Colorimetric Sensor Array Allows Earlier Detection and Simultaneous Identification of Sepsis-Causing Bacteria in Spiked Blood Culture

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Running Title: Colorimetric Sensor Array for blood culture workup

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Abstract

Sepsis is a medical emergency demanding early diagnosis and tailored antimicrobial therapy. Every hour of delay in effective therapy measurably increases mortality. Blood culture is currently the reference standard for diagnosing bloodstream infection, a multistep process which may take one to several days. Here, we report a novel paradigm for earlier detection and simultaneous identification of pathogens in spiked blood culture by means of a metabolomic “fingerprint” of the volatile mixture outgassed by the organisms. The colorimetric sensor array provided significantly faster detection of positive blood cultures than a conventional blood culture system (12.1 hours vs. 14.9 hours, \( p < 0.001 \)), while allowing identification of 18 bacterial species with 91.9% overall accuracy within 2 hours of growth detection. The colorimetric sensor array also allowed discrimination between unrelated strains of methicillin-resistant *Staphylococcus aureus*, indicating that the metabolomic fingerprint has potential to track nosocomial transmissions. Altogether, the colorimetric sensor array is a promising tool that offers a new paradigm for diagnosis of bloodstream infection.

Introduction

Bloodstream infection (BSI) due to bacteria and fungi is a medical emergency demanding early diagnosis and tailored antimicrobial therapy (21). Every hour of delay in diagnosis of sepsis increases mortality (10). Definitive diagnosis of BSI is achieved through recovery of the organisms from blood (17). Currently, clinical laboratories employ a two-step strategy: the first step involves the use of an
automated blood-culture system to detect the presence of growing organisms in blood culture. This is achieved through sensing a change in the pH or headspace pressure. In the second step, positive cultures are processed for species identification using phenotypic and genotypic assays. Phenotypic methods include coagulase tube test for Staphylococci, biochemical arrays in automated identification systems, and most recently, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (6, 27). Genotypic identification methods include fluorescence in situ hybridization (FISH) with peptide nucleic acid (PNA) probes for a limited number of species and several commercially available multiplex nucleic-acid amplification tests (NAATs) for a broad range of species recovered from blood cultures (1, 4, 7, 17, 27). With the existing blood-culture systems, the time to positivity typically ranges from 1 to 3 days, and an additional 1 to 2 days is needed for species identification with conventional methods, although MALDI-TOF MS and genotypic methods have shortened this time to hours. Meanwhile, patients are treated empirically with broad-spectrum antibiotics until the etiologic agent is identified and its susceptibility to antibiotics is determined (17). This approach puts the patient at risk for ineffective antimicrobial therapy and selection for antibiotic-resistant organisms.

Other assays used to speed up the diagnosis of BSI include NAATs for organisms and enzyme immunoassays for inflammatory biomarkers in whole blood and serum, respectively (17). However, NAATs do not have sufficient sensitivity and specificity to replace microbial cultures (22), while biomarkers are non-specific and unable to identify the etiology (18, 23). Thus, novel approaches are needed to reduce time to diagnosis of BSI.

The colorimetric sensor array (CSA) is an artificial olfaction technology used for rapid and accurate discrimination of complex mixtures or volatile organic compounds (VOCs) (15, 25). The CSA consists of a high-dimensional array of diverse chemically-responsive indicators embedded in a nanoporous matrix. Each indicator has distinct chemical reactivity with volatiles, and changes color
differently upon exposure to VOCs at concentrations as low as parts-per-billion. The resulting pattern
of color changes comprises a high-dimensional signature that is unique for each volatile mixture.
Moreover, the CSA includes rapidly reversible indicators that allow the sensor to track temporal
fluctuations in headspace VOCs. When placed in bacterial cultures growing on agar, the CSA can
distinguish between bacterial species with 98.8% accuracy (3). In this report, we describe the
application of the CSA for early detection and simultaneous identification of blood cultures inoculated
with sepsis-causing bacteria. We also describe the potential application of the CSA for discriminating
different strains of methicillin-resistant *Staphylococcus aureus* (MRSA).

**Materials and Methods**

**Blood culture preparation.** Bacterial species and strains were obtained from the Stanford University
Medical Center clinical microbiology laboratory strain collection. Eighteen species of bacteria
commonly recovered from blood cultures (28) were selected for building species-specific CSA
signatures (Table S1). Each species was represented by one type strain from the American Type
Culture Collection (ATCC) and two clinical strains; except for *S. aureus*, which had 11 clinical strains,
and for *Enterobacter cloacae*, which had only one clinical strain. For inoculum preparation, colonies
from sheep-blood agar (Difco) were suspended in sterile saline and turbidity was adjusted to 0.5
McFarland units. Each suspension was then serially diluted and plated to confirm the concentration.
A bacterial suspension of 0.5 ml from the appropriate dilution (see below) was injected into a
BacT/ALERT Standard Aerobic bottle (bioMérieux, Inc.), along with the 10 ml of whole blood
purchased from Stanford University Blood Center. For initial experiments, the content of blood
culture bottles was aseptically transferred to a Petri dish. For subsequent experiments, the inoculated
bottle was tested using either the BacT/ALERT system or the CSA system. For the CSA system, the
bottle septum was replaced with the CSA adaptor prior to incubation (Fig. 1). The inoculum
concentration was 10 CFU/ml in blood culture bottle trials and ranged from 2.5 to 1000 CFU/ml of blood in the Petri dish trials. Blood culture bottles inoculated with blood without organisms were included as negative controls. Upon completion of each trial, the blood culture medium was plated onto sheep blood agar to confirm purity. The detailed list of all trials, including technical and biological repetitions is shown in Table S1.

**CSA blood culture prototype instrument.** The CSA system has two major components: a disposable sensor and an optical scanner (Fig. 1). The CSA’s manufacturing procedures have been described previously (15, 16). A cap was custom designed to fit bioMérieux’s Standard Aerobic bottle due to availability of the BacT/ALERT 3D automated blood culture system in our laboratory. The sensor array was mounted inside the transparent cap or placed on a Petri dish cover with sufficient air gap to ensure adequate diffusion of headspace gases.

**Detection time.** The CSA was imaged every 20 minutes after inoculation using a flatbed scanner. For each image, RGB color values were extracted to create a time series of color changes. Using this time series, bacterial growth was detected by one of three trigger indicators in the sensor array. To identify each trigger indicator, the time series for each trial in the targeted species was reduced to the minimum and maximum slopes of the RGB color changes for each indicator during that trial. Each feature was ranked using Welch’s two-tailed t-test with false-discovery-rate correction, and logistic regression was used to fit a detection threshold for the feature with the lowest corrected p-value. Detection time was taken to be the earliest time that any of the three trigger indicator slopes crossed its detection threshold.

**Species classifier.** To create a CSA signature for each species, the slopes of the RGB color changes was sampled for each indicator at one-hour intervals, starting 3 hours before the detection time and extending up to 9 hours after. A species classifier was constructed by training a support vector
machine (SVM) on the time series of color changes using the e-SVC implementation in libSVM (5).

Ten repetitions of 10-fold stratified cross-validation was used to evaluate classifier performance on a test set that did not include the training set used to build the classifier. One-vs.-all classification performance for individual classes was constructed using sensitivity, specificity, and predictive value (2). Feature selection and classification were performed in R using the stats and e1071 packages as well as the data mining framework RapidMiner (20). Exact binomial confidence intervals were computed with the binom package (8).

Strain typing. PFGE genotyping was performed as previously described (26). The PFGE dendrogram was generated by BioNumerics (19). The similarity between patterns was calculated using the Dice coefficient with settings at 1.25% optimization, 1.25% tolerance, and a “change towards the end of the fingerprint” value of 0.5%. The cluster analysis was done using the unweighted pair group method with arithmetic averages. CSA strain typing was performed by inoculating 9 different strains of MRSA at 10 CFU/ml in our standard blood culture bottle and collecting the sensor response. For the CSA analysis, principal component analysis was performed and the resulting 10 most important principal components were used for hierarchical cluster analysis using the minimum variance method.

Results

Species-specific CSA signatures in blood culture. To establish that the CSA can detect species-specific VOC signatures in blood culture, we inoculated Petri dishes with BacT/ALERT aerobic blood culture medium, human whole blood and various concentrations of *S. aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *P. aeruginosa*, ranging from 2.5 to 1000 CFU/ml, and monitored CSA response over time. After an initial equilibration to the VOCs released by the growth medium itself, bacterial growth triggered a species-specific pattern of color changes. The CSA kinetic profile...
showed a highly dynamic pattern, which is reflective of the changes in the headspace VOC composition over time (Fig. 2). Using the kinetic profiles, the CSA was able to discriminate the four bacterial species with 96.8% accuracy (95% confidence interval (CI), 87.6-100%; Table S2). Detection times at lower concentrations lagged behind detection times at higher concentrations. However, the species-specific responses of key colorimetric indicators were independent of inoculum concentration.

**Library construction of bacterial CSA signatures.** Encouraged by the result in Petri dish, we constructed a library of CSA signatures for 18 bacterial species in the BacT/ALERT blood culture bottles using the CSA blood culture prototype instrument (Fig. 1). In total, we assembled a dataset of 1192 blood culture trials. Out of 73 different indicators in the sensor array, 53 had a very strong response to bacterial volatiles. Most indicators responded with dynamic patterns while a few indicators, such as Nile red, showed no response to any bacteria. Furthermore, one indicator, 5,10,15,20-tetrakis(4-methoxyphenyl)-21H,23H-porphine cobalt (II), was particularly important for consistently detecting *A. baumannii*, which generally had a weaker response than most other bacteria. A heat map used to visualize the average rates of color change with the CSA revealed unique signatures for the 18 species (Fig. 3). An accompanying dendrogram revealing the relationship between 18 bacterial species based on their VOC signatures (Fig. 3) (13).

**Detection time and identification accuracy.** The CSA detected the presence of bacteria in blood culture bottles (10 CFU/ml of blood) with 99.9% sensitivity. Most bacteria were detected within 13 hours, but some species, such as *Staphylococcus epidermidis* and *Staphylococcus lugdunensis*, required longer incubation periods. Furthermore, certain species, such as *Enterococcus faecium*, had a larger standard deviation for detection time due to strain variability (ATCC 27270, 10.4 hours ± 0.3 hour; IS-09, 20.1 hours ± 0.6 hour; and IS-23, 12.61 hours ± 0.6 hour), and had an average detection time of 14.2 hours with a standard deviation of ± 4.3 hours. Overall, the CSA provided significantly
faster detection of blood cultures when directly compared to the bioMérieux BacT/ALERT system (12.1 hours vs. 14.9 hours; \( p < 0.001 \); Fig. 4).

To evaluate the accuracy of CSA for species identification, the sensitivity and specificity of CSA were determined for each of the 18 species at the time of culture positivity and each subsequent hour, for a total of 9 hours (Table 1). Several species, such as *P. aeruginosa*, *S. lugdunensis*, and *A. baumannii*, were identified with >90% sensitivity at the time of blood culture detection while other species had moderate sensitivities with an overall sensitivity of 82.5%. However, within 2 hours after culture detection, sensitivity on average reached 91.9% (95% CI 90.2-93.4%). With each additional hour, the identification sensitivity improved and after 9 hours, it reached an overall sensitivity and specificity of 95.3% (95% CI 94.0-96.5%) and 99.7% (95% CI 99.7-99.8%), respectively (Tables 1, S3 and S4).

**Reproducibility of CSA signature.** Certain experimental parameters were not uniformly distributed for each species in our datasets, including blood source (donor variability), blood storage type (fresh vs. banked), inoculum concentration (Petri dish trials only), and sensor manufacturing lot. To check that the CSA’s high classification accuracy was not due to spurious correlations with these potential confounders, we constructed principal-component plots of *E. coli* and *S. aureus* and confirmed that varying blood source, inoculum concentration, and array manufacturing lots did not affect species separation (Fig. S1).

**Strain classification.** We investigated the CSA’s ability to discriminate between individual strains within a species. We used pulse-field gel electrophoresis (PFGE) genotyping as a reference standard to classify the degree of genetic relatedness for 9 clinical strains of MRSA comprising 3 genotypes (Fig. 5). Remarkably, the two unrelated strains (genotypes B and C) separated completely from the cluster of seven related strains in principal-component space and hierarchical cluster analysis dendrogram, while the genetically related strains (genotypes A and A\(^1\)) overlapped substantially,
indicating that the metabolomic fingerprint can discriminate between bacterial strains. However, further studies with more strains and species must be done to validate the preliminary results.

**Discussion**

We have demonstrated that the CSA system simultaneously detects and identifies bacterial species significantly faster than existing standard methods that only detect the presence or absence of positive culture. We report classification of all 18 clinically-relevant species with 91.9% overall accuracy within 2 hours of detection, approximately 1 hour before the BacT/ALERT system reports the mere presence of bacteria. The CSA system combines incubation, detection, and identification into a single step, thus both speeding time to positivity and furnishing a highly accurate species determination. The CSA also provides strain-specific data which may assist hospital epidemiologists in the identification and disruption of nosocomial outbreaks in real-time which is not presently possible (24). The CSA method is intrinsically simple, safe, robustly repeatable, and inexpensive; and thus offers an alternative platform for the characterization of blood cultures, particularly in resource-poor settings where technical expertise is limited. Most importantly, the CSA would facilitate earlier treatment with species-specific antibiotics leading to reduced utilization of unnecessary antibiotics and improved outcome in patients receiving tailored antibiotic regimens.

In recent years, MALDI-TOF MS has been developed for rapid and accurate identification of pathogens in blood cultures (30). Although MALDI-TOF MS reduces the time to identification when performed on blood cultures, it is not performed until a positive blood culture signal has been obtained, and therefore registers a result hours after the methods reported here. It also requires blood culture handling and processing which takes time, incurs labor cost, raises the possibility of human error in sample preparation, and can pose a biohazard risk to the operator. At present, MALDI-TOF successful identification rates are good for Gram-negative species for which adequate CFU/ml are present, but for Gram positive species or comparatively dilute positive cultures only 67% to 80%
reached acceptable confidence score results (6). Most recently, on demand multiplex NAATs have been developed and commercialized for identification of up to 92% of positive blood cultures (1, 17).

However, these tests also require minor sample handling and processing of one to four hours which add additional time to laboratory diagnosis of BSI.

Sources of error which must be assessed as this system is developed for clinical use include the potential misclassification of samples that contain bacterial species novel to the library, as well as mixed cultures and phenotypically distinct species that have very similar VOC patterns. The database in this study consisted of 1192 entries of bacterial signatures derived from 18 different species with an average of three different strains per species, and we believe that the system will become increasingly accurate with the inclusion of more strains per species in the database to account for the diversity that exists between strains in the population at large. Other diagnostic databases, such as MALDI-TOF MS address this issue by including at least 15 to 20 different isolates of one species to create a reference spectrum (11). It is likely that the clinical introduction of the CSA system will require generation of a pattern library built up with a similarly broad range of strains for each species classified.

Prior studies have indicated that the bacteria concentration in the blood of adult patients with BSI typically range from 1 to 30 CFU/ml (12, 14, 29). In this study, blood culture bottles were spiked with bacterial species at a clinically relevant inoculum concentration of 10 CFU/ml of blood. As demonstrated with four species, the classification ability is fairly robust to variations in inoculum concentrations. Some patients, however, may have <1 CFU/ml (9), which is outside the boundary of conditions evaluated here; future assessment of lower bacterial concentrations will be needed.

Although the results of this study are very promising, there is considerable room to improve the CSA technology to achieve even more rapid time to positivity of blood cultures. For example, the set of colorimetric indicators can be tuned to maximize sensitivity to the volatiles released by sepsis-
causing bacteria, and the growth media could be optimized so that bacterial VOC production during growth includes the classes of volatiles to which the CSA’s are most sensitive. Importantly, the BacT/ALERT bottles used in this study contain activated charcoal to absorb antibiotics, but charcoal is also well known to absorb VOCs. This may have limited our sensor’s ability to detect low concentration of VOCs at the early stage of the bacteria growth cycle. Replacing activated charcoal with polymer beads that are more selective for antibiotics (31) could enhance CSA sensitivity and result in even faster detection and characterization of bacterial infection.

In conclusion, the analysis of volatile metabolic biomarkers in the headspace of blood culture bottles with CSA allowed the earlier detection and simultaneous identification of sepsis-causing bacteria with great accuracy. This technology also demonstrated the ability to discriminate between bacterial strains. Altogether, the CSA holds promise for shortening the diagnosis of sepsis, and thereby improving clinical outcomes.

Acknowledgments

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References


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Figure legends

Fig. 1. (A) CSA with 73 indicators and 3 black fiducial marks, (B) cross section of the sensor cap adaptor, (C) CSA embedded blood culture bottle, and (D) the CSA prototype shaker and scanner.

Fig. 2. Bacterial VOC signatures with CSA (A) Color difference maps of four bacterial species cultivated in blood. Each color represents the difference between the indicator color intensity measured before and after culture positivity. (B) Selected indicator time response at various concentrations (2.5 – 1000 CFU/ml). Each line represents an independent trial. ZnTPP, 5,10,15,20-Tetraphenyl-21H,23H-porphine zinc; PV, pyrocatechol violet; NY, nitrazine yellow.
**Fig. 3.** Heat map of average CSA sensor patterns for 18 species of bacteria and an uninoculated blood. For each trial, a 213-feature vector of the 73 indicators was created using RGB-color-change slopes at 6 hours after the detection time. Each feature was standardized to have zero mean and unit variance across all trials, and assigned a column in the heat map. A cluster dendrogram displays the relationships to the right of the heat map.

**Fig. 4.** Comparison of time to detection by the CSA system (n = 1017) and bioMérieux’s BacT/ALERT system (n = 92). The detection time for the CSA system is based on average of minimum of 33 trials per species versus 3 to 14 trials with the BacT/ALERT system. Error bars represent the standard deviation of the detection time.

**Fig. 5.** Strain-specific CSA signatures of methicillin resistant *S. aureus* isolates. *(A)* Genotype data derived using pulse-field gel electrophoresis typing. Genotype A, consisting of definitely related strains (ST-43, -44, -45, and -64); Genotype A’ (ST-47, -48, and -54), closely related strains to genotype A; Genotypes B (ST-49) and C (ST-65), unrelated strains to genotype A. The similarity value at the root of the dendrogram is 40.09%. The similarity value at the node for the A and A1 genotypes is 95.65%. The similarity value at the node for the B and C genotypes is 45.46%. *(B)* Principal Component Analysis score plot and *(C)* Hierarchical Cluster Analysis dendrogram showing the separation of VOC signatures among different strains of methicillin resistant *S. aureus*. MWM, molecular weight marker.

**TABLE 1.** The sensitivity of CSA for species identification as a function of time following culture detection. At each hour (hr) after blood cultures were detected by CSA, the sensitivity of CSA for culture identification was calculated. The color map is based on sensitivity value in which red and green represent minimum (49.7%) and maximum (>93%), respectively.

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