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Rapid Diagnosis of Tuberculosis from Analysis of Urine Volatile Organic Compounds

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Abstract

The World Health Organization has called for simple, sensitive, and non-sputum diagnostics for tuberculosis. We report development of a urine tuberculosis test using a colorimetric sensor array (CSA). The sensor comprised of 73 different indicators captures high-dimensional, spatiotemporal signatures of volatile chemicals emitted by human urine samples. The sensor responses to 63 urine samples collected from 22 tuberculosis cases and 41 symptomatic controls were measured under five different urine test conditions. Basified testing condition yielded the best accuracy with 85.5% sensitivity and 79.5% specificity. The CSA urine assay offers desired features needed for tuberculosis diagnosis in endemic settings.

Keywords

colorimetric sensor array; electronic nose; chemical sensor; urine headspace; tuberculosis

Mycobacterium tuberculosis (MTB), the causative agent of tuberculosis (TB), is one of the leading causes of morbidity and mortality from infectious diseases worldwide, with an estimated 9.6 million new cases of active TB and 1.5 million deaths annually.¹ In the majority of endemic settings, diagnosis of TB is still based on microscopic visualization of bacilli.² This method has served the world for over 100 years, but it misses half of TB cases and requires an equipped laboratory and trained technologists. More recently, the development of various nucleic acid amplification tests (NAATs) for TB, such as the Xpert

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Additional experimental methods, demographic data of enrolled patients, sensor response figures, detailed statistical results, and the list of significant indicators (PDF)

Notes

P.R., R.A.M., M.E., and S.H.L. have an interest in Metabolomx, which engineers and supplies CSAs.

MTB/RIF and Line Probe Assays, have significantly improved the sensitivity and turnaround time of TB diagnosis.³ However, these assays still require sputum collection, 2+ hours of assay run time, and most importantly, financial and operational resources.^{4–7} Hence, in its consensus target product profile for better TB diagnostics, the World Health Organization has called for sensitive, non-sputum sample-based, and simple diagnostics.⁸

There is increasing interest in application of metabolic profiling for diagnosing invasive infectious diseases and treatment response monitoring. The potential of a metabolic approach to TB diagnosis has been demonstrated in sputum,^{9, 10} blood,¹¹ breath,¹² and urine-based tests.^{13, 14} These studies suggest both the host and the pathogen contribute to the metabolomic TB signature. Several metabolic-based applications for TB diagnosis already exist. For instance, African pouched rats are able to diagnose TB by sniffing sputum samples.^{9, 10} Attempts to develop non-invasive breath-based tests have resulted in moderate sensitivity and specificity.¹²

Several studies have identified urine as a rich source of TB biomarkers.^{13–18} Banday and colleagues showed that TB patients have increased level of *o*-xylene and isopropyl acetate and decreased levels of 3-pentanol, dimethylstyrene, and cymol in their urine headspace compared to healthy controls.¹⁴ Lipoarabinomannan (LAM), a cell wall lipoglycan of MTB, can also be found in urine of TB patients, although with poor sensitivity.¹³ Cannas reported small fragments of MTB DNA were found in the urine of 79% of TB patients studied.¹⁵ The Campos-Neto group reported a list of TB-diagnostic urine biomarkers,¹⁶ among which the Rv1681 protein was clinically validated with 44% sensitivity for TB diagnosis.¹⁷ More recently, Young *et al.* reported the identification of tuberculosis protein biomarkers in human urine samples and concluded that three of ten mycobacterial proteins identified in the urine of TB patients are promising biomarkers for POC diagnostics for TB.¹⁸

Given the abundance of TB biomarkers in urine and the fact that urine can be collected noninvasively, there is an opportunity to apply novel diagnostic tools for noninvasive diagnosis of TB. A CSA is a cross-responsive chemical sensor that can identify high-dimensional signatures of volatile organic compounds (VOCs), which is analogous to biological olfaction.¹⁴ The core sensing element is a broad spectrum of indicators, which participate strong indicator-analyte interactions, such as metal-ion-containing dyes to sense Lewis basicity (e.g. amines), pH indicators to sense Brønsted acidity/basicity (e.g. amines and organic acids), dyes with large permanent dipoles to sense local polarity (e.g. alcohols), redox indicators to sense redox sensitive analytes (e.g. sulfides and nitric oxide), and nucleophilic indicators to sense electrophilic analytes (e.g. aldehydes and ketones). Each indicator has distinct chemical reactivity with volatile molecules, and changes color differently upon exposure to analytes. The resulting pattern of color changes comprises a high-dimensional fingerprint, which has been demonstrated to enable the identification of both single component analytes (e.g., TICs,¹⁹ VOCs²⁰ and explosives^{21, 22}) and complex mixtures (e.g. urine,²³ exhaled breath,²⁴ and bacterial culture headspace^{25, 26}). We report here the application of a CSA for diagnosis of TB via urine headspace analysis. The CSA used in this study was developed previously during our work to identify sepsis-causing bacteria in blood culture by their metabolic VOC fingerprint.²⁶ While our prior works on

culture headspace involved measurement of metabolic VOC fingerprints during bacterial growth in spiked cultures, the current study presents the first application of the CSA for direct urine-based diagnosis of a bacterial infection based on detection of disease-specific VOCs in urine samples from patients with suspected TB. Further, we demonstrate that the CSA performance can be enhanced by pretreating urine samples with an additive to enhance release diagnostic volatile mixtures.

For this study, we collected urine samples from patients admitted to Highland Hospital in Oakland, California for suspected TB to determine whether CSA can distinguish TB cases from controls. Informed consent was obtained from all study participants under an institutional review board protocol at Highland Hospital and Stanford University. Microbiological culture was performed per standard of care and used as the reference standard in this study. Patients were excluded from the study if they had already started TB treatment. Overall, 22 cases and 41 controls were enrolled. Five patients had extrapulmonary TB (Table S1). Control patients included patients with diverse diseases, including community-acquired pneumonia (CAP). Table 1 compares the clinical features between two groups and shows no significant difference in terms of their sex, age and smoking status. Numerical age data was compared using *t*-tests, and categorical sex and smoking data were analyzed by χ^2 test.

The complexity of urine samples due to various confounding clinical variables (e.g., urine concentration, pH and sampling method) poses a challenge for any urine-based TB diagnosis. As such, urine samples have been pretreated to differentially liberate urine volatiles in a search for a TB-specific VOC fingerprint. A detailed experimental procedure can be found in the supporting information. To foster differential liberation of volatiles, the urine sample was divided into five test conditions, including a neat sample and mixtures with chemical additives known to liberate additional volatiles from urine.^{15,20} In brief, the pH of healthy human urine can range from 6.5 to 8.0, rendering amines non-volatile. At high pH (i.e., addition of basic additives), these amines are converted to their volatile free base form. Conversely, organic acids and nitric oxide are more easily volatilized from acidified urine.^{14, 27, 28} Elevated levels of nitric oxide metabolites have been found in urine from patients with TB.²⁹ Salt was added to increase the ionic strength to enhance the release of VOCs from urine to the cartridge headspace. The final test condition incorporated a pre-oxidation step, accomplished by passing urine volatiles through a chromium (VI)-packed tube to convert aldehydes, esters, alcohols, and hydrocarbons into more reactive species that could be better detected by the sensor array. Lin *et. al.* have shown that a vapor stream through such a pre-oxidation tube before presentation to the CSA can substantially improve the sensitivity to less-reactive VOCs.³⁰

For each urine test, the CSA was deployed inside a urine headspace cartridge comprised of a plastic container with a viewing window and an absorbent paper disk for urine deposition (Figure 1). The CSA's manufacturing procedures have been described previously.^{19, 26} Teflon rings on each side of the sensor were used to prevent the urine sample from coming in direct contact with the sensor array. Urine sample (200 μ L) was loaded through a small hole onto a filter paper, quickly saturating the cartridge headspace (about 5 mL) with urine volatiles. The sensor cartridge remained sealed with a septum, except during sample loading.

Additives were mixed with urine in a 1:1 volume ratio, and each biological sample was tested in duplicate under each of the five conditions: 1) neat urine, 2) urine + 1 M tosylic acid aqueous solution, 3) urine + 1 M NaOH aqueous solution, 4) urine + saturated sodium chloride aqueous solution, and 5) passed through a pre-oxidation tube containing chromic acid.

An Epson V600 scanner was used to image the CSA at 3-minute intervals for 4 hours with both scanner and sensor cartridges kept inside an incubator at 37 °C. Running urine headspace analysis under constant temperature ensured highly reproducible evaporation of urine volatiles, and allowed low-noise colorimetric data. For statistical analysis, images from 7 time points were selected (9 min, 18 min, 27 min, 1 h, 2 h, 3 h, and 4 h). For each image, the red, green, and blue values of 73 indicators in the sensor array under the five conditions were extracted, and color difference maps were constructed by subtracting the color vector of the initial image from the color vectors of all subsequent images in the trial. Thus, for each of five test conditions, 1,533 features (73 indicators $\times$ 3 RGB colors, 7 time points) were extracted for statistical analysis. A visualization of the color difference maps for each test condition is presented in Figures S2.

Principal component analysis (PCA) was performed using data from all test conditions to reduce a high dimensional feature space into a far smaller number of optimally informative orthogonal feature vectors. The resulting principal components are the coefficients of these new features, and plotting our samples in the space of the first few principal component coefficients reveals the separation between sensor patterns for the TB and suspected TB cases (Figure 2). Note that the first three principal components represent only 57.7% of total variance, but that already shows moderately good separation between TB (red dots) and control patients (blue).

For supervised statistical analysis, a random forest (RF) model was trained and validated using repeated stratified cross-validation (10 $\times$ 10-fold). RF is a nonlinear classification and regression algorithm that operates by constructing multiple decision trees.³¹ First, the dataset was broken into non-overlapping training and test sets. The RF model was built based on the training set alone using statistically relevant features extracted by performing a *t*-test (threshold = 0.05). A heat map of selected sensor features for all test conditions is shown in Figure S3. The trained classifier was then evaluated using the hold-out test dataset for each test condition individually.

Sensor responses for each of the five urine conditions were individually used to predict TB vs. no TB, and to assess their relative value as well as the combination of all in an attempt to further increase accuracy by combining the data. Evaluation of the impact of additives on the sensor performance revealed that addition of basic additive led to the highest performance with 85.5% sensitivity and 79.5% specificity (Table 2), while the salt additive and the pre-oxidation treatment reduced the sensor performance (56.2% and 52.1% accuracies, respectively) from the level achievable from urine alone. Banday *et al.* previously reported that TB patients could be differentiated from healthy controls via headspace analysis of acidified urine samples.¹⁴ While we also observed that acidified samples nominally improved the accuracy, the best sensitivity and specificity were achieved when the urine was

treated with a strong base (NaOH), which suppresses volatilization of acids while increasing the concentration of basic compounds in the headspace. When all test conditions were combined in the analysis, our accuracy diminished slightly to 75.4%, which may be due to a small sample size in our training set.

Consequently, a permutation test was also carried out to evaluate the robustness of our classifier. The urine labels associated with the sensor data were randomly shuffled, and the above described statistical analysis was repeated. As expected, analysis results obtained from scrambled labels drop down to near random chance (Table S2–4). We also performed the statistical analysis as a function of sensor exposure time using the basified urine data only. As shown in Table 3, the sensor had already achieved 80% sensitivity within 20 mins of exposure. Near maximum performance was achieved within 60 mins with nominal improvement of performance in the following 3 hours, suggesting that the headspace gas and sensor response are equilibrated under an hour. A receiver operating characteristic (ROC) curve was plotted to visualize how our prediction model performed at different cut-offs, and the area under ROC curve was calculated to be 0.904, supporting the validity of the sensor technology (Figure 3).

We also determined the list of significant indicators identified by the RF classifier during classifier training. Out of 73 different indicators in our sensor array, 14 indicators were identified in every cross validation permutation as important for detecting TB signature (Table S5). This list of indicators represents a diverse set of indicators that includes that includes metalloporphyrins, pH indicators, solvatochromic dye, Zn (II), lithium, and Hg (II) salts combined with pH indicators that respond to metal complexation, and a nucleophilic indicator that responds to electrophilic analytes. We suspect that this broad spectrum of sensitive chemical interactions is responsible for the CSA's ability to classify TB-specific VOCs in the urine headspace robustly in the face of an otherwise highly diverse cohort of TB suspects.

In this work, we have demonstrated that the CSA is capable of identifying the VOC signature from the urine headspace of individuals with TB. Our study included patients with extrapulmonary TB, which require invasive sampling with the conventional diagnostic methods. The current study has focused on identifying a TB-specific metabolic signature rather than the identification of individual VOC biomarkers. Future work should include identification and quantification of volatile biomarkers that give rise to the TB-specific CSA signature using mass spectrometry. Furthermore, in the time since this study was performed, we have determined that standardization of sample collection and sample processing impact sensor responses. Hence, controlling for these pre-analytical factors is likely to improve the quality of the results; accordingly, we consider the accuracies in TB discrimination reported here are likely a lower bound. Further, in our study, urine samples were collected and frozen within a few hours. However, the tests were batched and some samples were tested several months after collection. Therefore, we also need to evaluate the impact of the storage on assay results. Future studies to enhance the sensor performance will include expansion of TB-responsive CSA indicators, evaluation of other urine additives, optimization of test conditions (e.g., urine volume, test temperature), and normalization of CSA response to

urine concentrations. Further validation studies involving larger cohorts of TB suspects are currently underway to confirm the initial findings.

The results from this suggest that the disposable CSA can be developed into an effective diagnostic tool for TB based on detection of VOC fingerprint in urine. While our initial study consisted of five different test conditions and four hours of exposure time, our results indicate that a single condition (urine alkalization) and an hour of headspace exposure are sufficient for an optimal sensor performance. Thus, we are optimistic that a noninvasive, simple, and inexpensive diagnostic device can be further developed for rapid diagnosis of patients infected with TB. Given that the disposable CSA cartridges can be manufactured inexpensively; the CSA is ideal for use in low resource settings. We suspect that the low cost and simplicity of this sensor technology may also make it a platform very well suited to other applications, including monitoring of TB treatment response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Image of the disposable urine headspace sensor used in the urine headspace analysis. The CSA contains 73 chromogenic indicators.

Figure 2.
Principal component analysis score plot of urine headspace analysis showing discrimination of TB and high risk control patients.

Figure 3. Receiver operating characteristic (ROC) curve demonstration performance of the RF model on the hold-out data set. The average false positive rate (1-specificity) is shown on the x-axis and the average true positive rate (sensitivity) on the y-axis.

Table 1

Baseline demographics for urine headspace analysis of Highland Hospital Patients.

	TB cases (n)	TB suspect controls (n)	p-value
Sample size	22	41 ^a	–
HIV coinfection	4	3	–
Smear-positive	13	0 ^b	–
Male/Female	14/8	27/13	0.758
Age, mean (SD)	43.2 (16.5)	47.8 (11.6)	0.208
Range	19–73	24–71	
# of Smokers (%)	3 (13.6%)	9 (22.5%)	0.398

^aOne subject was enrolled twice.^bSmear test was not performed on 2 control subjects.

Table 2

The CSA performance in detection of TB using 10×10-fold cross validation broken down by test conditions.

Condition	Accuracy (%)	Sensitivity (%)	Specificity (%)
1. Neat urine	63.9	64.8	63.5
2. Acidic additive	64.3	65.5	63.8
3. Basic additive	81.5	85.5	79.5
4. Salt additive	56.2	47.7	60.9
5. Pre-oxidation	52.1	45.2	56.0
All conditions	75.4	80.2	73.0

Table 3

The CSA performance in detection of TB as a function of exposure time for condition 3.

Exposure time (min)	Accuracy (%)	Sensitivity (%)	Specificity (%)
9	69.0	72.2	67.5
18	77.7	81.2	75.8
27	77.2	80.8	75.5
60	78.6	83.7	76.2
120	80.8	86.2	78.0
180	79.8	85.0	77.5
240	81.5	85.5	79.5