first one, which tried to test DMS, or IMS for that matter, with a larger base of bacteria species without any single clinical subgroup in mind. Our results reinforce the promising results of the *in vitro* studies conducted previously and also show the potential of DMS to distinguish the correct species of bacteria even from a vast set of possible species. Largest difficulties arose in distinguishing bacteria of the same genus. This was most clearly seen in *Streptococci*, where many measurements of *pyogenes, agalactiae, pneumoniae* and *milleri* were mixed with each other in classification. However, sometimes when the number of possible bacteria can be narrowed to a handful through clinical information and reasoning, even the identification of the right genus or whether the studied bacteria are gram-positive or negative can greatly help in selecting the right antibiotic. In these kinds of cases, for instance with suspected tonsillitis, an input could be given to DMS that limits the possible bacteria to those typically causing upper respiratory tract infections. Different bacteria could also be weighed by their Bayesian *a priori* probability gained by clinical information, which in turn would guide the analytics of DMS measurements. [17] Another approach would be a decision tree type solution, where DMS first determines the gram status of the bacteria and then only includes bacteria of the determined gram in the following classification, does the same with genera and finally determines the species.

The most evident limitation in our study was the asymmetric distribution of different measured species of bacteria. This was due to the bacteria being obtained from the regular flow of hospital patients, in which some species of bacteria are much more common than others. With more symmetric and larger sample base, better results could possibly be obtained. One technical challenge was the carry-over of the previous sample affecting the measurement of the next sample. After each measured bacterial plate, we attempted to clean the DMS system by measuring a water sample, but in some cases, even with multiple measurements of water, some carry-over could still be seen when the DMS dispersion plot was analysed by comparing it to that of a typical water sample. Improvements in the sampling system - for example, the use of heated sample lines, sample dilution, and minimized sampling time - could be tried to attenuate this issue in the future.

The use of bacterial culture is expected to remain the most common method for bacterial recognition in the future. One of its biggest advantages is the ability to find the sensitivities of the studied bacteria to different antibiotics - something that is at the moment hard, although not impossible, to see DMS achieving. However, bacterial culture takes time and, therefore, the greatest possibility of DMS in clinical use lies, perhaps, in acute situations, where the quick recognition of bacterial species straight from clinical samples of patients is hugely beneficial. DMS could also possibly, in some cases, eliminate the need for acquiring hard-to-obtain or invasive samples from patients and/or offer an easy option for screening of different infectious diseases. The latter ability could prove valuable, for example, with difficult-to-treat hospital-acquired bacteria such as MRSA, extended-spectrum beta-lactamase (ESBL) and vancomycin-resistant *enterococcus* (VRE).

With bacterial growing *in vitro*, the overwhelming smell of the sample is that of the bacteria. In clinical samples, urine for example, the smell of the bacteria is only a small part of overall spectrum and for this reason the great challenge for DMS in clinical use is to be able to distinguish the smell of bacteria from all the surrounding confusing irrelevant smells. There's also a possibility that, in certain situations, current sensors can't even detect any of the smell of the bacteria, because its signal is so well hidden by the noise of rest of the sample. Perhaps the most obvious way in trying to overcome these possible obstacles may be to develop the measurement systems to be even more sensitive and at the same time create more sophisticated analytical methods of eliminating any irrelevant smell from the overall spectrum. The sensitivity could possibly be improved by a device capable of higher frequency and duty cycle for the separation

voltage, which might allow a wider spectrum of ions to reach the detector plate. Other solutions might involve the use of dopants [18] or diathermy, which could be applied to the sample and possibly transform the chemistry of the VOC's and their ions preferable for detection. This is supported by previous evidence, which demonstrates that rapid evaporative ionisation mass spectrometry (REIMS) has been shown to be accurate in the detection of at least *Candida* species and multiple bacteria. [19-21] Similar results could also perhaps be achieved by adjusting the pressure, temperature and humidity of the measurement chamber or the composition of the carrier gas in the drift tube.

It is worth noting that DMS and our current methods of analytics don't have knowledge of the distinct VOCs that create the differences in dispersion plots between species of bacteria. The classification is made completely "blind". Nonetheless, it might be useful to research and discover those difference-making VOCs, because with that information it could, for example, be easier to promote the essential VOC's and reduce the non-essential ones during measurements.

We were able to find one study involving detection of bacteria from clinical samples with DMS. More specifically, in it, *Clostridium difficile* was attempted to be diagnosed from stool samples. [22] With a set of 213 samples, a sensitivity of 92.3% and a specificity of 86.0% were achieved, which tentatively show the ability of DMS to detect bacteria outside of *in vitro* conditions. In this case, though, DMS was used only to distinguish between *C. difficile* positive and negative samples, so it remains to be seen how well DMS can recognize between multiple different bacteria from clinical samples.

Conclusions

Microorganism diagnostics could greatly benefit from a device that is at the same time accurate, fast, affordable and easy-to-use. An electronic nose utilizing DMS can provide feasible measurements within seconds to minutes without laborious sample handling. The results of our study also indicate that DMS is capable of identifying bacteria from a wide selection of species and genera with a moderate accuracy *in vitro*. Newer sensors, more sophisticated methods of analytics and more balanced study designs can possibly improve classification accuracy in the future. The next essential step for DMS technology in microorganism detection is to move out of *in vitro* conditions and attempt to identify pathogens straight from clinical samples.

Acknowledgements

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Future perspective

In the field of ion mobility spectrometry, advances are expected to be made both in the available hardware and in the methods of analytics. Currently, numerous studies have shown the preliminary abilities of IMS and DMS to be of use in multiple aspects of medical diagnostics. In the future, the studies are bound to move closer to clinical

settings with realistic clinical use in mind. In some cases, these kinds of studies have already been conducted and commercial use is about to be implemented.

Summary points

- Rapid identification of bacterial species would facilitate timely initiation of therapy, quarantine and improve cost-effectiveness of treatment.
- Traditional methods (culture, PCR) require reagents, consumables and hours to days to complete while state of the art time-of-flight mass spectrometry (MALDI-TOF MS) is expensive and requires several hours to complete the identification.
- In this study, we aimed to test whether atmospheric pressure operating differential mobility spectrometry (DMS) could classify most common bacterial species, genera and between gram-status within minutes.
- Cultured bacterial sample gaseous headspaces were analysed with DMS and data analyzed using dimension reduction followed by K-nearest neighbour analysis and the results were validated using leave-one-out cross-validation (LOOCV).
- DMS achieved correct a classification rate 70.7% for all bacterial species.
- When trying to classify bacterial genera, the rate was 77.6% and between gram+ and gram-, 89.1%.
- Largest difficulties arose in distinguishing bacteria of the same genus. This was most clearly seen within *Streptococci*, where several measurements of *pyogenes, agalactiae, pneumoniae* and *milleri* were mixed with each other in classification.
- Future improvement of the sensor characteristics may improve the classification accuracy.
- The next essential step for DMS technology in microorganism detection is to move out of *in vitro* conditions and attempt to identify pathogens straight from clinical samples.

Figure legends

Figure 1: Example dispersion plots of a single measurement of *S. aureus*. Positive channel is on the left and the negative channel on the right. The color represents the amount of current that is generated with each combination of compensation voltage (Vc) and separation voltage (Vrf).

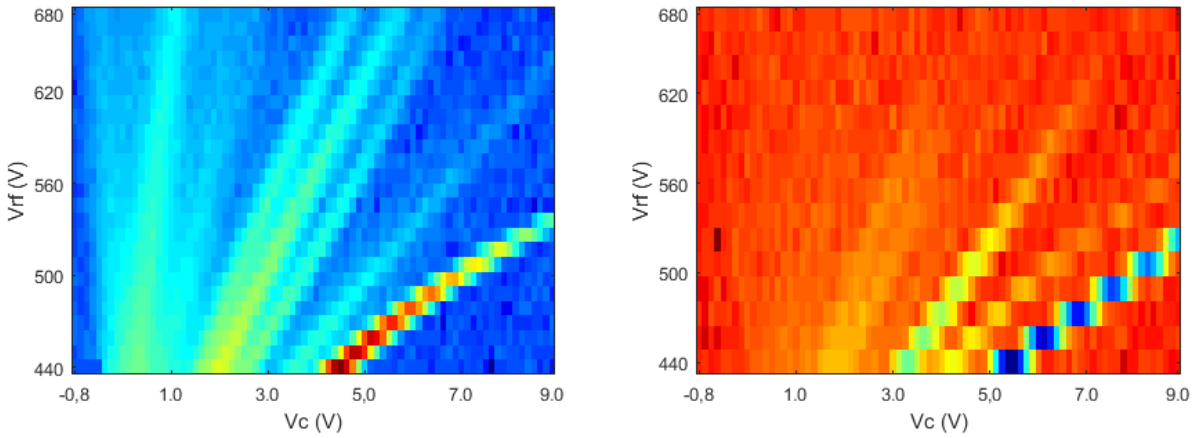

Figure 2: Average dispersion plots for the positive channels of selected bacterial species. From left to right: *S. aureus, E. coli, Str. agalactiae.*

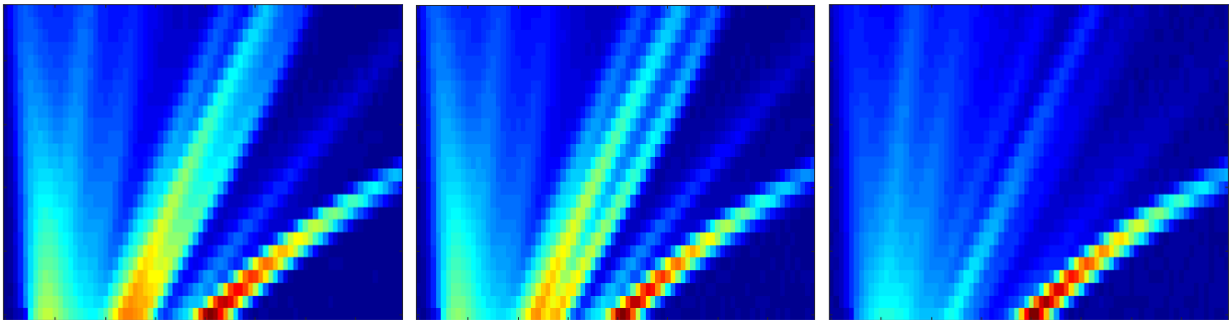

Figure 3: Classifications with k = 1 in the first scenario. To be read as follows: for example, of the 80 measurements of *S. aureus*, 56 were correctly identified as *S. aureus,* 2 incorrectly as *E. coli*, 2 incorrectly as *Str. pyogenes*, and so forth.

	S. aureus	*E. coli*	*Str. pyog.*	*P. mirab.*	*P. aerug.*	*Str. agal.*	*Str. pneum.*	*E. faec.*	*K. pneum.*	*E. cloacae*	*C. koseri*	*S. haemol.*	*Str. milleri*	*M. catar.*	*S. marcec.*
S. aureus	56	2	2	0	7	0	0	0	3	5	0	5	0	0	0
E. coli	4	56	0	0	2	0	1	0	1	0	4	1	0	0	0
Str. pyogenes	1	0	72	1	1	1	12	1	0	0	0	2	5	0	0
P. mirabilis	0	0	0	24	1	0	1	0	0	0	0	0	0	0	1
P. aeruginosa	2	4	0	2	49	0	0	0	1	2	1	1	0	2	0
Str. agalactiae	0	0	0	0	0	13	5	2	0	0	0	1	0	0	0
Str. pneumoniae	2	0	7	0	0	5	19	2	0	0	0	1	0	0	0
E. faecalis	0	0	0	0	0	2	4	18	0	0	0	0	1	0	0
K. pneumoniae	4	0	0	0	5	1	0	0	3	1	0	0	0	0	0
E. cloacae	3	0	0	0	3	0	0	0	1	22	0	1	0	0	0
C. koseri	0	0	0	0	2	0	0	0	0	0	10	0	0	0	0
S. haemolyticus	3	1	1	0	1	1	1	0	0	2	0	10	0	0	0
Str. milleri	0	0	4	0	0	0	0	0	0	0	0	0	10	0	0
M. catarrhalis	4	0	1	0	2	0	1	0	1	0	0	0	0	8	0
S. marcecens	0	0	0	2	0	0	0	0	0	0	0	0	0	0	10

Figure 4: Classifications with k = 1 of both the second and the third scenario. To be read as the matrix in figure 3.

	Streptoc.	*Stafyloc.*	*Enteroc.*	*Pseudom.*	*Escherc.*	*Morax.*	*Klebsiella*	*Citrob.*	*Enterob.*	*Proteus*	*Serratia*	Gram pos.	Gram neg.
Streptococcus	156	8	5	1	0	0	1	0	0	1	0		
Stafylococcus	6	106	1	9	5	1	3	0	6	0	0		
Enterococcus	7	0	18	0	0	0	0	0	0	0	0		
Pseudomonas	0	7	0	46	4	1	1	1	2	2	0		
Escherichia	1	6	0	2	55	0	2	3	0	0	0		
Moraxella	2	6	0	1	0	6	2	0	0	0	0		
Klebsiella	2	4	0	5	0	0	5	0	5	1	0		
Citrobacter	0	0	0	2	0	0	0	12	0	0	0		
Enterobacter	0	5	0	3	0	0	4	0	24	0	0		
Proteus	1	0	0	1	0	0	1	0	0	25	1		
Serratia	0	0	0	0	0	0	0	0	0	2	12		
Gram positive												312	39
Gram negative												21	260

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Reference annotations

13. ** Study showed the ability of IMS to detect most common urinary pathogens *in vitro*

14. ** Study showed the ability of IMS to detect most common wound pathogens *in vitro*

15. ** Study showed the ability of DMS to identify common pathogens involved with rhinosinusitis *in vitro*

22. ** In the study, DMS was shown to be capable of detecting *clostridium difficile* from stool samples