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ORIGINAL ARTICLE

Electronic nose analysis of bronchoalveolar lavage fluid

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ABSTRACT

Background Electronic nose (E-nose) technology has been successfully used to diagnose a number of microbial infections. We have investigated the potential use of an E-nose for the diagnosis of ventilator-associated pneumonia (VAP) by detecting micro-organisms in bronchoalveolar lavage (BAL) fluid in a prospective comparative study of E-nose analysis and microbiology.

Materials and methods BAL samples were collected using a blind technique from 44 patients following a minimum of 72 h mechanical ventilation. Control samples were collected from six patients mechanically ventilated on the intensive care unit (ICU) immediately following elective surgery. Quantitative microbiological culture and E-nose headspace analysis of the BAL samples were undertaken. Multivariate analysis was applied to correlate E-nose response with microbiological growth.

Results E-nose fingerprints correctly classified 77% of the BAL samples, with and without microbiological growth from patients not on antibiotics. Inclusion of patients on antibiotics resulted in 68% correct classification. Seventy per cent of isolates, cultured in the laboratory from the clinical samples, were accurately discriminated into four clinically significant groups.

Conclusions E-nose technology can accurately discriminate between different microbial species in BAL samples from ventilated patients on ICU at risk of developing VAP with accuracy comparable with accepted microbiological techniques.

Keywords Electronic nose, mechanical, complications, ventilation, ventilator associated pneumonia.

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Introduction

Ventilator associated pneumonia (VAP) is the most common health care associated infection in the intensive care unit (ICU), affecting up to 28% of patients receiving mechanical ventilation [1]. Patients with VAP have a longer duration of mechanical ventilation and hospital stay [2] and a 2–10 fold increased risk of death [1]. Subsequently, VAP has major cost implications, of up to $25,072 (2005 US Dollars) in hospital charges per patient [3].

Determining when a mechanically ventilated patient develops VAP remains difficult. A variety of techniques exist, using a combination of clinical symptoms and signs and the results of microbiological analysis. Microbiological culture of bronchoalveolar lavage (BAL) fluid is probably the best technique, but results are usually not available for 24–48 h after sample collection. A number of means of sample collection exist, including the aspiration of tracheal secretion, blind or formal BAL, or protected specimen brush; however, the preferred method of sample collection remains uncertain [4,5]. The Clinical Pulmonary Infection Score (CPIS) has been proposed as a way of combining the clinical parameters with microbiological findings into a more accurate clinical tool for the diagnosis of VAP [6], but some studies suggest that it is of limited use [7].

Electronic nose (E-nose) technology was developed in the early 1980s in an attempt to replicate the mammalian olfactory system's ability to detect volatile organic compounds (VOC's) [8]. VOC's are organic compounds that can easily become gases or vapours; E-noses can also detect other types of volatile compounds. VOC's produced by metabolic or microbiological

activity are in part excreted via the lungs and can be detected in exhaled breath. The potential of E-nose technology has been investigated for the diagnosis of a number of infections [9]. Studies have shown accurate diagnosis of urinary tract infection (UTI) [10] and mycobacterium tuberculosis infection [11]. E-noses are easily portable, suited to near patient testing and have the potential to give a diagnosis of infection with minutes or hours as opposed to days with conventional means. This in turn may allow earlier initiation of antibiotic therapy with the potential to improve patient outcome as well as avoiding inappropriate antibiotic use. In this preliminary study, we have investigated the use of E-nose technology to detect the presence of micro-organisms in BAL fluid from mechanically ventilated patients by comparing E-nose analysis of BAL samples with quantitative microbiological culture.

Materials and methods

With local research ethics committee approval, we studied 50 patients receiving mechanical ventilation on the ICUs at Cheltenham General and Gloucestershire Royal Hospitals. If informed consent could not be obtained from the patient, assent from a relative was obtained and patient's consent was sought at the earliest possible opportunity. In cases where the patient subsequently refused consent, the relevant samples were destroyed and the data were not analysed. In accordance with the standard definition of VAP, sample collection did not commence until patients had been receiving a minimum of 72 h of mechanical ventilation [12]. Samples were collected three times per week until mechanical ventilation was discontinued. Exclusion criteria were lack of patient consent to sample collection and an inspired oxygen concentration of more than 80%. Patients requiring an inspired oxygen concentration of more than 80% were felt to be too oxygen dependent to tolerate BAL sampling safely. Control samples were collected from patients mechanically ventilated following elective surgery within 24 h of admission to the ICU with no evidence of pulmonary problems. It is extremely difficult to find a suitable control group for critically ill, mechanically ventilated patients. For this reason, we have used a small number of controls to see if any obvious discrimination occurred with the E-nose. In a larger study, the issue of adequate controls will need to be carefully considered. For a statistical power of 80%, with 5% significance, a sample size of 100 samples was calculated assuming an incidence of microbiological growth of 25% within our patient population.

Bronchoalveolar lavage collection

BAL was performed using a blind technique as previously described by Garrard [13]. The patient was ventilated with 100% oxygen prior to sampling. The patient was positioned 30 degrees head-up and disconnected from the ventilator circuit. A 14 Fr suction catheter was then introduced down the endotracheal or tracheostomy tube into the bronchial tree until it could be advanced no further. Twenty millilitres of sterile normal saline was then injected through the catheter and immediately aspirated back into the syringe. Care was taken to stop aspiration before the tip of the suction catheter was withdrawn into the endotracheal or tracheostomy tube. The sample was divided in two and placed in sterile sample containers for microbiological and E-nose analysis. Quantitative microbiological culture was performed according to the standard hospital protocol.

E-nose analysis

BAL samples collected from patients were stored at 4 °C and allowed to warm to room temperature prior to analysis. The samples were then pipetted into 25 mL glass vials and allowed to equilibrate at room temperature for 1 h. The headspace gas was then analysed using the E-nose (NST 3320; Applied Sensor, Linkoping, Sweden) comprising a hybrid metal oxide semiconductor sensor array of 24 individual sensors. The headspace gas is drawn over the sensor array via a sampling port which pierces a diaphragm on the vial containing the sample for analysis.

To remove patient related confounding factors and to construct a laboratory-based model to assess the ability of the E-nose to discriminate between microbiological species; bacterial slopes taken from the BAL samples were created. These were created from the portion of the BAL samples sent to the hospital microbiology laboratory for standard microbiology and culture. The purpose of this laboratory-based model was to validate the performance of the E-nose to discriminate between micro-organisms grown in culture media, without the effect of patient confounding factors. The micro-organisms used were all isolated from BAL samples from patients. These were then used to culture the organisms in question in a laboratory setting without the presence of the BAL fluid. Organisms isolated from the bacterial slopes were maintained on nutrient agar plates. One colony per species was inoculated in 10 mL sterile nutrient broth (LabM, Bury, Lancs, UK) and incubated for 4 h at 37 °C in a rotary shaker at 0.3 *g*. Thereafter 100 μL of each microbial suspension was transferred into a fresh 10 mL sterile nutrient broth and incubated for 18 h at 37 °C in a rotary shaker at 0.3 *g*. Subsequently, 5 mL from each suspension was transferred into 25 mL glass vials and left for 1 h at 37 °C for headspace generation. Uninoculated nutrient broth was used as a control and five replicates per treatment were analysed in a random order using the E-nose. These studies were repeated at least twice. The same manufacturer's nutrient broth was used to culture all the organisms used in this part of the study to avoid the potential for a different volatile fingerprint being generated by different

culture media. It is important to note that the clinical samples were the patient's BAL fluid which was not cultured in broth prior to headspace analysis. This maintains the aim of near patient testing with the E-nose.

Data analysis

The response generated by the E-nose sensors in the form of normalized, mean-centred data were analysed using Matlab 7.2 (MathWorks Inc., Natick MA, USA). The response is defined as the change in resistance of each of the 24 individual sensors in the detection array to the adsorption and desorption of VOC's in the headspace of the sample being analysed. Multivariate analysis techniques were applied to the data. Principal component analysis (PCA) was used as a data reduction technique to explore the variance in the dataset and fed into linear discriminant analysis (LDA) to correlate E-nose response with the findings of culture of the lavage samples. LDA is a statistical technique used to classify samples into one of two or more groups based on a set of features which describe the samples. The samples are assigned to one of a number of predetermined groups based on observations made about the sample. Because of the sample size, leave-one-out cross-validation was used to evaluate the how the results would translate to an independent dataset. The same analysis technique was applied to the BAL samples from patients and to the model constructed using species grown in culture media from the bacterial slopes.

Results

Ninety-six samples were obtained from 44 patients and six samples from six control subjects. There were 32 men and 18 women. Full details of the organisms isolated following microbiological growth of the lavage samples are shown in Table 1. In summary, there were eight Gram-positive, seven Gram-negative, nine fungal, 10 mixed growth (more than one species isolated) and 67 samples with no growth. Fourteen samples were excluded from the data analysis because of technical problems with the sample processing and analysis. These were problems with storage and transport as opposed to inherent problems with the E-nose itself. The mixed growth samples were excluded from the four group data analysis.

E-nose discrimination of isolated microbiological species

Discrimination of the individual microbial species using E-nose signatures, measured from isolates grown from the clinical samples, was not attempted because of the small numbers of any single species. We therefore grouped the isolated microorganisms into four clinically significant groups (Gram-positive, Gram-negative, fungi and no microbiological growth), according to their classification, PCA fed LDA was shown to accurately classify 81/98 of the samples (83%), with sensitivity of 74–95% and specificity of 77–100%. Testing the performance with a leave-one-out cross-validation demonstrated correct classification of 70% of samples with sensitivity of 56–84% and specificity of 81–97%. Figure 1 shows the distribution of the E-nose response with samples coded according to the microbiological growth. Clustering of the E-nose data into the four groups is clearly seen.

Table 1 Number of isolates of the species grown from the bronchoalveolar lavage samples, in order of pathogenic potential. The total includes those found in mixed growth samples

Organism isolated	Number of isolates
Gram positive	
Staphylococcus aureus (all MRSA)	5
Coagulase negative Staphylococci	13
Streptococcus species	6
Enterococcus species	1
Aerococcus species	1
Gram negative	
Klebsiella species	6
Enterobacter species	4
Pseudomonas aeruginosa	4
Proteus species	1
Stenotrophomonas species	1
Acinetobacter species	2
Neisseria species	1
Bacteroides thetaiotaomicron	1
Fungi	
Candida species	20

E-nose discrimination of clinical samples

A rudimentary classification differentiating samples with and without microbiological growth would allow the clinician to introduce empirical antimicrobial therapy earlier than would otherwise be possible. Therefore, a two-group PC-fed LDA classification model was developed, to evaluate the discrimination of E-nose fingerprints from 88 samples; 58 with no microbiological growth and 30 with microbiological growth (Fig. 2). Sixty-eight per cent of samples were correctly classified by the leave-one-out cross-validated model, with sensitivity and specificity of 67–69%.

Twelve samples were collected from patients who had not received antimicrobial therapy within 48 h of sampling. Nine

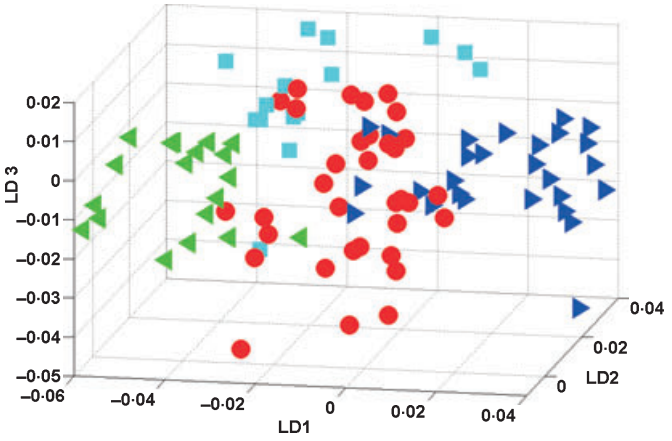

Figure 1 Scatter plot of linear discriminant scores of the electronic nose response to micro-organisms isolated from clinical bronchoalveolar lavage samples. Each measurement is coded according to the microbiological classification into four clinically significant groups, Gram-positive (▶), Gram-negative (◀), Fungi (●) and no microbiological growth (■).

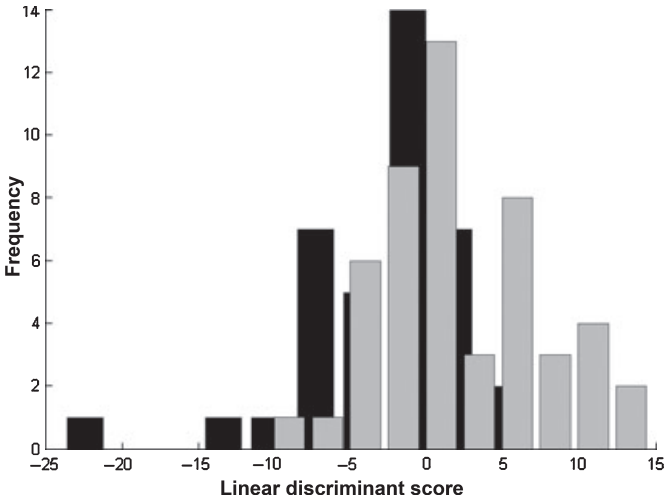

Figure 2 Histogram of linear discriminant scores differentiating samples with microbiological growth (black) and samples without microbiological growth (grey). The E-nose fingerprints from samples with no microbiological growth are seen to cluster in two sub-groups and a significant number of them are misclassified (to the left of zero) overlapping samples with microbiological growth.

of these samples were culture negative and three grew organisms. A further two-group PC-fed LDA classification model was developed to evaluate the discrimination of E-nose fingerprints of these nine no growth samples, from patients not on antibiotics and 30 samples with microbiological growth

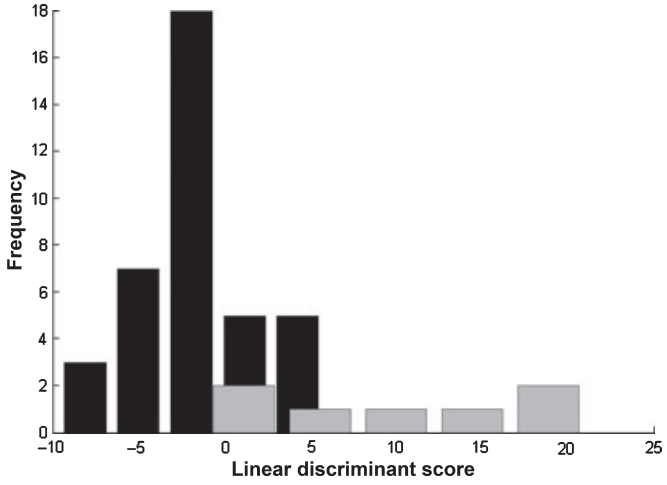

Figure 3 Histogram of linear discriminant scores differentiating E-nose fingerprints measured from samples with microbiological growth (black) and samples without microbiological growth taken from patients not on antibiotics (grey).

(Fig. 3). Seventy-seven per cent of samples were correctly classified by the leave-one-out cross-validated model, with sensitivity and specificity of 56–83%.

A clinically significant four group classification (Gram-positive, Gram-negative, fungi and no microbiological growth) was then developed, to evaluate the discrimination of E-nose fingerprints from 29 samples; nine with no microbiological growth, from patients not on antibiotics, five samples with Gram-positive growth, seven samples with Gram-negative growth and eight with fungal growth. Samples exhibiting mixed microbiological growth were excluded from this analysis. Seventy-six per cent of samples were correctly classified by the model, with sensitivity of 60–100% and specificity of 81–100%.

Discussion

This study demonstrates that E-nose technology has the ability to accurately discriminate between different groups of micro-organisms isolated from clinical BAL samples grown in nutrient broth, with 83% of samples being correctly classified in a clinically significant four group model (Gram-positive, Gram-negative, fungi and no microbiological growth). These findings suggest an E-nose may be able to detect the presence of and type of micro-organisms in BAL fluid, thereby potentially allowing for earlier initiation of targeted antibiotics in patients with clinically suspected VAP. We believe this is the first time that E-nose technology has been used to analyse BAL samples for the presence of micro-organisms.

There are various types of E-nose available commercially but they all share the same basic design. This consists of a sample delivery system, a sensor array over which the volatiles pass; and some form of data processing software which produces an output. This is an attempt to replicate the chemical sensor array found in the mammalian nose, signal processing in the olfactory bulb and pattern recognition performed by the brain.

The sample delivery system facilitates the generation of a headspace (containing the volatile compounds). The headspace is then injected into the detection system. An efficient sample delivery system is vital to ensure constant operating conditions. The sensor array (or detection system) reacts when in contact with volatile compounds. This reaction takes the form of a change in electrical properties. Each individual sensor reacts to all volatile compounds, but in a different way. The adsorption of the volatile compound onto the sensor causes a physical change; this is then recorded as a digital value. The data are then fed into the data processing software. As previously stated, we have used an E-nose with a hybrid MOS sensor array. Another type of E-nose with a different sensor array (for example, conducting polymer) may have produced different discrimination results. To ensure consistent results, we use substances such as acetone and ethanol at different concentrations that act as controls prior to analysing study samples.

We have demonstrated that E-nose analysis of BAL samples from ventilated patients has the ability to discriminate between different species (Fig. 1). This is in agreement with other published work on clinical infections. Pavlou showed that an E-nose could discriminate between different organisms causing UTIs [14] and different species of mycobacterium [11]. The only published studies investigating the diagnosis of VAP using an E-nose have compared E-nose analysis of breath samples with either computed tomography scans of the chest or a CPIS to diagnose VAP [15,16].

In the study comparing E-nose analysis of exhaled breath samples from mechanically ventilated patients with CT scanning of the chest, 25 sets of exhaled gas samples were taken from 25 patients. Each set consisted of five consecutive breath samples. The samples were taken within 48 h of CT scanning (average time 17·3 ± 14·5 h). Twenty-five CT scans were performed in 23 patients; two patients underwent two CT scans each. CT detected 12 cases of pneumonia and 13 pneumothoraces. No explanation for this high rate of pneumothorax is given. The authors do not state the time interval between the initiation of mechanical ventilation and CT scanning or whether any of the patients were receiving antimicrobial therapy prior to samples being taken. When using a prediction set different to the training set a diagnostic accuracy of 80% was achieved for E-nose breath analysis compared with chest CT. These results are promising and suggest that E-nose technology is able to discriminate between the breath of those with pneumonia and those without; but as with our study the sample size is small. The basis on which this discrimination occurs is unknown.

CPIS has been shown to have a moderate performance when compared with pathological diagnosis with a sensitivity of between 72% and 77% and a specificity of between 42% and 85% [17,18]. The inter-observer agreement in calculating CPIS has been shown to be poor [19]. Comparison of the E-nose fingerprints measured in this study with recorded CPIS will be reported elsewhere.

The instrument used to analyse breath in the above study was a Cyranose 320 (Cyrano Sciences, Pasedena, CA, USA). This is a hand held, portable device as opposed to the workbench based E-nose we used. Collecting breath samples for analysis with our system would have been difficult and prone to contamination. We were particularly concerned about the high levels of VOC's given off by the materials used in the construction of endotracheal tubes and breathing circuits. For these reasons, we elected not to attempt breath analysis using the E-nose.

The VOC's of interest detected from our BAL samples are likely to be alcohols, phenols and ketones. To gain more information on the nature of these VOC's and changes in the breath of mechanically ventilated patients, we have performed analysis of the breath of our study patients using Gas Chromatography-Mass Spectrometry. These results will be reported elsewhere.

In the clinical setting a simple two group model differentiating BAL samples with and without microbiological growth correctly classified 68% of samples in a leave-one-out cross-validated analysis. Crossover of a significant number of E-nose fingerprints from samples without demonstrated microbiological growth were seen to be overlapping the samples with microbiological growth, as classified by their E-nose signature. Indeed the no growth samples appear to cluster into two sub-groups, one of which overlies the growth samples (with negative discriminant scores). There are likely to be a number of reasons for this finding; however, the most obvious of these is the use of antimicrobial therapy prior to sampling. Eighty-nine of 102 (87%) of the samples were taken from patients who had been given antibiotics for at least 24 h prior to sampling. Other factors such as host response could also influence the results.

Deeper scrutiny of the misclassified samples in the clinical model (Fig. 2) indicated that most of these were in the 'no growth' group. These are samples that had no microbial growth during the culture period. Previous studies have shown that even 24 h of prior antimicrobial therapy can affect culture results [20]. Torres demonstrated that prior antibiotic use considerably decreased the sensitivity of BAL culture [5]. Montravers showed that 72 h of antimicrobial therapy could result in complete eradication of causative organisms [21]. As quantitative microbiological culture of BAL is used as the gold

standard for evaluation of the E-nose performance in this study, this false negative rate is of great concern. By this we believe that samples are correctly classified into a 'growth' group by the E-nose but microbiological culture has failed because of the use of antibiotics. This has led to the accuracy of the E-nose on the clinical samples appearing to be much lower than expected in comparison with the performance of the E-nose identification of species isolated in the laboratory.

The effect of antibiotics on the microbiological culture of the BAL samples was the motivation behind the development of the second two-group model shown in Fig. 3, as the use of antimicrobial therapy prior to sampling appears to affect the measured volatile signature. Samples collected from patients who had received antimicrobial therapy within 48 h of sampling were removed from the analysis. An improvement to 77% for the two group leave-one-out cross-validated classification was demonstrated, with little overlap between the two groups. However, removal of a large number of samples leads to a diminished statistical significance. Development of a four group classification, mirroring that constructed with the E-nose fingerprints measured from the species isolated in the laboratory, resulted in the correct classification of 76% of samples. This classification rate is comparable with current microbiological culture and sensitivities. Microbiological culture of BAL has been shown to have a sensitivity of between 19% and 83% and specificity of between 36% and 83% [22].

The accepted cutoff for the presence of infection as opposed to colonization in the use of quantitative BAL culture is usually $> 10^4$ CFU mL^{-1}. Five of our positive growth samples achieved 10^4 CFU mL^{-1} and 10 of the samples achieved 10^5 CFU mL^{-1}. All other positive samples achieved 10^3 CFU mL^{-1}. There appears to be no difference in the E-nose's ability to discriminate based on the level of growth. Our positive growth samples occurred in patients who had been receiving prior antimicrobial therapy and in one control patient who had not. Souweine suggest that in this situation the diagnostic threshold for infection should be reduced to 10^3 CFU mL^{-1} for BAL [20]. At present, we are unable to differentiate between colonization and infection. However, we have considered the presence of any growth in the BAL samples to have been significant, as the identification of any growth with surveillance sampling allows earlier identification of likely infecting organism in suspected VAP and earlier initiation of appropriate antibiotics.

The clinical signs and symptoms of VAP can be vague and unreliable. Clinical manifestations in combination with other diagnostic modalities are the usual method of diagnosing VAP. Chest radiography may be sensitive but typically is non-specific. Quantitative cultures obtained by various methods including BAL can be equivocal in the diagnosis of VAP. They can also take up to 48 h to yield results. Lung histology has been considered to be the gold standard but this also has inherent problems. Corley showed the prevalence of pneumonia in open post-mortem lung biopsies determined by each of four pathologists varied from 18% to 38% [23]. Various biomarkers including CRP, Procalcitonin and soluble triggering receptor (sTREM) in BAL fluid have all been evaluated as novel ways of diagnosing VAP [24]. We have shown that E-nose technology has the potential to offer rapid, near patient analysis of BAL samples with an accuracy level comparable with that of accepted microbiological techniques. However, larger multicentre studies are required to validate these preliminary results. The biggest advantage of E-nose diagnosis is its ability to give results within minutes or hours compared with days for microbiological methods. It has been shown that delayed treatment with appropriate antibiotics increases the risk of death from VAP by up to seven fold [25]. It is also important to establish the absence of infection rapidly as this decreases antibiotic usage, with associated side effects, and the development of resistance. The ability to institute early, targeted antimicrobial therapy has been shown to improve patient outcome and reduce rates of antimicrobial resistance and side effects. [1,26].

In conclusion, we have demonstrated that E-nose technology can be used to provide rapid, near patient analysis of BAL fluid to identify the presence of micro-organisms. The accuracy is comparable with that of current microbiological techniques and a result can be obtained in minutes or hours. This may permit more rapid diagnosis of VAP, thereby allowing earlier institution of antibiotic therapy where necessary, which has been shown to improve patient outcome. Prevention of the use of antibiotics in equivocal cases where patients may receive unnecessary antibiotic therapy pending a negative culture result could also be achieved. The ability to recognize the presence of Gram-positive, Gram-negative or fungal infection would enable the use of more targeted therapy prior to culture results, leading to reduced incidence of antibiotic resistant organisms. Further work is required to refine our models towards a target of accurate identification of the infecting organism.

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