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Diagnosis of Bacteriuria by Detection of Volatile Organic Compounds in Urine Using an Automated Headspace Analyzer with Multiple Conducting Polymer Sensors

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The Osmetech Microbial Analyzer (OMA) is an automated headspace analyzer fitted with a novel detector system consisting of an array of polymer sensors, each of which responds to different volatile organic compounds. The system can be used for screening clinical urine specimens for significant bacteriuria by sampling urine headspace and subjecting the output of the multiple-detector response to principal component analysis. The OMA readily distinguished artificially infected urine samples from sterile controls. The OMA was then used to analyze 534 unselected clinical urine specimens, of which 21.5% had significant bacteriuria (containing >10^5 CFU of bacteria/ml). The sensitivity and specificity of the OMA compared with conventional culture were 83.5 and 87.6%, respectively. The OMA is a promising automated system for the rapid routine screening of urine specimens, and further clinical trials are in progress.

Urines for bacterial culture are among the most common specimens submitted to clinical microbiology laboratories. In our own laboratory we receive up to 500 specimens a day, but only 10 to 20% of these are subsequently found to be positive for bacteria. A rapid screening method to exclude probable negatives would save time and money as well as provide an improved clinical service. Many rapid screening methods have been proposed, including the chemical detection of products of bacterial metabolism.

Analysis of these bacterial compounds has usually been performed by gas chromatography (GC) or GC-mass spectrometry (GC-MS). Nonvolatile compounds have been analyzed after chemical derivatization and more volatile ones have been analyzed after organic extraction. Headspace analysis is an adaptation of the latter strategy which eliminates the need for extraction and simplifies sample handling. However, detection in clinical samples (without culture) is difficult since bacterially derived chemicals are present at low concentrations and easily swamped by the chemical noise from the patient's body fluid. Previous work in this area using GC headspace analysis (2–4, 6–8, 11) has not led to a practical application of this method, and the more sensitive and specialized technique of GC-MS has not been applied to urine screening.

Industrial methods of direct headspace analysis have been improved with the introduction of new types of conducting polymer sensors. When used in multiple arrays and combined with computer pattern analysis of the output data, these instruments can discriminate complex volatile mixtures (14), and they have been used for the diagnosis of a variety of clinical infections. Parry et al. (12, 13) could identify the presence of β-hemolytic streptococci by analyzing contact dressings from chronic leg ulcers; Greenwood et al. (5) showed that the pattern of volatile compounds released from dressings of infected chronic wounds could be used to monitor the progress of wound healing; Chandiok et al. (1) analyzed volatile compounds from high vaginal swabs and, in a small group of patients, were able to distinguish between patients with and without bacterial vaginosis.

In this study, we investigated the use of one such instrument, the Osmetech Microbial Analyzer (OMA), for the analysis of infected and uninfected human urine. The device samples the headspace above the surface of the specimen and detects volatile compounds by using an array of four conducting polymer sensors. Each sensor interacts with different adsorbed volatile chemicals, depending on their size, shape, and functional groups. We report here the results of analyses of samples of reconstituted human urine (RHU) experimentally infected with common urinary bacterial pathogens and of 534 clinical urine specimens sent to the clinical laboratory for investigation of suspected bacteriuria.

MATERIALS AND METHODS

Bacterial strains used in preliminary studies. These were strains of *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Staphylococcus aureus, Staphylococcus saprophyticus*, and *Enterococcus faecalis*. There was one reference strain from each species (shown in Table 1), together with five recent clinical isolates of each species obtained from patients with urinary tract infection. Organisms were identified by standard laboratory methods and by using an API 20E system (bioMerieux, Basingstoke, Hampshire, United Kingdom).

Chemicals and reagents. The following items were purchased from Sigma Aldrich Co. Ltd. (Dorset, United Kingdom): 0.1 M hydrochloric acid, high-performance liquid chromatography-grade water, 0.1 M sodium hydroxide solution, 0.1 M ammonium hydroxide solution, and sodium sulfate. Nutrient broth, nutrient agar, and phosphate-buffered saline were obtained from Oxoid Ltd. (Basingstoke, United Kingdom) and made up according to the manufacturer's instructions. Control chemicals for calibrating the analyzer (USBlk, US1, US2, US3) were supplied by Osmetech plc (Crewe, United Kingdom).

RHU. Protein lyophilizate and urinary metabolite lyophilizate of male urine were obtained from Sigma Aldrich Co. Ltd. RHU was made by reconstituting these lyophilized fractions in an appropriate volume of sterile water as recommended by the supplier, pooling the two components, and finally filtering the

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TABLE 1. PCA scores for reference and clinical bacterial isolates cultured in RHU

Organism	Mean score	
	PC1	PC2
E. coli		
Reference NCTC 10418	−14.4	−0.23
Clinical isolate 1	−14.4	−0.20
Clinical isolate 2	−13.8	−0.38
Clinical isolate 3	−13.2	−0.25
Clinical isolate 4	−14.0	−0.25
Clinical isolate 5	−15.3	−0.27
Mean (SD)	−14.2 (0.7)	−0.26 (0.06)
E. faecalis		
Reference NCTC 775	−14.0	−0.08
Clinical isolate 1	−14.5	−0.18
Clinical isolate 2	−13.8	−0.18
Clinical isolate 3	−13.4	−0.20
Clinical isolate 4	−8.2	−0.06
Clinical isolate 5	−15.9	−0.31
Mean (SD)	−13.3 (2.7)	−0.17 (0.09)
S. aureus		
Reference NCTC 8530	−8.7	0.05
Clinical isolate 1	−8.6	−0.57
Clinical isolate 2	−8.1	−0.44
Clinical isolate 3	−8.5	−0.39
Clinical isolate 4	−10.2	−0.59
Clinical isolate 5	−10.1	−0.53
Mean (SD)	−9.0 (0.87)	−0.41 (0.24)
Klebsiella spp.		
Reference NCTC 009633	−11.5	−0.01
Clinical isolate 1	−11.6	−0.10
Clinical isolate 2	−11.5	−0.13
Clinical isolate 3	−10.7	−0.14
Clinical isolate 4	−14.8	−0.75
Clinical isolate 5	−9.7	−0.08
Mean (SD)	−11.6 (1.7)	−0.20 (0.27)
S. saprophyticus		
Reference NCTC 007292	−1.9	0.26
Clinical isolate 1	−2.8	0.14
Clinical isolate 2	−1.8	0.06
Clinical isolate 3	−1.4	0.10
Clinical isolate 4	−2.9	0.07
Clinical isolate 5	−2.0	0.10
Mean (SD)	−2.1 (0.60)	0.12 (0.07)
P. mirabilis		
Reference NCTC 011938	12.0	−52.4
Clinical isolate 1	7.0	−29.0
Clinical isolate 2	11.3	−41.1
Clinical isolate 3	11.6	−39.6
Clinical isolate 4	11.9	−38.5
Clinical isolate 5	12.2	−37.7
Mean (SD)	11.0 (2.0)	−39.7 (7.5)
RHU sterile controls [Mean (SD)]	0.99 (0.53)	0.13 (0.53)

resulting solution through a 0.2-μm-pore-size microbiological filter (Gelman Science, Northampton, United Kingdom).

Reconstituted urine specimens. Cultures of the reference strains and clinical isolates described above were made up in RHU to produce artificial urine specimens for testing. The bacterial strains were grown in nutrient broth overnight at 37°C without shaking. Serial dilutions of the cultures were made in phosphate-buffered saline to obtain a concentration of ~10^2 CFU/ml. One hundred microliters of this suspension was added to 10 ml of sterile RHU and cultured overnight without shaking. The bacterial concentration (total viable count) was determined by serial dilution and spread plate culture prior to analyzing in the OMA system. Each organism was cultured four times, and the results were expressed as an average of the four replicates. Blank control specimens were uninfected samples of RHU. Four blank controls were run with each organism.

Clinical urine specimens. Clinical urine specimens were sent to the microbiology laboratory from patients with suspected urinary tract infection at Guy's and St. Thomas' Hospitals. Conventional diagnosis of urinary tract infection was made by using standard methods of microscopy and semiquantitative culture. Negative specimens were defined as those containing $<10^5$ CFU of any organism per ml as shown by conventional culture. Positive specimens contained $\geq 10^5$ CFU of one or more strains of bacteria per ml. These specimens were stored at 4°C and processed with the OMA within 24 h of culturing. Preliminary experiments showed excellent reproducibility when clinical specimens containing $\geq 10^5$ CFU of *E. coli* per ml were repeatedly analyzed. For this reason, clinical samples were analyzed only once.

Instrumentation and operation. The OMA consists of a sample carousel which maintains sample vials at a constant temperature of 30 ± 0.5°C and presents the headspace to a sensor array for analysis. The system is computer controlled, and data are captured to files on a computer hard disk. Urine samples are analyzed in the following way. One milliliter of culture or urine is transferred to a 22-ml sample vial containing 0.2 g of sodium sulfate and 0.1 ml of 1 M HCl. The vial is capped with a polytetrafluoroethylene-lined silicone septum, placed in the carousel of the machine, and allowed to equilibrate at 30°C for 5 min. The machine then automatically inserts a needle through the sample vial septum, in order to analyze the headspace. Nitrogen gas at 50% relative humidity is introduced above the surface of the urine via the inner lumen of the coaxial needle. The outer needle lumen allows the sample headspace to be delivered across the sensor array for 3 min at a flow rate of 60 ml/min. The sensor is then allowed to recover before humid nitrogen gas is passed over the sensor for a 4-min wash. The resistance of each of the polymer sensors is measured during the sampling period, and the change (ΔR) from the initial resistance (base resistance R) is calculated. The needle is then removed, the carousel moves the next sample into position, and the process is repeated. Each reconstituted urine specimen in this study was analyzed four times, and the results were recorded as the means of the four replicates. The clinical specimens were analyzed once.

Data handling. The percentage resistance change output from the sensors was measured and recorded every second. An average of the ΔR values over a 30-s optimal time period (for example, the period of 150 to 180 s) was used as the raw data.

(i) Calibration of the sensor. The performance of the sensor array was characterized by running a set of controls. In preliminary experiments with artificially infected RHU, the controls were sterile (uninoculated) RHU samples. In subsequent tests on clinical specimens using a more sensitive instrument, the controls were chemical standards provided by the manufacturer, which had been shown to produce results identical to those of a more expensive sterile RHU control and allowed calibration of the sensors. The sensor raw data for the controls were transformed into a reference map using principal component analysis (PCA) (9, 15). PCA reduces the control chemical data matrix into a set of scores and loading vectors. Once the system had been calibrated with the controls, scores for subsequent samples were calculated by multiplying the sample analysis data by the reference loadings calculated in the calibration step.

(ii) Classification of samples. In the preliminary studies, artificially infected RHU samples were classified by comparing their projected PCA scores against thresholds set by 24 negative-control RHU samples. Thresholds were set on the PC1 and PC2 axes at 3 standard deviations (SD) from the average PC1 or PC2 scores of the control RHU. Samples outside either or both ranges were classified as positive. In subsequent studies, clinical specimens were classified according to their projected response on a reference map that had been calibrated with chemical controls provided by the manufacturer. Sensitivities and specificities were calculated on the premise that the culture result was the true result.

RESULTS

Artificially infected RHU. These all had bacterial concentrations of $\geq 10^7$ CFU/ml. Table 1 shows the PCA scores for the RHU samples; each result for cultured urines is the mean of four replicates. The blank controls had a mean PC1 score of 0.99 and a mean PC2 score of 0.13. This gives a PC1 threshold of −0.6 to +2.58 and a PC2 threshold of −0.65 to +0.91. The RHU medium inoculated with reference or clinical bacterial

TABLE 2. Results of analysis of clinical urine specimens

Organism(s)	No. of specimens with Osmetech result		Total no. of specimens
	Negative	Positive	
Culture negative			
Acinetobacter spp.	0	1	1
Candida spp.	1	0	1
Contaminants	71	19	90
Enterococcus faecalis	1	0	1
Enterococcus spp.	1	1	2
Escherichia coli	4	1	5
Klebsiella spp.	5	0	5
Nongrowers[a]	280	27	307
Proteus mirabilis	1	0	1
Others	3	3	6
Total	367	52	419
Culture positive			
Candida spp.	1	2	3
Contaminants	2	8	10
Enterobacter cloacae	0	2	2
Enterococcus faecalis	0	1	1
Enterococcus spp.	1	6	7
Escherichia coli	9	58	67
Group B streptococci	2	0	2
Klebsiella spp.	0	6	6
Mixed	0	1	1
Morganella morganii	0	1	1
Proteus mirabilis	1	0	1
Proteus vulgaris	0	1	1
Providencia spp.	0	1	1
Pseudomonas aeruginosa	3	1	4
Staphylococcus aureus	0	2	2
Others	0	6	6
Total	19	96	115

[a] Specimens exhibiting no growth.

isolates gave mean PC1 and PC2 scores ranging from −39.7 to 11.0, respectively, for *P. mirabilis*, and −2.1 to +0.12, respectively, for *S. saprophyticus*. The other species gave mean PC1 and PC2 scores ranging from approximately −14 to −8 and −0.4 to −0.2. When these results were plotted on the PC1 and PC2 axes, all the infected RHU results fell outside the thresholds delineated by the controls.

Clinical urine specimens. A total of 534 clinical samples were analyzed both by culture and by a more sensitive OMA instrument than that used to analyze the artificially infected urine. When significant bacteriuria was defined as $\geq 10^5$ CFU/ml by conventional culture, there were 115 positive and 419 negative samples, giving a positive prevalence of 21.5% (Table 2). When specimens were classified by the OMA as positive or negative relative to thresholds set on the PC1 and PC2 axes by the control chemical calibrators, the sensitivity and specificity of the instrument were 83.5 and 87.6%, respectively. As would be expected, when the CFU-per-milliliter cutoff was lowered, the sensitivity fell and the specificity rose. Thus, when significant bacteriuria was defined as $\geq 10^4$ CFU/ml, the sensitivity and specificity were 72.3 and 89.4%, respectively. The detailed results are shown in Table 3.

DISCUSSION

The detection of volatile organic compounds in urine by gas-liquid chromatography (GLC) was used some years ago to detect bacteriuria. Manja and Rao (11) performed conventional GLC on urine samples incubated with appropriate supplements and showed that *E. coli* could be identified by the production of ethanol from lactose and *Klebsiella* spp. by the production of ethanol from adonitol. Hayward and colleagues (7–8) and Coloe (2, 3) utilized headspace GLC to identify volatile bacterial metabolites in artificial cultures and urine. *Proteus* spp. characteristically produced dimethyl disulfide and methyl mercaptan from L-methionine and trimethylamine from acetylcholine; *E. coli* and other coliforms produced ethanol from lactose or arabinose. This system was moderately successful in distinguishing infected and noninfected urine by direct analysis, but better results were obtained after incubation with arabinose and acetylcholine.

GC detection of bacteriuria, with or without MS, was not developed into a practical diagnostic tool. In the present study we investigated a new type of instrument fitted with a novel multiple polymer sensor array and an automated headspace sampler for the direct detection of volatile bacterial compounds in clinical urine specimens. In order to encourage volatile compounds to enter the headspace, specimens were acidified and were maintained at 30°C. The addition of sodium sulfate further enhanced volatility and increased sensitivity (10). The analyzer delivered a sample of headspace gas to an array of sensors, and the mean ΔR of each of the sensors was analyzed by PCA. Threshold PCA values were set by analysis of a series of controls, and these were used to classify test specimens as positive (infected) or negative (noninfected).

This technique was used to analyze artificially infected and uninfected RHU. At this early stage of development of the instrument, commercial pooled RHU was chosen as the test medium because, unlike clinical urine specimens, different samples from the same RHU batch have a uniform chemical composition, and other workers can use this medium to repeat our experiments. The instrument we used initially was less sensitive than the later one, and all the infected RHU specimens tested contained $\geq 10^7$ CFU/ml. Infected specimens were readily distinguished from negative controls, with PCA scores all more than 3 SD away from the mean of the controls. The

TABLE 3. Evaluation of samples[a] based on bacterial concentrations and comparison of OMA system and comparative culture

Bacterial concn cutoff (CFU/ml)	No. of samples with: Culture result		No. of samples with: OMA result				Comparison of OMA with culture	
	Positive	Negative	True positive	False negative	True negative	False positive	Sensitivity (%)	Specificity (%)
$\geq 10^4$	148	386	107	41	345	41	72.30	89.38
$\geq 10^5$	115	419	96	19	367	52	83.48	87.59

[a] A total of 534 samples were evaluated.

different PCA scores produced by the different species suggest that with refinement the analyzer may have the potential to distinguish different organisms associated with urinary tract infection.

For the analysis of clinical urine specimens, a more sensitive instrument was used, and the sensors were calibrated each day with a set of chemical standards. It is not practical to use negative urine controls in routine clinical analyses, because clinical specimens vary greatly in their chemical composition.

When 534 clinical urine specimens were analyzed, the OMA was found to have a sensitivity of 84% and a specificity of 88% relative to standard culture results when significant bacteriuria was defined as $\geq 10^5$ CFU/ml. When the cutoff was defined as $\geq 10^4$ CFU/ml, not unexpectedly, the sensitivity fell and the specificity rose.

There are a number of possible reasons for the false-negative results. Firstly, natural volatile compounds in human urine, caused by disease or diet, might saturate the sensor detectors and block the response to bacterial compounds by competitive inhibition. Secondly, bacterial volatile products might be lost, either by adsorption onto urinary cells or protein or by dissipation during any prolonged delay between specimen collection and analysis. Finally, some bacterial species may not produce volatile compounds that can be detected by the present sensor array; this may be the case for the group B β-hemolytic streptococci that were missed.

At the present time we do not know exactly to which of the volatile compounds out of the complex mixture in the headspace the instrument is responding; therefore, we cannot be sure that the present sensors are optimized for urine analysis. Furthermore, we do not know if there are other significant volatile compounds, presently undetected, which could be included in the analysis by the addition of other sensors to the array. Analysis of headspace by MS might reveal metabolic products that could be targeted by specially designed arrays in order to improve both the sensitivity and specificity of urinalysis. The present speed of analysis, although faster than that of GLC, is limited by the need for the sensors to recover after each sample. However, speed can be improved by using several sensor arrays in sequence, so that samples can be processed continuously without waiting for sensor recovery.

There is a need in the clinical laboratory to rapidly screen out culture-negative urine specimens, so that time and resources can be directed at further analysis of doubtful or possibly positive ones. The OMA shows considerable promise for automated screening, and more-extensive clinical trials with more-refined versions of the instrument are in progress.

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