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Sniffing Out Resistance – Rapid Identification of Urinary Tract Infection-Causing Bacteria and their Antibiotic Susceptibility Using Volatile Metabolite Profiles

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Highlights

- **Antibiotic susceptibility** of bacterial cultures were rapidly analysed by **TD-GC-MS**

- Cultures were analysed in under 30 minutes vs. 24 hours for traditional tests

- Differences in **volatile profiles** found between **resistant** and **sensitive** bacteria

- Differences in **volatile profiles** found between three **UTI-**causing bacterial species

Abstract

Antibiotic resistance is set to be an unprecedented threat to modern medicine. 'Sniffing' bacteria potentially offers a rapid way to determine **susceptibility**. A successful proof-of-principle study is described, using **thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS)** to 'smell' **cephalexin** and **ciprofloxacin** resistant and sensitive **Urinary Tract Infection (UTI)**-causing bacteria. 578 peaks at unique retention times were detected from 86 chromatograms of 18 bacterial isolates (*E. coli, K. pneumoniae* and *P. aeruginosa*). The isolates were grown with and without the presence of **antibiotic.** Chi-square analysis found 9 compounds that differed significantly between cephalexin sensitive and resistant isolates, and 22 compounds that differed significantly between ciprofloxacin sensitive and resistant isolates, at $p \leq 0.05$. When antibiotic was added to the media, more differences were found in the cephalexin group, attributed to lysis, but not in the ciprofloxacin group. Further work with large sample sizes will potentially enable the development of diagnostic algorithms using presence/absence of particular compounds of interest.

Graphical abstract
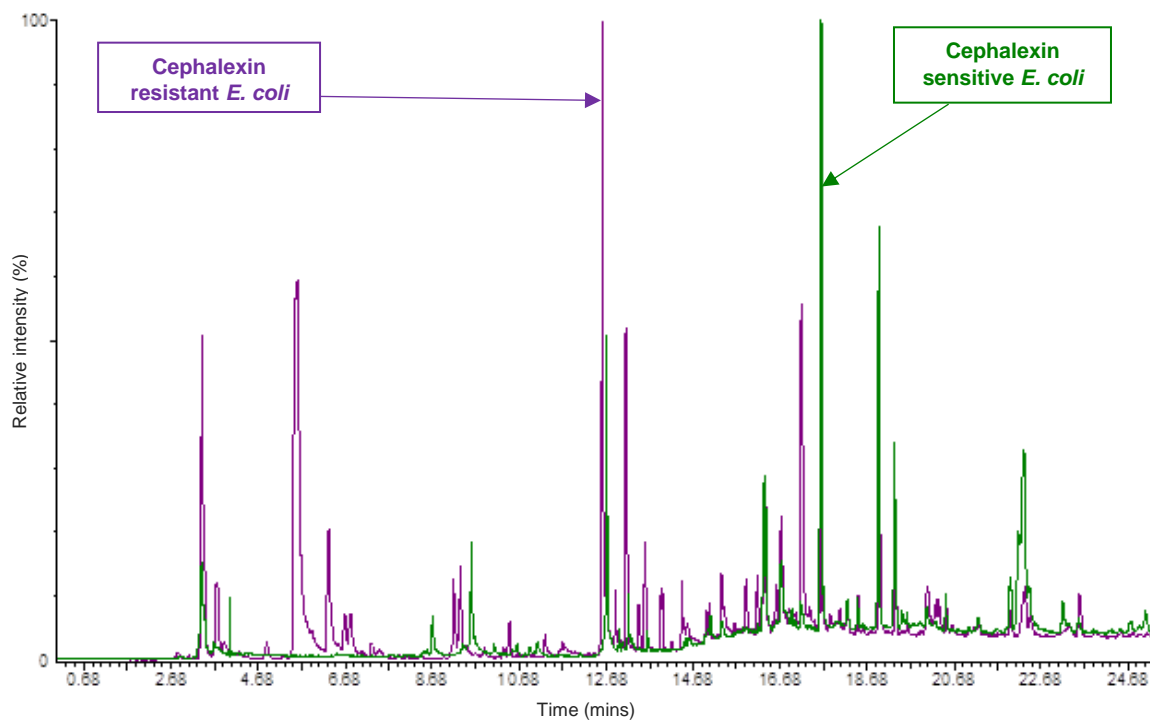
Keywords:

Volatile, metabolite, profiles, smell, thermal desorption, gas chromatography, mass spectrometry, antibiotic, resistance, urinary tract infection, susceptibility, cephalexin, ciprofloxacin

1. Introduction

There is a need for more rapid determination of antibiotic resistance (ABR) in bacteria isolated from clinical samples. ABR is becoming more prevalent, as are urinary tract infections (UTIs) due to an aging population. The WHO states up to 8 million urinary-tract infections occur in the United States each year, and each year, a growing and significant proportion – hard to measure, but probably 1 in 10, or 800,000 – are antibiotic-resistant [1]. Rapid diagnosis and appropriate treatment will decrease morbidity and healthcare costs.

The aim of any rapid diagnostic is to confirm infection and then susceptibility. Species identification is not always necessary, but can allow predictions about susceptibility in the absence of any other test, and might help suggest source of infection, or likely prognosis. Current time to confirm culture positivity, bacterial identification and susceptibility is 48-72 hours from urine [3], and 72 hours from blood [4] depending on starting culture density (susceptibility testing from culture takes 24 hours).

The WHO stated that the lengthy turnaround time for microbiological testing means practitioners often forgo it and prescribe broad-spectrum antibiotics [2]. Inappropriate prescribing of antibiotics in human and veterinary medicine is arguably the biggest driver of resistance, and is potentially avoidable. However, rapid bacterial identification direct from clinical samples, and more importantly, rapid antibiotic susceptibility testing is essential to make significant impact on inappropriate antibiotic prescribing.

Certain resistance mechanisms (e.g. production of enzymes/efflux pumps/altered binding sites) may use valuable energy and resources, and in the absence of a selection pressure, bacteria may stop expressing these resistance mechanisms. Therefore, the metabolism of a bacterium conferring resistance should be different from its sensitive counterpart, and these changes may be detectable using gas chromatography-mass spectrometry (GC-MS) to measure volatile metabolites.

Bacteria produce a wide and diverse range of volatile compounds. These compounds may be produced as by-products of metabolism, but they can also act as signalling molecules for communication between bacteria, or between bacteria and host. Although these interactions aren't fully understood, they are thought to play an important role in antibiotic resistance, as described in a review by Schmidt *et al.* (2015) [5].

Much research has been conducted into analysing the volatile compound (VC) profiles of common infectious bacteria [6]; and the aim has often been differentiation between species. For example, McGuire *et al.* (2014) rapidly differentiated *C. difficile* from other diarrhoea causing aetiologies using a custom-made GC-sensor system featuring a short multi-capillary column [7] and Shestivska *et al.* (2015) found *in vitro* data indicating methylbutanol isomers may be exhaled breath biomarkers of *S. maltophilia* lung infection in patients with cystic fibrosis [8].

UTIs are most frequently caused by *Escherichia coli*, as well other enteric bacterial pathogens such as *Enterococci*, *Klebsiella*, *Staphylococci*, and *Proteus* species, and fungal pathogen such as *Candida albicans* [9]. The first review on the potential applications of VOCs for identifying such UTIs was in 2001 by Guernion *et al.* [10]. Since then, there have been various reports showing that this approach has been promising, for instance as described in the review by Capelli *et al.* (2016) on electronic nose technology [11], and Storer *et al.* (2011) using selected ion flow tube-mass spectrometry [12]. More recently, Rees *et al.* (2018) used gas

chromatography mass spectrometry to identify pathogen-specific volatile metabolite profiles for 10 organisms responsible for 90% of urine and bloodstream infections [13]. It remains the case that more analytical work is needed on VOC profiling of urine headspace, in terms of sensitivity and reproducibility, so that research results can be transformed into routine clinic use.

Few groups have looked at differences between antibiotic resistant and sensitive strains. Zhu *et al.* (2013) showed methicillin-resistant *Staphylococcus aureus* (MRSA) can be detected in lung infections in mice via breath analysis [14]. Boots *et al.* (2014) found differences in the VCs produced by methicillin resistant and sensitive *Staphylococcus aureus* using a similar methodology as employed in this investigation [15], showing that GC-MS can be used effectively for this purpose. Most notably the compounds 1,1,2,2-tetrachloroethane, 2-heptanone and 1,4-dichlorobenzene showed significant differences. Weisner *et al.* (2014) used ion/molecule reaction-MS to analyse bacterial VCs, to differentiate the susceptibility of *E. coli* and *S. aureus* to ampicillin and oxacillin. They identified methanethiol as a marker for bacterial growth, however, only one sensitive and one resistant isolate of each species was studied. The authors then investigated the use of a metal oxide sensor to detect this compound, with promising initial results [16].

There is still a lack of published literature on rapid identification of antibiotic resistant bacteria relevant to UTIs using volatile profiling. This proof of principle paper explores the potential of volatiles to address this, by determining the volatile markers involved using GC-MS, it could lead to low-cost vapour detection technologies at point of care.

2. Experimental section

18 bacterial isolates (from University of Bristol School of Cellular and Molecular Medicine) were studied, which included three UTI-causing bacterial species with focus on the commonest aetiological agent (*E. coli*). Details are in table 1.

Table 1. Bacterial isolates showing number of repeats (n) with and without the addition of antibiotics

E. coli	n, n with cephalexin, n with ciprofloxacin	K. pneumonia	n, n with cephalexin, n with ciprofloxacin	P. aeruginosa	n, n with cephalexin, n with ciprofloxacin
17 1	2, 3, 0	K30	3, 0, 0	PA01 13	2, 0, 0
IR10	3, 3, 3	R1	2, 0, 3	301-5473 218	3, 0, 0
IR60	3, 3, 3	R2	3, 0, 3	-	-
PSA 1	3, 3, 0	NLTC505514	2, 0, 0	-	-
RKV 29	3, 3, 0	-	-	-	-
VCE 17	3, 2, 0	-	-	-	-
CDG	2, 0, 0	-	-	-	-
DH5α (PYT vector)	4, 2, 0	-	-	-	-
DH5α (PYT vector + CTXM)	4, 2, 0	-	-	-	-
DH5α (PYT vector + NDM1)	3, 2, 0	-	-	-	-
17 (PSU18 vector + CTXM)	0, 3, 0	-	-	-	-
17 (PSU18 vector + NDM1)	0, 3, 0	-	-	-	-

Overnight cultures were set up by using 10ml of nutrient broth in 10ml universal containers (Oxoid), inoculated with a 10 μl loop of frozen glycerinated stock culture, and then grown in a shaking incubator at 180 rpm and 37°C). The overnight cultures were then sub-cultured into 10 ml headspace vials with silicone rubber PTFE septa (Sigma-Aldrich), (60 μl inoculated into 3 ml of Muller-Hinton broth (Oxoid)) and grown for three hours as before. The optical densities of the cultures were between 0.7 and 1.1 at 600 nm (exponential growth phase). In some cases, cephalexin or ciprofloxacin antibiotics (both Sigma-Aldrich) were added to the media after 2.5 hours. Kanamycin was added to all cultures of DH5α (PYT vector) for plasmid retention. A media control of Muller-Hinton broth, incubated for three hours at 180 rpm and 37°C, was also analysed by GC-MS.

Susceptibility to cephalexin and ciprofloxacin was determined for each of the isolates by performing disc diffusion tests on Mueller-Hinton agar (Oxoid) at minimum inhibitory concentrations. The isolates were assigned sensitive, intermediate or resistant to cephalexin and ciprofloxacin, based on the measurements of the zones of inhibition. Intermediate isolates were not included for analysis of that antibiotic.

2.2. Analytical techniques

Volatile metabolites in the vial headspace were loaded onto stainless steel TD tubes with appropriate adsorbents (TENAX TA 68 mg for C6 to C30 compounds and Sulficarb 26 mg for C3 to C8 compounds (Markes International Ltd)), by pumping purified room temperature lab. air (pumped through an Activated Charcoal Purifier (Alltech®) containing 4-12 mesh activated charcoal (DARCO®)) at 80 ml/min for two minutes using a custom-built tube loading rig, as described in Gould *et al.* (2018) [17]. Tubes were analysed the following day if possible, or stored at -80°C and analysed within three weeks. Tubes were analysed on a TD-GC-MS system (Unity model 1 TD with Ultra 2 auto-sampler (Markes International ltd.), 6890N-GC 5973N-MS GC-MS system (Agilent)). Volatiles were separated using an RTx-624SilMS column (60 m x 0.32 mm x 1.8 μm (Restek)).

The TD-GC-MS method was taken from a recommended application by Agilent Technologies Inc. for the analysis of VCs [18]. Detailed parameters are in table 2.

<u>Table 2. TD-GC-MS parameters</u>

Parameters	Setting
GC	
Carrier gas	Helium in a constant pressure mode
Initial oven temperature	45°C
Temperature ramp(s)	Ramped by 10°C per minute to 190°C, then further ramped by 20°C per minute to 250°C
MS	
Mode	Scan mode
Scan range and rate	33-300 m/z (8 scans per peak)
Source temperature	230°C
Quadrupole temperature	150°C
Transfer line temperature	260°C
TD	
Pre-purge time	1.0 minutes
Primary desorb	200°C for 10 minutes
Trap desorb	250°C for 5 minutes (Trap heating rate 100°C/s)
Trap (low temperature)	-7°C

Trap type	General purpose hydrophobic (C4/5-C26) (tenax/graphitised carbon black)
Flow path temperature	140°C
Nominal carrier gas pressure	30.9 psi
Desorb flow	20 ml/min

2.3. Data analysis

Data files were initially converted to .CDF files using Agilent Chemstation® software. Chromatograms were opened using Perkin-Elmer Turbomass software and peak areas calculated. Peak areas with a total ion current (TIC) of less than 10,000 were not included in the data analysis. Compounds were identified using the NIST 2014 library.

Background subtraction was undertaken manually for each of the peaks, to reduce background "noise" and spectral overlap due to partial co-elution of compounds. NIST's suggested general guidelines were taken into account for assignations; match factor scores >900 are considered an excellent match, 800- 900 as a good match, 700-800 as a fair match, and <600 as a poor match [19]. Many of the compounds had an excellent match, compounds that had a reverse fit NIST library score <800 were not assigned a chemical identity and were assigned a unique number, starting with the shortest retention time. These were referred to as 'unknowns'. Siloxane-containing compounds and suspected plasticisers were not included in data analysis, as these do not have a biological origin or may be attributed to breakdown of the GC column or septa.

Retention time matching of 7 compounds (acetaldehyde, 2-butanone, dimethyl disulphide, decane, 2-ethyl-1-hexanol, decanal and indole) was undertaken. The compounds were selected as they are readily commercially available; all compounds were supplied by Sigma-Aldrich. A standard mix of the compounds dissolved in methanol was directly injected onto the TD tubes used previously (1 µl injections), and analysed using the GC-MS method as before, in duplicate. The chromatograms were analysed using Turbomass software and compounds were identified using the NIST library and matched on retention time (within 1% or 6 seconds of the retention time, whichever is greater) [20].

Statistical differences were assessed with chi-square tests, using presence and absence of compounds (absence = peak area <10,000 TIC). Statistical analysis taking into account relative differences in the amount of each compound, using peak area as a measure of abundance, was also performed for each compound with independent samples T-tests and Mann-Whitney-Wilcoxon tests.

3. Results and discussion

578 peaks at unique retention times were detected above the threshold peak area from 86 chromatograms of 18 bacterial isolates. Example chromatograms are shown in figure 1.
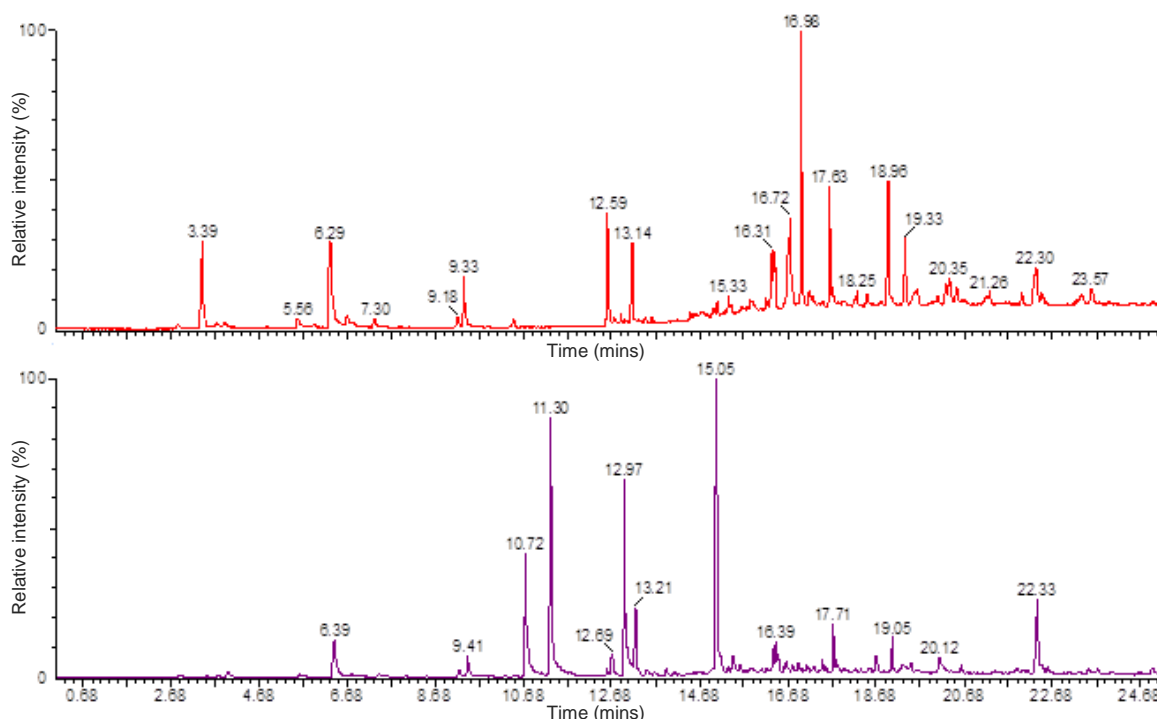
Figure 1. A typical chromatogram of a P. aeruginosa isolate (top) and of an E. coli isolate (bottom)

A media control was analysed, and 21 peaks were identified above the threshold of 10,000 TIC. Since all bacteria were grown in Mueller-Hinton broth, differences in compounds also present in the media can be attributed to bacteria either depleting or producing the compounds at different rates.

Statistical analysis using chi-square tests found 31 compounds whose presence/absence differed significantly between species (*E. coli, K. pneumoniae* and *P. aeruginosa*) at p ≤0.05, shown in table 3 and figure S1 in supplementary material.

Table 3. Compounds which are different in *E. coli* (EC), *K. pneumoniae* (KP) or *Pseudomonas aeruginosa (PA)*, and the percentage occurrence of compounds in samples

Compound	RT (mins)	In % of EC	In % of KP	In % of PA	More likely present or absent in PA (than in EC or KP)	More likely present in KP (than in PA or EC)	More likely absent in EC (than in PA or KP)
Unknown 1	3.39	3.3	13.3	40.0	More likely present in PA	-	More likely absent in EC
Butane	3.72	6.7	20.0	0.0	-	More likely present in KP	-
Unknown 2	4.27	0.0	13.3	0.0	-	More likely present in KP	More likely absent in EC
Ethanol	5.56	0.0	0.0	60.0	More likely present in PA	-	More likely absent in EC
Acetone	6.29	0.0	0.0	40.0	More likely present in PA	-	More likely absent in EC
2-Butanone	9.33	6.7	6.7	60.0	More likely present in PA	-	-
3-Methylbutanal	10.83	0.0	13.3	0.0	-	More likely present in KP	-

Compound	RT						
Dimethyl disulphide	12.59	13.3	26.6	60.0	More likely present in PA	More likely present in KP	More likely absent in EC
3-Methyl-1-butanol	12.64	0.0	13.3	0.0	-	More likely present in KP	More likely absent in EC
3-Methylbutyl acetate	14.55	0.0	20.0	0.0	-	More likely present in KP	More likely absent in EC
Unknown 3	14.68	0.0	0.0	20.0	More likely present in PA	-	-
α-Pinene	15.4	0.0	13.3	0.0	-	More likely present in KP	More likely absent in EC
Unknown 4	15.89	0.0	0.0	20.0	More likely present in PA	-	-
Trimethylbenzene	15.92	0.0	6.7	40.0	More likely present in PA	-	More likely absent in EC
Dimethyl trisulphide	16.31	13.3	13.3	60.0	More likely present in PA	-	-
4-Methylstyrene	16.53	0.0	6.7	20.0	-	-	More likely absent in EC
Octyl ether	16.69	3.3	0.0	20.0	More likely present in PA	-	-
2-Ethyl-1-hexanol	16.72	13.3	26.7	60.0	-	-	More likely absent in EC
Unknown 5	16.82	0.0	0.0	20.0	More likely present in PA	-	-
Unknown 6	16.91	0.0	0.0	20.0	More likely present in PA	-	-
Undecane	16.98	0.0	0.0	60.0	More likely present in PA	-	More likely absent in EC
2-Methyl-1-hexadecanol	17.07	0.0	0.0	40.0	More likely present in PA	-	More likely absent in EC
Isopropyl benzene	17.69	26.7	6.7	0.0	More likely absent in PA	-	More likely absent in EC
Dodecane	18.25	3.3	0.0	40.0	More likely present in PA	-	-
Decanal	18.96	20.0	33.3	60.0	-	-	More likely absent in EC
Tridecane	19.61	0.0	0.0	60.0	More likely present in PA	-	More likely absent in EC
Cyclohexyl isothiocyanate	20.27	0.0	0.0	20.0	More likely present in PA	-	-
Benzothiazole	20.34	6.7	6.7	40.0	More likely present in PA	-	-
Pentadecane	23.35	0.0	6.7	40.0	More likely present in PA	-	More likely absent in EC
Unknown 7	23.62	0.0	0.0	40.0	More likely present in PA	-	-
Unknown 8	24.97	0.0	0.0	20.0	More likely present in PA	-	-

EC = E. coli, KP = K. pneumoniae and PA = P. aeruginosa. RT = retention time. Additional spectral information for unknowns presented in table S1 in supplementary information.

These findings were compared to the literature, using the recently published microbial volatile database (mVOC 2.0) [21]. *P. aeruginosa* was shown to be much more likely to bio-synthesise organo-sulphides, particularly dimethyl disulphide and dimethyl trisulphide. Dimethyl disulphide has been found previously in this species [22] and benzothiazole, pentadecane and dodecane have been found in several *Pseudomonas* species [23]. Isopropyl benzene, which was more likely to be absent in *P. aeruginosa* (found in 0% of isolates) has also never been found previously in *P. aeruginosa*. Of the compounds more likely to be present in *K.*

pneumoniae, only 3-methylbutanal [24], and 3-methyl-1-butanol [25-26] have been found before. This study has potentially identified new VCs which are descriptive for these two species. It is worth noting that 3-methyl-1-butanol, 3-methylbutanal and 3-methylbutyl acetate are related; 3-methylbutanal can be readily produced by facile oxidation from 3-methyl-1-butanol and esterification will produce the acetate. All 3 compounds were found in *K. pneumoniae* and not in *E. coli* or *P. aeruginosa.* For *E. coli,* of the compounds more likely to be absent, undecane, 2-methyl-1-hexadecanol, isopropyl benzene, 4-methylstyrene and decanal have also never been identified from this species before.

Chi-square analysis identified 9 compounds whose presence/absence differed significantly between cephalexin sensitive and cephalexin resistant bacterial isolates at p ≤0.05, without cephalexin added to the media, shown in table 4. When cephalexin was added to the media containing bacterial isolates, 16 compounds were identified that differed significantly in terms of presence/absence at p = ≤0.05, shown in table 5.

Table 4. Compounds which are different in cephalexin sensitive and resistant *E. coli* and *K. pneumoniae* isolates, and the percentage occurrence of compounds in samples

More likely in Cephalexin S				More likely in Cephalexin R			
Compound	RT (mins)	In % of S	In % of R	Compound	RT (mins)	In % of S	In % of R
Carbon dioxide	3.64	15.8	0.0	Unknown 1	3.39	0.0	19.2
1-Dodecanol	24.03	15.8	0.0	Acetone	6.30	0.0	23.1
-	-	-	-	2-Butanone	9.33	0.0	23.1
-	-	-	-	4-Cyanocyclohexane	17.19	0.0	19.2
-	-	-	-	Isopropyl benzene	17.69	0.0	19.2
-	-	-	-	Benzothiazole	20.34	0.0	19.2
-	-	-	-	2,6-Bis(1,1-dimethylethyl)-1,4-benzenediol	20.51	0.0	19.2

S = sensitive and R = resistant. RT = retention time. Additional spectral information for unknowns presented in table S1 in supplementary information.

Table 5. Compounds which are different in cephalexin sensitive and resistant *E. coli* isolates with antibiotic added to culture media, and the percentage occurrence of compounds in samples

More likely in Cephalexin S (+AB)				More likely in Cephalexin R (+AB)			
Compound	RT (mins)	In % of S	In % of R	Compound	RT (mins)	In % of S	In % of R
Ethanol	5.54	23.1	0.0	Dimethyl trisulphide	16.30	7.7	56.3
Acetone	6.29/ 6.30	61.5	0.0	-	-	-	-
Dimethyl sulphide	6.51/ 6.52	61.5	0.0	-	-	-	-
Isopropyl alcohol	6.67	23.1	0.0	-	-	-	-
Methyl ethyl sulphide	9.30	61.5	0.0	-	-	-	-
Trichloromethane	9.88	46.2	0.0	-	-	-	-
Dimethyl disulphide	12.59-12.70	100	6.3	-	-	-	-
Decane	15.82	53.8	6.3	-	-	-	-
5-Hepten-2-one, 6-methyl-	16.17	23.1	0.0	-	-	-	-
Octanal	16.36/ 16.37	84.6	18.8	-	-	-	-
2-Ethyl-1-hexanol	16.71	53.8	12.5	-	-	-	-
1-Propenethiol	17.16	23.1	0.0	-	-	-	-

Decanal	18.95	23.1	0.0	-	-	-	-
Undecanal	20.49	38.5	0.0	-	-	-	-
Dodecanal	22.42/ 22.43	76.9	6.3	-	-	-	-

S = sensitive and R = resistant. RT = retention time. AB = antibiotic (cephalexin).

With the exception of acetone, the compounds found to be different in isolates grown without antibiotic, were not found to be different once cephalexin was added to media. A greater number of new compounds were found to be different with the addition of cephalexin to the media, and unlike previously, the majority of these compounds were more likely to be present in sensitive isolates and absent in resistant ones. The only exception was dimethyl trisulphide, which was more likely to be present in resistant isolates. Of the compounds significantly associated with sensitive isolates, there were three medium-chain aldehydes (octanal, undecanal and dodecanal) and three volatile sulphur compounds (dimethyl sulphide, dimethyl disulphide and methyl ethyl sulphide). A proposed explanation is that sensitive bacterial cell walls lysed, and resistant cells did not. Lysis causes cell contents to be released into the media, thus increasing the differences between sensitive cells and their resistant counterparts. Cephalexin is a cephalosporin class beta-lactam, whose mode of action is the prevention of the formation of the bacterial cell wall, leaving sensitive cells vulnerable to lysis without the protection of an intact cell wall. As a note, trichloromethane described in table 5, was once considered to be man-made however, it is now known that it can also be biosynthesised by some microorganisms [27].

Chi-square analysis found 22 compounds whose presence/absence differed significantly between ciprofloxacin sensitive and ciprofloxacin resistant bacterial isolates at p ≤0.05, without ciprofloxacin added to the media, shown in table 6. With the addition of ciprofloxacin to the media, 5 compounds were identified whose presence absence differed under the same parameters, shown in table 7.

Table 6. Compounds which are different in ciprofloxacin sensitive and resistant *E. coli* and *K. pneumoniae* isolates, and the percentage occurrence of compounds in samples

More likely in Ciprofloxacin S				More likely in Ciprofloxacin R			
Compound	RT (mins)	In % of S	In % of R	Compound	RT (mins)	In % of S	In % of R
Carbon dioxide	2.87	40	0.0	Dimethyl trisulphide	16.32	13.3	47.1
Acetaldehyde	3.97	33.3	0.0	-	-	-	-
Acetone	6.39	40	0.0	-	-	-	-
2-Butanone	9.41	26.7	0.0	-	-	-	-
Benzene	10.72	26.7	0.0	-	-	-	-
Cyclobutene-2-propenylidene	12.98	33.3	0.0	-	-	-	-
Butanoic acid	13.92	33.3	5.9	-	-	-	-
p-Xylene	15.05	33.3	0.0	-	-	-	-
Octanal	16.45	33.3	0.0	-	-	-	-
Diethylene glycol ethyl ether	16.58	33.3	0.0	-	-	-	-
2-Ethyl-1-hexanol	16.78	33.3	0.0	-	-	-	-
Heptanoic acid	17.47	26.7	5.9	-	-	-	-
Acetophenone	17.78	33.3	0.0	-	-	-	-
Nonanoic acid	20.12	26.7	0.0	-	-	-	-
Undecanal	20.62	33.3	0.0	-	-	-	-
2-Undecenal	22.03	26.7	0.0	-	-	-	-
Indole	22.33	33.3	5.9	-	-	-	-
Dodecanal	22.41	26.7	0.0	-	-	-	-
Tetradecanal	22.57	26.7	0.0	-	-	-	-

Hexadecane	23.51	33.3	0.0	-	-	-	-
1-Dodecanol	24.04	26.7	0.0	-	-	-	-

S = sensitive and R = resistant. RT = retention time.

Table 7. Compounds which are different in ciprofloxacin sensitive and resistant *E. coli* and *K. pneumoniae* isolates with antibiotic added to culture media, and the percentage occurrence of compounds in samples

More likely in Ciprofloxacin S (+AB)			
Compound	RT (mins)	In % of S	In % of R
Carbon dioxide	2.81	42.9	0.0
Methyl ethyl sulphide	9.54	42.9	0.0
Dimethyl disulphide	12.58	42.9	0.0
Undecanal	20.48	42.9	0.0
Dodecanal	22.41	42.9	0.0

S = sensitive and R = resistant. RT = retention time. AB = antibiotic (ciprofloxacin).

When ciprofloxacin was added to the media, many of the compounds identified previously as being different between sensitive and resistant isolates were no longer found to be significantly different. Ciprofloxacin is a broad-spectrum fluoroquinolone class antibiotic, with a different mechanism of action to cephalexin. Rather than threatening the integrity of the cell wall, ciprofloxacin prevents DNA synthesis by inhibiting DNA gyrase (prevents uncoiling of DNA) and topoisomerase IV (interferes with cell division). This mechanism would not necessarily cause cells to lyse.

Dodecanal and carbon dioxide were more likely to be found in sensitive isolates, both with and without the addition of antibiotics. However, methyl ethyl sulphide, dimethyl disulphide, and undecanal were only found to be different between sensitive and resistant isolates when antibiotic was added.

Statistical analysis taking into account the differences in peak areas i.e. the abundance of each compound, was performed using independent T-tests and Mann-Whitney-Wilcoxon tests. However, the results of these tests did not qualitatively differ from the results of the chi-square tests and for this reason are not reported.

From a starting culture, TD-GC-MS offers rapid profiling of bacteria (2 minutes for tube loading plus 26 minutes for GC-MS analysis), which is far quicker than traditional microbiological methods of susceptibility testing (around 24 hours). Once biomarkers have been unambiguously assigned, the analytical method would be refined and would be expected to be much more rapidly undertaken.

Additional work with a greater number of isolates, and the addition of standard compounds, may enable the development of diagnostic algorithms to predict species and susceptibility using the presence/absence of particular compounds of interest. The creation of diagnostic algorithms are possible using statistical modelling and/or pattern recognition software. This methodology could also be applied to other clinically relevant bacteria.

There is potential to use volatile profiles for faster and lower cost analysis using a shorter column and different chromatographic conditions and detection systems. Once compounds of interest have been identified and diagnostic algorithms created using gold-standard GC-MS, gas sensors specific to these compounds could be put together in an array. This type of sensor system offers online rapid analysis, as well as being relatively inexpensive, portable and user-friendly. Gould *et al.* (2017) recently reported use of heated metal oxide semiconductor sensors to detect VCs; unlike mass spectrometry, this type of sensor has negligible responses to siloxanes, and thus offers a solution to siloxane contamination adversely affecting the VC

profile [28]. For UTIs, the high sensitivity of this type of sensor array may remove the need to culture bacteria prior to analysis, allowing direct analysis of urine for both species identification and susceptibility testing.

4. Conclusions

Significant differences in volatile profiles were found between cephalexin resistant and sensitive isolates of *E. coli* and *K. pneumoniae*, and between ciprofloxacin sensitive and resistant isolates of the same bacterial species. Differences were found with and without the addition of antibiotic. Bacteria were rapidly analysed by TD-GC-MS, in less than 30 minutes from a starting culture, as opposed to 24 hours for traditional susceptibility tests. This technology if developed further could be used as a diagnostic tool to reduce inappropriate prescribing of antibiotics.

Significant differences in volatile profiles were also found between three UTI-causing bacterial species. Another potential application of this technology could be rapid differential diagnosis between UTI-causing bacteria.

Further work is required to validate these findings on a larger data set particularly using clinically obtained urine samples. This will allow robust statistical models to be built and tested against new samples, with the end goal being a clinically useful analytical method.

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