Evaluation of multiplex tandem PCR (MT-PCR) assays for the detection of bacterial resistance genes among Enterobacteriaceae in clinical urines

K. Schmidt[1]*, K. K. Stanley[2], R. Hale[2], L. Smith[2], J. Wain[1,3], J. O'Grady[1,3] and D. M. Livermore[1,4]

Abstract

Background: Increasing resistance drives empirical use of less potent and previously reserved antibiotics, including for urinary tract infections (UTIs). Molecular profiling, without culture, might better guide early therapy.

Objectives: To explore the potential of AusDiagnostics multiplex tandem (MT) PCR UTI assays.

Methods: Two MT-PCR assays were developed successively, seeking 8 or 16 resistance genes. Amplification was tracked in real time, with melting temperatures used to confirm product identity. Assays were variously performed on: (i) extracted DNA; (ii) cultured bacteria; (iii) urine spiked with reference strains; and (iv) bacteria harvested from clinical urines. Results were compared with those from sequencing, real-time SybrGreen PCR or phenotypic susceptibility.

Results: Performance was similar irrespective of whether DNA, cultures or urines were used, with .90% sensitivity and specificity with respect to common b-lactamases, dfr genes and aminoglycoside resistance determinants except aadA1/A2/A3, for which carriage correlated poorly with streptomycin resistance. Fluoroquinolonesusceptible and -resistant Escherichia coli (but not other species) were distinguished by the melting temperatures of their gyrA PCR products. The time from urine to results was ,3 h.

Conclusions: The MT-PCR assays rapidly identified resistance genes from Gram-negative bacteria in urines as well as from cultivated bacteria. Used directly on urines, this assay has the potential to guide early therapy.

Introduction

Emergency hospital admissions of elderly UK patients for complicated urinary tract infections (cUTIs) doubled from 2002 to 2012.[1] These infections are the source of most septic episodes with Escherichia coli, which is now the most common agent of bacteraemias.[2] As elsewhere in the world, the spread of MDR E. coli, often of ST131, complicates treatment and drives the wider use of previously reserved antibiotics, including carbapenems. Meanwhile, increasing trimethoprim resistance in the community has led to a switch to nitrofurantoin for the treatment of uncomplicated cystitis,[3] despite nitrofurantoin having poorer pharmacodynamics, tolerability and (for ascending infection) efficacy.

A potential way to overcome these problems is to move from empirical to early targeted therapy, by profiling resistance directly from clinical samples, without culture. This can be comprehensively achieved by metagenomic sequencing,[4] although there is scepticism around implementation, based on cost and workflows.[5] PCR methods are more immediately deployable, cost less, and have been widely adopted to seek mecA or carbapenemase genes, informing infection control.[6,7] PCR is less commonly used to guide treatment, except for rifampicin in the case of tuberculosis,[8] but its potential is clear. Accordingly, we evaluated two multiplex tandem PCR (MT-PCR) panels, seeking common resistance genes in Enterobacteriaceae. These were applied to both urines and cultivated isolates. The prototype panel sought eight resistance genes commonly responsible for resistance to trimethoprim, aminoglycosides and fluoroquinolones. The second panel also sought important b-lactamase genes.

MT-PCR has two stages. In the first step, samples are amplified in a multiplex PCR with primers for all targets in the panel. This is allowed to proceed for only 10–18 cycles and, because very little dNTP is thereby consumed, each PCR is independent of all others, preserving the relative quantification of each target. The Step 1 products are then diluted and divided into individual real-time PCR reactions, one for each target. The Step 2 reactions use primers

Table 1. Target genes sought by the 8-plex panel

No.	Target gene	Enzyme name	Resistances conferred
	dfrA1	dihydrofolate reductase (DHFR)	trimethoprim
	dfrA5/A14		
	dfrA12		
4	dfrA7/A17		
5	aac(6′) (including Ib, Ic, Ig, Iy, Iq II, IIc)	acetyltransferase [AAC(6′)-I]	aminoglycoside (amikacin, tobramycin)
6	aadA (including aadA1/A2/A3)	adenyltransferase [ANT(3″)-I]	aminoglycoside (streptomycin)

1
2
3

7	gyrA (including gyrA/S[a], gyrA/R[a], gyrKlebR/S[b])	DNA gyrase (Ser83 and Asp87)	fluoroquinolone (ciprofloxacin)
8	KPparC[c]	topoisomerase IV (Ser80)	fluoroquinolone (ciprofloxacin)
	SPIKE	internal control	

[a] This assay was designed to detect point mutations in gyrA affecting Ser83 and Asp87 and associated with high-level ciprofloxacin resistance; these were distinguished by PCR products with different T_m values for E. coli only.
[b] This assay was designed to identify Klebsiella spp. but not discriminate between resistant and susceptible types.
[c] This assay was designed to identify fluoroquinolone-resistant/-susceptible Klebsiella spp. based on PCR product T_m.

nested inside the Step 1 primers, preventing further amplification of any non-specific products from Step 1. MT-PCR can thus amplify many targets simultaneously, whilst preserving their relative quantities.[9]

Materials and methods

AusDiagnostics assays

MT-PCR was performed using two sequentially developed research panels (AusDiagnostics, Sydney, Australia): (i) an 8-plex (catalogue number 17412) to seek eight genes involved in resistance to trimethoprim, aminoglycosides and fluoroquinolones (Table 1); and (ii) a 16-plex (catalogue number 2741201) additionally seeking b-lactamase gene targets (Table 2).

Sample processing for MT-PCR assays

Tests were performed on extracted DNA from 7 reference strains, 42 overnight bacterial cultures from CLED Agar (Becton Dickinson, Oxford, UK), 5 urines from a healthy volunteer spiked with 10^8cfu/mL of reference strains from overnight broth cultures and 76 clinical urines from the Norfolk and Norwich University Hospital (NNUH).

Purified DNA was extracted from reference E. coli and Klebsiella pneumoniae grown on CLED Agar (Becton Dickinson). Harvested bacteria were suspended in a mixture of 200lL of lysis buffer (Roche, Basel, Switzerland), 180lL of PBS (Sigma–Aldrich) and 20lL of proteinase K (20 mg/mL) (Roche) and incubated for 10 min at 65C. DNA was then extracted using the MagNA Pure Compact Nucleic Acid Isolation Kit (Roche) with the DNA Bacteria v3_2 protocol.

Bacterial colonies were picked from agar, suspended in 300 lL of water and denatured at 95C for 4 min. The suspensions were then diluted 10fold in water, with 10lL volumes used for MT-PCR.

In the case of spiked and clinical urines, 1–1.5mL volumes were first centrifuged for 2 min at 300g to remove human cells. The supernatants were then centrifuged for 5 min at 12500g to pellet the bacteria, which were thereafter treated as for bacterial isolates, as described above.

Data analysis for MT-PCR

Data analysis was performed automatically using the integrated MT-Plex Result software (AusDiagnostics) for samples meeting the criteria of: (i) a cycle threshold (CT), in the second PCR, ,20; and (ii) a correct melting temperature (T_m) for the amplified gene. For the 16-plex panel only we added a further criterion (iii) of detection of .1000 Step 2 PCR product copies. Quantitative analysis of the Step 2 PCR product was performed by comparison with an internal control (SPIKE) containing 10000 copies of a synthetic oligonucleotide template with corresponding primers.

Reference methods

Results from the MT-PCR assays were variously compared with phenotypic susceptibility data, SybrGreen real-time PCR for the corresponding gene, or with WGS data, determined as below.

Phenotypic characterization

Isolates were grown on CLED Agar (Becton Dickinson) at 37C overnight. Species were identified by MALDI-TOF (Bru¨ker Daltonics), with susceptibility testing performed by BSAC disc diffusion.[10]

Molecular characterization

Extracted DNA was used for WGS on MinION or Illumina instruments, as described.[4] Alternatively, SybrGreen real-time PCR, with appropriate primers (Table 3) and a LightCycler 480 (Roche) were used to seek b-lactamase genes. A single colony from overnight culture was resuspended in 100lL of water, denatured at 95C for 4 min and used as a template (2lL). The PCR programme comprised denaturation at 95C for 5 min, followed by 35 cycles of denaturation at 95C for 20s, annealing at 60C for 20s and primer extension at 72C for 30s. After the last cycle, the melting curve analysis was followed by denaturation at 95C, then cooling to 72C. Fluorescence signals were collected continuously at k 530nm from 72C to 99C at 0.2C/s.

Ethics

Ethics approval was not required, because tests were performed, as method development, on excess samples submitted to the NNUH clinical microbiology laboratory. No patient data were collected.

Results

Resistance genes were readily detectable by both the 8- and 16-plex assays (Tables 4 and 5 respectively), regardless of whether extracted DNA, cultured bacteria or bacteria harvested from urine were used.

Table 2. Target genes sought by the 16-plex panel

	Target gene	Alleles sought
b-Lactamases		
1	bla_{TEM}	including 1, 3, 10, 104–106, 71, 76–84, 138, 143, 150, 155
2	bla_{SHV}	including 1, 2, 5, 11, 12, 25, 26, 38, 56
3	$bla_{CTX\text{-}M\ group\ 1}$	including 1, 3, 15, 28, 29, 32, 36, 58, 79, 103
4	$bla_{CTX\text{-}M\ group\ 9}$	including 9, 13, 14, 24, 27, 38
5	bla_{CMY}	including 2, 4, 16, 31, 73, combined with assay to detect CMY-1
6	$bla_{OXA\text{-}1}$	including 1, A1, 4, 30
7	$bla_{OXA\text{-}48}$	including 48, 163, 162, 181, 204, 244, 245, 247, 370, 405
8	bla_{KPC}	including 1, 2, 3
9	bla_{NDM}	including 1, 2, 3, 4, 5, 6, 7, 8
10	bla_{VIM}	including 1, 2, 3
Trimethoprim resistance determinants		
11	dfr	including A1, A5/A14
12	dfr	including A12, A7/ A17
Aminoglycoside resistance determinants		
13	aac(6^0)	including Ib, Ic, Ig, Iy, Iq II, IIc and aac(6^0)-Ib-cr
14	aadA	including A1, A2, A3
Fluoroquinolone resistance mutations		
15	gyrA	including gyrA/S[a], gyrA/R[a], gyrKlebR/S[b]
16	KPparC[c]	
Controls		
	tuf	Enterobacteriaceae, with T_m varying according to genus
	SPIKE	internal control

[a] This assay was designed to detect point mutations in gyrA affecting Ser83 and Asp87 and associated with high-level ciprofloxacin resistance; these were distinguished by PCR products with different T_m values for E. coli only.
[b] The assay was designed to identify Klebsiella spp. but not discriminate between resistant and susceptible types.
[c] This assay was designed to identify fluoroquinolone resistant/susceptible Klebsiella spp. based on PCR product T_m.

Table 3. Primers used for the SybrGreen real-time PCR assays seeking b-lactamase genes

Primer	Primer sequence (5^0–3^0)	GenBank	Product size (bp)
TEM_F	CAGCGGTAAGATCCTTGAGAG		
TEM_R	GAGTTACATGATCCCCCATGTT	KU376497.1	326
SHV_F	CGCCTGTGTATTATCTCCCTGT		
SHV_R	CAAGGTGTTTTTCGCTGACC	EU586041.1	316
CMY_F	GAGTTACGAAGAGGCAATGACC		
CMY_R	CCAGCCTAATCCCTGGTACATA	GQ351345.1	310
OXA1_F	AGACGTGGATGCAATTTTCTGT		
OXA1_R	GCACCAGTTTTCCCATACAGTT	J02967.2	319
CTX-M gr1_F	GCAAAAACTTGCCGAATTAGAG		
CTX-M gr1_R	GCTTATTCATCGCCACGTTATC	AJ549244.1	320
CTX-M gr9_F	CTTTCCAATGTGCAGTACCAGT	AF252623.2	320
CTX-M gr9_R	CGGTATTCAGCGTAGGTTCAG		

8-Plex panel

The initial 8-plex panel sought common trimethoprim and aminoglycoside resistance determinants as well as mutations associated with fluoroquinolone resistance. It was applied to 21 samples, including 7 extracted DNAs, 7 bacterial isolates and 7 urines (2 clinical and 5 spiked with known bacteria). Water was used as negative control. A total of 15 different isolates (9 E. coli and 6 K. pneumoniae), all trimethoprim resistant, were represented across the various formats. Trimethoprim determinants (dfrA1/A5/A7 or A12) were found in all 21 samples (100% sensitivity), consistent with universal phenotypic trimethoprim resistance. WGS for 12 organisms, representing 18 samples, confirmed the presence of the same dfr genes as found by the MT-PCR assay. aac(6^0)-Ib was detected in 14/21 examined samples and agreed with observed tobramycin resistance and sequencing in the 13 cases in which this was performed, with 100% sensitivity and specificity. Streptomycin adenyltransferase aadA1/A2/A3 genes were found by MT-PCR in 8/21 analysed samples, including 5/14 streptomycin-resistant isolates but also 3/7 streptomycinsusceptible isolates, with 35.7% sensitivity and 57.1%

specificity. WGS confirmed the presence of aadA1/A2/A3 in 7/8 PCR-positive isolates; including three that were susceptible to streptomycin.

The gyrA assay (Tables 1 and 2) amplified across the region encoding the mutations (Ser83 and Asp87) responsible for most high-level ciprofloxacin resistance, leading to a shift in the T_m of the resulting amplicon in E. coli. Thus, the T_m for the gyrA product of ciprofloxacin-resistant E. coli (n " 10, identified as 'gyrA/R' by the assay) was between 85C and 86C (mean 85.5C) but was between 86C and 87C (mean 86.5C) for ciprofloxacin-susceptible

Table 4. Comparison of results between the 8-plex MT-PCR panel and reference methods

	No. of samples with corresponding combinations of results (among 7 DNAs, 7 cultivated isolates and 7 urines)													
	trimethoprim						tomycin		obramycin		ciprofloxacin			
Gene sought by MT-PCR (and found/not found):	dfrA1	dfrA5/A14	dfrA7/A17	dfrA12	no dfr gene found	aadA1/A2/A3	aadA1/A2/A3 not found	aac(6 0) - b	c(60)-Ib not found	gyrA/R	gyrA/S	gyrKlebR/S	KPparC
Total no. samples with indicated result	4	6	8	3	0	8	13	14	7	10[a]	4[a]	7[b]	7[b]
Of which: gene confirmed by sequencing; resistant to corresponding antibiotic	3	5	7	3	–	4	–	13	–	–	–	–	–
gene confirmed by sequencing; susceptible to corresponding antibiotic	–	–	–	–	–	3	–	–	–	–	–	–	–
resistant to corresponding antibiotic[c]	1	1	1	–	–	1	9	1	–	10	–	6	6
susceptible to corresponding antibiotic[c]	–	–	–	–	–	–	4	–	7[d]	–	4	1	1
Sensitivity			100%				35.7%		100%			100%	
Specificity			100%				57.1%		100%			100%	

[a] All E. coli.
[b] All Klebsiella spp.
[c] Phenotypic data available only. [d] 5/7 samples were sequenced but genes were not found.

organisms (n " 4, identified as 'gyrA/S'). The gyrA assay also identified K. pneumoniae, giving a product (gyrKlebR/S) with a T_m of 88C–89C (mean 88.5C), but did not distinguish ciprofloxacin resistance or susceptibility in this species. A gyrKlebR/S product was seen for all 7 K. pneumoniae samples and for none of the 14 E. coli. The KPparC assay aimed to predict fluoroquinolone resistance in Klebsiella based on the T_m shift contingent on the Ser80 mutation but failed to do so, with identical products from all seven isolates irrespective of phenotypic ciprofloxacin resistance.

16-Plex panel

Seventy-four infected urines were tested with the 16-plex assay, selected based on phenotypic testing of the corresponding isolates. Identification of b-lactamase genes from these urines by MT-PCR was in good agreement with real-time PCR on the corresponding isolates, with 100% sensitivity and, according to the enzyme, 95.3%–100% specificity (Table 5), though without discrimination of whether bla_{TEM} and bla_{SHV} genes encoded classic, inhibitor-resistant or ESBL types. The 16-plex assay found dfrA1/A5/A7/A12 in 38/41 urines containing isolates resistant to trimethoprim and in 1/33 urines containing a trimethoprimsusceptible organism, giving sensitivity 92.7% and specificity 97%. aac(6^0)-Ib was detected in nine urines, though only eight of these contained bacteria resistant to tobramycin, giving 100% sensitivity and 98.5% specificity. aadA1/A2/A3 genes were detected in 9/24 urines containing bacteria resistant to streptomycin, but also in 8/50 urines containing streptomycin-susceptible bacteria, giving sensitivity 37.5% and specificity 86%.

A gyrA/R product (T_m " 85.5C) was obtained from 25/28 urines containing ciprofloxacin-resistant E. coli whereas the gyrA/S (T_m " 86.5C) product was obtained from 22/25 urines containing ciprofloxacin-susceptible E. coli. On this basis, the E. coli gyrA fluoroquinolone resistance assay was 89.3% sensitive and 100% specific for urines (Table 6). The gyrA/R and gyrA/S were not, however, E. coli specific: corresponding PCR products were also obtained from 15 urines containing other Enterobacteriaceae, including 3 Citrobacter freundii, 8 Enterobacter spp., 2 Serratia marcescens, 1 Klebsiella oxytoca, 1 Proteus spp. and 1 with a Pseudomonas spp. Nevertheless, E. coli was distinguished from other Enterobacteriaceae, except Citrobacter spp. based on a lower T_m for the tuf product (83.5 versus 84.0C) (Figure 1).

Assays for gyrA and parC were less discriminatory among other species. Thus: (i) a gyrKlebR/S product was obtained from all K. pneumoniae-containing urines (n" 5) but also from one containing S. marcescens and from two containing Pseudomonas spp.; and (ii) T_m values for tuf products did not distinguish among Enterobacteriaceae species in these cases.

Thirty-five cultivated isolates were also tested with the 16-plex assay, selected as phenotypically multiresistant or based on sequencing data. Detection of b-lactamase genes agreed perfectly with reference molecular methods, giving 100% sensitivity and specificity (Table 5). Dihydrofolate reductase genes dfrA1/A5/A7/A12 were identified in 30/32 trimethoprim-resistant isolates, with sensitivity and specificity (93.7% and 100%, respectively) nearly identical to values for clinical urines. aac(6^0)-Ib was found in 22/24 isolates with phenotypic tobramycin resistance, whereas aadA was detected in 10/18 streptomycin-resistant isolates and in 6/17 streptomycin-susceptible ones, a mismatch rate similar to that for urines, with sensitivity 55.7% and specificity 64.7%. The fluoroquinolone gyrA/R product (T_m " 85.5C) was generated from 9/10 ciprofloxacin-resistant E. coli isolates, whereas gyrA/S (T_m " 86.5C) was obtained from 6/7 ciprofloxacin-susceptible E. coli isolates, with 90% sensitivity and 100% specificity for prediction of ciprofloxacin resistance in the species (Table 6). gyrA/R and gyrA/S PCR products were also obtained from two Enterobacter spp. and one K. oxytoca, with no correlation to phenotypic ciprofloxacin resistance; nevertheless, as with urines, E. coli could be distinguished from other species by the T_m for the tuf product. The gyrKlebR/S PCR product was obtained from all K. pneumoniae isolates (n" 15) and one nonfermenter (93.8% sensitivity); resistant and susceptible profiles were not distinguished for K. pneumoniae.

The KPparC product was obtained from 19 urines and 19 isolates containing or representing 2/3 Citrobacter spp., 9/10 Enterobacter spp., 2/2 K. oxytoca, 20/20 K. pneumoniae, 3/3 Serratia spp., 1/1 Proteus spp. and 1/1 Acinetobacter, whereas no signal was seen for any of 66 E. coli or 3 Pseudomonas spp. There was no difference in the T_m (88.5C) for the KPparC product between susceptible and resistant isolates of any species.

Table 5. Comparison of results between the 16-plex panel and reference methods

Resistance gene target	Clinical urines (n " 74) 16-plex, no. positive	reference, no. positive	sensitivity; specificity, %	Isolates (n " 35) 16-plex, no. positive	reference, no. positive	sensitivity; specificity, %
b-Lactamase genes versus molecular reference genes detected by real-time PCR bla$_{TEM}$ 33 31 100; 95				24	24	100; 100
bla$_{SHV}$	6	6	100; 100	16	16	100; 100
bla$_{CTX-M\ group\ 1}$	24	23	100; 98	18	18	100; 100
bla$_{CTX-M\ group\ 9}$	13	13	100; 100	4	4	100; 100
bla$_{CMY}$	9	9	100; 100	7	7	100; 100
bla$_{OXA-1}$	8	8	100; 100	18	18	100; 100
bla$_{OXA-48/181/244}$	–	–	–	7	7	100; 100
bla$_{KPC}$	–	–	–	5	5	100; 100
bla$_{NDM}$	–	–	–	10	10	100; 100
bla$_{VIM}$	–	–	–	1	1	100; 100
Trimethoprim gene versus phenotypic trimethoprim resistance dfrA1/A5/A7/A12 39 41			92.7; 97	30	32	93.7; 100
Aminoglycoside genes versus phenotypic tobramycin and streptomycin resistance aac(6^0)-Ib 9 8 100; 98				22	24	91.7; 100
aadA1/A2/A3	9	24	37.5; 86	10	18	55.7; 64.7

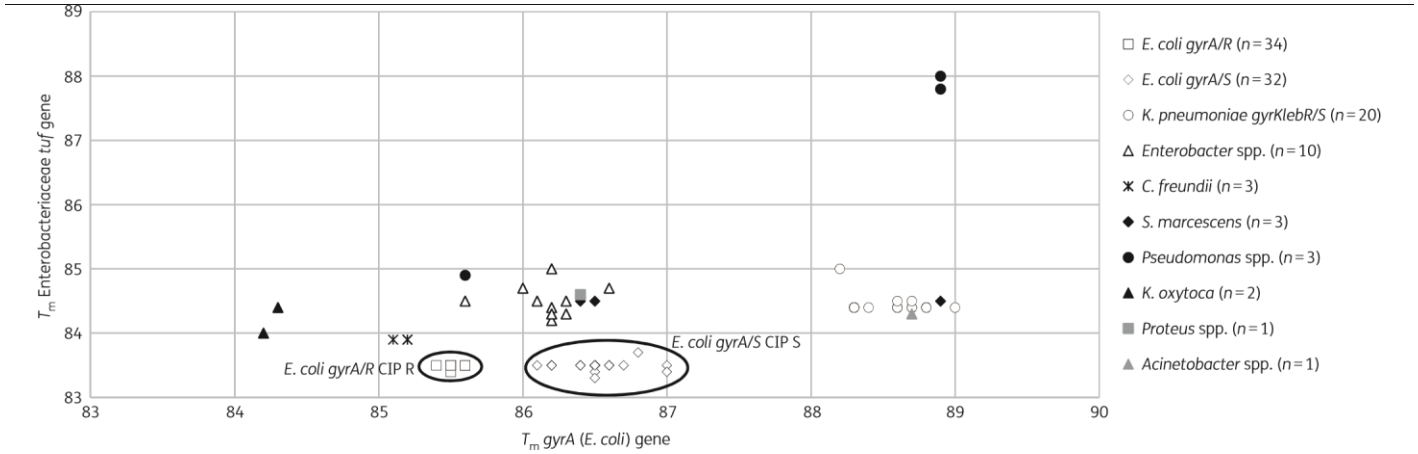

Figure 1. Melting ranges for gyrA and Enterobacteriaceae (tuf) genes in the High-Plex UTI assay as an aid to genus identification.

Table 6. Detection of fluoroquinolone-relevant genes by the 16-plex panel in relation to bacterial species

	Clinical urines (n " 74)				Isolates (n " 35)			
Samples (n " 109)	gyrA/R	gyrA/S	gyrKlebR/S	KPparC	gyrA/R	gyrA/S	gyrKlebR/S	KPparC
E. coli (n " 66)								
CIP R	25	3	–	–	9	1	–	–
CIP S	–	22	–	–	–	6	–	–
K. pneumoniae (n " 20)								
CIP R	–	–	4	4	–	–	13	13
CIP S	–	–	1	1	–	–	2	2
Non-Enterobacteriaceae (n " 4)[a]								
CIP R	–	–	–	–	–	–	1	1
CIP S	1	–	2	–	–	–	–	–
Other Enterobacteriaceae species (n " 19)[b]								
CIP R	2		–	3	–	–	–	–
CIP S	8		1	11	1	2	–	3
Sensitivity, %	89.3		62.5	–	90		93.8	–
Specificity, %	100		–	–	100		–	–

CIP, ciprofloxacin; R, resistant; S, susceptible.

[a]Acinetobacter spp. (n " 1), Pseudomonas spp. (n " 3). [b]C. freundii (n " 3), Enterobacter spp. (n " 10), S. marcescens (n " 3), K. oxytoca (n " 2), Proteus spp. (n " 1).

Discussion

Rapid molecular identification of resistance genes in clinical samples could guide early therapy for UTIs, improving patient outcomes and antimicrobial stewardship. PCR has been widely employed to seek resistance genes in clinical samples, but mostly to support infection control rather than to guide therapy.[11–17] We explored its potential to detect important enterobacterial resistance genes in clinical urines without culture. Two iterations of an MT-PCR assay were tested, expanding the number of resistance targets. Similar sensitivity and specificity was achieved for both urines and cultured bacteria, demonstrating proof of principle, although further validation of the assays directly on urine samples is recommended.

Both assays sought four widespread trimethoprim determinants (dfrA1, dfrA5/14, dfrA7/A17 and dfrA12), two aminoglycoside resistance genes [aadA1/A2/A3 and aac(6^0)-Ib] and sequence variants of gyrA and parC, where mutations confer high-level fluoroquinolone resistance. The 16-plex panel additionally sought b-lactamase genes, including common penicillinases (bla_{TEM} and bla_{SHV}), inhibitor-resistant penicillinase (bla_{OXA-1}), acquired ampC (bla_{CMY}), ESBLs ($bla_{CTX\text{-}M\ group\ 1}$ and $bla_{CTX\text{-}M\ group\ 9}$) and carbapenemases ($bla_{OXA-48}$, bla_{KPC}, bla_{NDM} and bla_{VIM}).

Despite including only the most prevalent dfr alleles, the tests were good predictors of trimethoprim resistance. With the 16-plex assay, dfr alleles were found in 68/73 urines and isolates with trimethoprim resistance: dfrA7/A17 predominated in E. coli and dfrA5/A14 in K. pneumoniae, as also found in Sweden.[18] Negative results for some trimethoprim-resistant isolates are likely to reflect the presence of other unsought dfr determinants, e.g. dfrA8, dfrA9, dfrA24, dfrA25 and dfrA26,[19–24] or mutations in chromosomal folA, although these are rare.[14]

The 16-plex assay achieved 100% sensitivity and 95%–100% specificity for b-lactamase genes in both clinical urines and cultivated isolates. This performance, using urines directly, is comparable to that found by others using similar methodology on cultivated isolates. Singh et al.[15] developed a multiplex real-time PCR assay to successfully detect 10 b-lactamases, including ESBLs, AmpC and carbapenemases, by T_m analysis, as here. The diversity of allelic variants sought was greater than in our study, though $bla_{CTX\text{-}M\ group\ 9}$ (a common ESBL group) was omitted. Chavada and Maley[25] used MT-PCR, as here, to seek 12 b-lactamase genes in cultivated Gram-negative isolates, achieving 95% sensitivity and 96.7% specificity, and Willemsen et al.[26] evaluated a commercial real-time PCR (Check-MDR ESBL PCR) to seek $bla_{CTX\text{-}M\text{-}like}$ along with ESBL-encoding alleles of bla_{TEM} and bla_{SHV}, which (unlike here) were discriminated from classic forms, achieving 98.9% sensitivity and 100% specificity against a reference microarray.

Detection of aac(6^0)-Ib was reliable by both MT-PCR panels (100% sensitivity for the 8-plex and 91.7%–100% sensitivity for the 16-plex); the main limitations were: (i) that tobramycin resistance can be caused by other unsought aminoglycoside resistance determinants; (ii) that amikacin resistance is a variable correlate of aac(6^0)-Ib carriage, though EUCAST counsels against its use whenever aac(6^0) variants are present; and (iii) that resistance to gentamicin, the most-used aminoglycoside in the UK, is associated with other enzymes, not aac(6^0)-Ib. A 24-plex assay variant, in early development, aims to address these aspects by adding further aminoglycoside-modifying gene targets (not shown).

High-level fluoroquinolone resistance in Enterobacteriaceae is mainly via mutations in gyrA or parC.[27] The 8- and 16-plex MTPCRs accurately predicted ciprofloxacin resistance for E. coli from the T_m of the gyrA product. With the 16-plex assay, 89.3% sensitivity and 100% specificity were achieved for prediction of resistance in E. coli in urine; however, resistance was not predictable for other Enterobacteriaceae, some of which also gave products for gyrA in the assay. Parallel rapid identification of the pathogen by other techniques, e.g. MALDI-TOF MS directly from urine,[28–30] may be

prudent; alternatively detection of a low-T_m (83.5C) tuf product in the 16-plex assay strongly predicted E. coli.

In contrast to good concordance for dfr, b-lactamase genes, aac(6^0)-Ib and E. coli gyrA, there was poor agreement between carriage of aadA1/A2/A3 and streptomycin resistance, with these adenyltransferase determinants variably present in both streptomycin-resistant and -susceptible bacteria. Other mechanisms (e.g. strA/strB and aadA5) likely explain streptomycin resistance in aadA-negative isolates but, in addition, it is apparent that aadA is often carried without resistance, as noted also by others.[31] This might be explained by (i) the aadA gene being well separated from its common promoter in the 5^0-conserved segment of integrons, leading to poor expression,[32] or (ii) 'gene silencing'.[33] In practical terms this failure matters little, as streptomycin is not ordinarily used to treat UTIs. The simplest answer would be to remove the aadA target from the assay.

Given the performance demonstrated here, the AusDiagnostics MT-PCR tests have the potential for use in community clinics for the rapid (,3 h) investigation of UTIs, helping determine whether to treat with trimethoprim or ciprofloxacin rather than nitrofurantoin, which is currently favoured owing to a lower resistance rate, despite being inferior in tolerability, pharmacokinetics and efficacy. The patient's midstream urine would be tested at the clinic visit and, if no dfr determinant is found, a prescription for trimethoprim would be electronically issued to a pharmacy for collection on the same day. If a trimethoprim resistance determinant is found, results for tuf and gyr would be reviewed and, if susceptible E. coli is predicted, ciprofloxacin would be prescribed. If resistance to both trimethoprim and ciprofloxacin is predicted, prescription would default to nitrofurantoin (or, possibly, pivmecillinam or fosfomycin). Assuming 25% trimethoprim resistance and 92.7% sensitivity/97% specificity for detection of resistance, as with the 16-plex panel, 72% of patients would receive trimethoprim and only 6% would do so inappropriately. This compares favourably with the previous maxim that trimethoprim (or co-trimoxazole) could be used empirically in cystitis, up to a resistance rate of 15%– 20%,[34] and to the present policy of preferring empirical nitrofurantoin, despite its limitations and a resistance rate of 6%.[35]

For cUTI hospital admissions, or patients with hospital-acquired UTIs, the 16-plex assay could reasonably be used to distinguish patients: (i) with pathogens likely to be resistant to cephalosporins (i.e. with bla$_{CTX-M}$ or bla$_{CMY}$ or any carbapenemase gene); (ii) with pathogens likely to be resistant to penicillin/b-lactamase inhibitor combinations (with bla$_{OXA-1}$, bla$_{CMY}$ or any carbapenemase); or (iii) with pathogens likely to be resistant to carbapenems (any carbapenemase). Detection of aac(6^0)-Ib should warn against use of tobramycin and amikacin. Such information, available within ,3 h, would reduce pressure to use carbapenems in patients developing sepsis, based on the suggestion that 'the patient has risk factors for ESBL producers'. The cost of the equipment is 30000 GBP and the cost per sample is 12GBP.

Challenges remain. First, although sensitivity .90% was achieved for key resistances, the system does not detect rare determinants, reflecting limits on the number of targets that can and non-ESBL variants of bla$_{TEM}$ be multiplexed. However, should the frequency of new or currently rare determinants increase over time, assays to detect them could be incorporated. Secondly, prediction of fluoroquinolone resistance was possible only for E. coli, limiting treatment guidance for UTIs due to other species. Thirdly, the system could not distinguish ESBL and bla$_{SHV}$, though these are 10fold rarer than bla$_{CTX-M}$ ESBLs among urinary E. coli.[36,37] Fourthly, the system does not provide guidance for all antibiotics: for example, it does not seek the major determinants of gentamicin or fosfomycin resistance, and the latter would be difficult given that most resistance is mutational. Lastly, MT-PCR cannot predict an MIC, and resistance to cephalosporins and carbapenems among Enterobacteriaceae depends not only on b-lactamase type but also on permeability and efflux traits.[38,39] These limitations must be balanced against those of standard culture, which does not deliver a result until 48 h after the urine is taken. If resistance is prevalent this means (i) that many patients are undertreated if a compromised agent is retained as empirical therapy or (ii) that an agent with limitations but little resistance (e.g. nitrofurantoin) or one that would ordinarily be reserved (e.g. ertapenem) becomes the standard of empirical care. This far-from-ideal situation is accepted only because 'that's how it has always been done'.

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Author contributions

Study conception and design: K. S., D. M. L., K. K. S., R. H., L. S., J. O., J. W. Acquisition of data: K. S. Analysis and interpretation of data: K. S., D. M. L., K. K. S., L. S. Drafting of manuscript: K. S., D. M. L. Critical revision: D. M. L., K. K. S., R. H., L. S., J. O. and J. W.

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