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Predicting antimicrobial susceptibilities for *Escherichia coli* and *Klebsiella pneumoniae* isolates using whole genomic sequence data

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Objectives: Whole-genome sequencing potentially represents a single, rapid and cost-effective approach to defining resistance mechanisms and predicting phenotype, and strain type, for both clinical and epidemiological purposes. This retrospective study aimed to determine the efficacy of whole genome-based antimicrobial resistance prediction in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*.

Methods: Seventy-four *E. coli* and 69 *K. pneumoniae* bacteraemia isolates from Oxfordshire, UK, were sequenced (Illumina HiSeq 2000). Resistance phenotypes were predicted from genomic sequences using BLASTn-based comparisons of *de novo*-assembled contigs with a study database of >100 known resistance-associated loci, including plasmid-associated and chromosomal genes. Predictions were made for seven commonly used antimicrobials: amoxicillin, co-amoxiclav, ceftriaxone, ceftazidime, ciprofloxacin, gentamicin and meropenem. Comparisons were made with phenotypic results obtained in duplicate by broth dilution (BD Phoenix). Discrepancies, either between duplicate BD Phoenix results or between genotype and phenotype, were resolved with gradient diffusion analyses.

Results: A wide variety of antimicrobial resistance genes were identified, including bla_{CTX-M}, bla_{LEN}, bla_{OKP}, bla_{OXA}, bla_{SHV}, bla_{TEM}, *aac(3′)-Ia*, *aac-(3′)-IId*, *aac-(3′)-IIe*, *aac(6′)-Ib-cr*, *aadA1a*, *aadA4*, *aadA5*, *aadA16*, *aph(6′)-Id*, *aph(3′)-Ia*, *qnrB* and *qnrS*, as well as resistance-associated mutations in chromosomal *gyrA* and *parC* genes. The sensitivity of genome-based resistance prediction across all antibiotics for both species was 0.96 (95% CI: 0.94–0.98) and the specificity was 0.97 (95% CI: 0.95–0.98). Very major and major error rates were 1.2% and 2.1%, respectively.

Conclusions: Our method was as sensitive and specific as routinely deployed phenotypic methods. Validation against larger datasets and formal assessments of cost and turnaround time in a routine laboratory setting are warranted.

Keywords: antibiotic, phenotype, Gram-negative

Introduction

The advances in sequencing technology over the last decade promise a potential revolution in clinical microbiology, with the cost-effective use of pathogen whole-genome sequence data for species identification, antimicrobial susceptibility prediction and outbreak detection having been proposed as applications of bench-top sequencers, such as the MiSeq (Illumina, San Diego, CA, USA) or Ion Torrent (Life Technologies Corp., Carlsbad, CA, USA), in routine laboratories.[1] Conceivably, this could enable a 'one-stop' approach to the microbiological analysis of cultured bacterial isolates with turnaround times of <1 day.

At present, routine antimicrobial susceptibility testing is undertaken using a variety of approaches, including disc diffusion,

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gradient diffusion and broth dilution methods, the latter being automated as part of commercial platforms such as BD Phoenix (BD, Franklin Lakes, NJ, USA) or Vitek 2 (bioMérieux, Marcy l'Etoile, France).[2–4] Despite extensive efforts to standardize laboratory assays, problems with particular test methods for certain organism–antimicrobial combinations are well recognized and may relate to inherent properties of the organism or antimicrobial being tested.[5,6] Other errors can arise in inoculum preparation, culture conditions or data entry.

Susceptibility phenotyping errors are typically classified as very major, resulting from a false-susceptible result, or major, resulting from a false-resistant result.[2] The US FDA stipulates rates must be <1.5% for very major errors and <3% for major errors prior to authorizing marketing approval for new susceptibility testing devices; similar cut-offs have been proposed by others.[2] In controlled research studies, overall error rates are 0%–8%,[7] but in routine settings the actual error rates are not generally known.

Routine genotypic prediction of bacterial antimicrobial susceptibility is currently used only in limited contexts, typically with single gene targets known to be highly associated with resistance, such as *mecA* assays to determine methicillin resistance in *Staphylococcus aureus*. The prevailing view has been that genotypic assays would be too difficult to implement for complex patterns of antimicrobial resistance, e.g. those in major Gram-negative pathogens such as *Escherichia coli* or *Klebsiella pneumoniae*.[2] However, recent data investigating whole-genome sequencing approaches to identifying susceptibility phenotypes of porcine *Salmonella* Typhimurium, *E. coli*, *Enterococcus faecium* and *Enterococcus faecalis* isolates for resistance surveillance purposes showed high concordance between phenotypic and predicted antimicrobial susceptibilities.[8] Caveats to this acknowledged by the authors include the low complexity of the resistance genotypes in the bacterial populations studied (i.e. small numbers of resistance genes per isolate conferring resistance to the same antimicrobial class) and that no assessment of some important chromosomal markers of resistance, such as *gyrA* mutations for fluoroquinolones, was made.

E. coli and *K. pneumoniae* are the Gram-negative species most commonly identified in bacteraemic patients in the UK,[9,10] with increases in incidence noted across Europe.[11] As such, these organisms, in which multidrug resistance is increasingly recognized,[12,13] represent species for which accurate and rapid antimicrobial susceptibility testing has the potential to deliver direct clinical benefit. Consequently, in this study we aimed to assess the feasibility of using whole-genome sequence data from human blood culture isolates of *E. coli* and *K. pneumoniae* representative of those seen in clinical practice to predict susceptibility phenotypes for antibiotics commonly used to manage infections caused by these organisms.

Materials and methods

Clinical isolate selection and in vitro antimicrobial susceptibility testing

We selected all retrievable extended-spectrum cephalosporin-resistant (commonly representative of multidrug-resistant phenotypes)[14] *E. coli* and *K. pneumoniae* blood culture isolates obtained from patients at the Oxford University Hospitals NHS Trust, Oxford, UK, between January 2008 and November 2010 (*E. coli*) or June 2011 (*K. pneumoniae*). Time-matched (by calendar year) susceptible control blood culture isolates were also selected at random and retrieved (Figure 1).

Isolates were recultured from frozen stocks (−80°C) and underwent automated susceptibility testing in duplicate with the BD Phoenix system using EUCAST breakpoints,[15] to allow comparisons with genotypic data. Intermediate BD Phoenix susceptibilities were considered as resistant (Tables 1 and 2). In cases where duplicate BD Phoenix runs were concordant regarding an isolate's resistance category [susceptible/susceptible (S/S), resistant/resistant (R/R)], the consensus BD Phoenix phenotype was compared with the genotype. Discrepancies, defined as discordance between BD Phoenix runs (S/R), or between predicted genotypic susceptibility and concordant BD Phoenix phenotype (S+R/R, or R+S/S respectively), were further investigated using gradient diffusion testing (Etest, bioMérieux, Basingstoke, UK; M.I.C. Evaluator, Fisher Scientific UK, Loughborough, UK) on Iso-Sensitest agar in accordance with BSAC guidelines.[16] In such cases, regardless of the nature of the discrepancy, the gradient diffusion result was adopted as the comparison standard phenotype; in all other cases, the concordant BD Phoenix phenotype was the comparison standard.

Reference gene database

Genetic loci and sequence variants known to be associated with resistance to antimicrobial agents commonly used in our hospital to treat *E. coli* and *K. pneumoniae* infections were identified from published reviews and web-based resources and were compiled as a reference gene database.[17–25] Chromosomal and plasmid-mediated loci conferring resistance to amoxicillin, co-amoxiclav, ciprofloxacin, gentamicin, ceftriaxone, ceftazidime and meropenem were included (full details of all mechanisms included in the algorithm are provided in Tables S1, S2 and S3, available as Supplementary data at *JAC* Online). An additional search of complete coding sequences annotated as being members of relevant bacterial (other than mycobacterial) resistance gene families deposited at the National Centre for Biotechnology Information was performed, using the following search terms: (i) 'lactamase', (ii) 'carbapenemase', (iii) 'aminoglycoside'+'resistance' and (iv) 'fluoroquinolone'+'resistance' (December 2012; see Supplementary data for additional references).

DNA extraction and whole-genome sequencing

DNA was extracted using a commercial kit (QuickGene DNA Tissue Kit S, Fujifilm, Japan) as per the manufacturer's instructions, with an additional mechanical lysis step (FastPrep, MP Biomedicals, USA) immediately following chemical lysis. A combination of standard Illumina and in-house protocols was used to produce multiplexed paired-end libraries of extracted DNA with an average insert size of ∼200 bp. Sequencing was performed on the Illumina HiSeq 2000, generating 100 bp paired-end reads. Reads were mapped against reference sequences [CFT073 for *E. coli* (RefSeq: NC_004431.1) and MGH78578 for *K. pneumoniae* (RefSeq: NC_009653)] using Stampy.[26] *De novo* assembly, for the purposes of resistance locus identification, was performed using Velvet,[27] with automated optimization of assembly parameters using VelvetOptimiser,[28] including the selection of *k*-mer length (length of overlapping read fragments), expected coverage (which assists in minimizing the impact of repetitive regions on the assemblies) and coverage cut-off [which minimizes the impact of areas of low sequencing coverage and repetitive regions (areas of high coverage) on assemblies]. *De novo* assembly quality was ensured by requiring >4 megabases (Mb) to be assembled into contigs and contig n50 values of >30000 bp (n50 is the longest contig length such that 50% of the assembled genome is represented in contigs of this length or longer). Sequencing data files have been deposited at the European nucleotide archive (ENA) and are available using the following URL: http://www.ebi.ac.uk/ena/data/view/ERP002642.

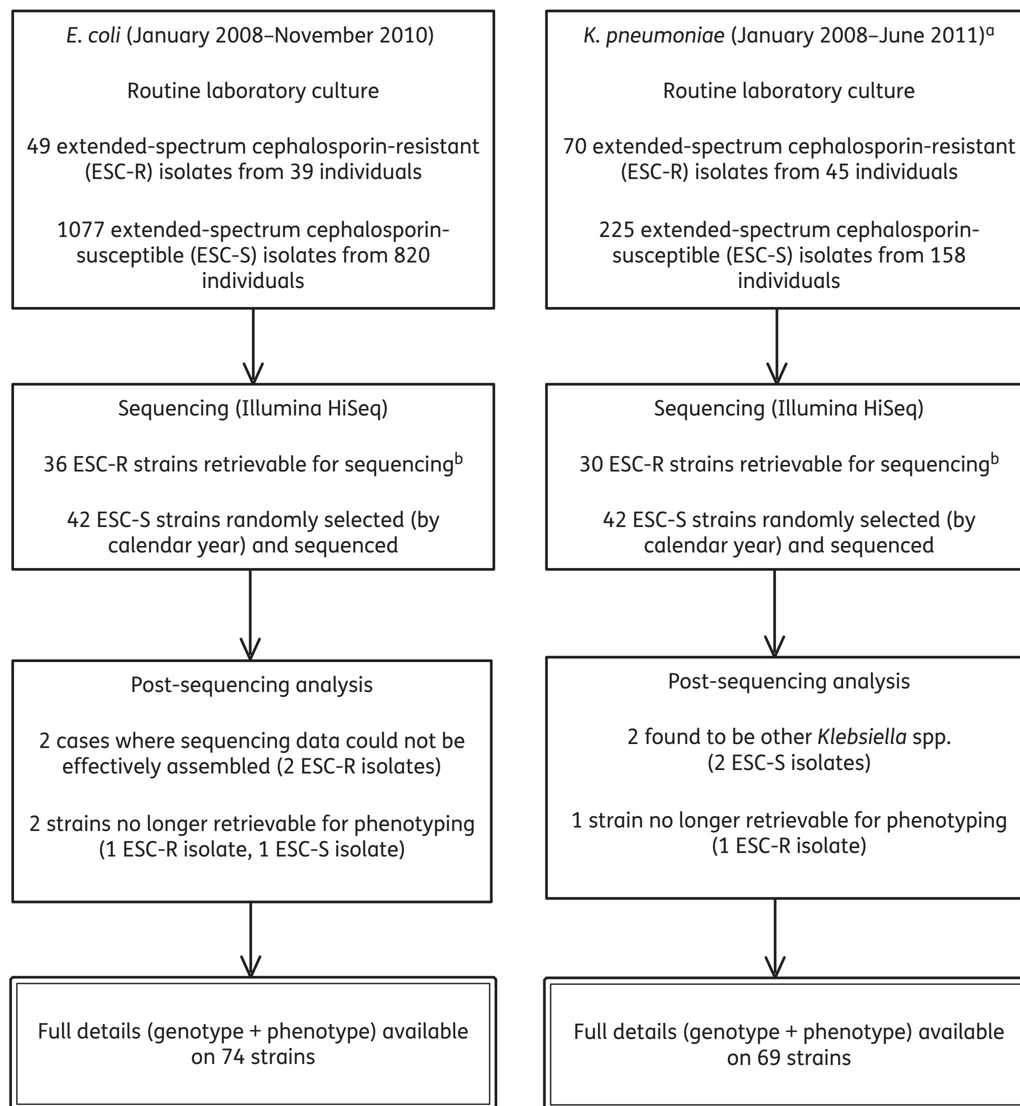

Figure 1. Sampling frame and processing of isolates. [a]The study time period for *K. pneumoniae* was extended to find similar numbers of organisms across both species groups. [b]Losses in retrieval rates were mostly due to the fact that repeat isolates from individuals were not routinely stored; other missing isolates could not be found in the routine laboratory freezer.

In silico prediction of antimicrobial susceptibility phenotypes

BLASTn was used to identify the presence of relevant resistance gene loci (from the reference database) in the *de novo*-assembled contigs for each clinical isolate, with a word length of 11 and an Expect value (*E*) cut-off of 1×10^{-4}. All matches were visually inspected for confirmation. Matches with >80% identity at the nucleotide level and representing a match of >80% of the reference gene length were retained; this included partial matches with >80% sequence homology over 80% of the reference gene length, but distributed over several contigs. Overlapping fragments were then aligned in SeaView[29] and combined to give a single sequence. Chromosomal resistance gene sequences were analysed to identify mutations, including those known to be associated with resistance.

Each isolate's susceptibility phenotype was predicted from the genetic data on the basis of published associations with phenotypic resistance for each locus, without reference to the BD Phoenix phenotype [details for susceptibility predictions for all profiles found are shown in Tables S4, S5 and S6 (*E. coli*) and S7, S8 and S9 (*K. pneumoniae*); Supplementary data available at *JAC* Online]. For any novel sequence variants identified, the genotypic susceptibility prediction mirrored that of the closest reference database variant. Discrepancies between the BD Phoenix phenotype and genotype were then investigated using gradient diffusion, as described above.

The sensitivity, specificity and rates of major and very major errors for genotypic susceptibility predictions were calculated for each antibiotic and species against the comparison standard (determined as above). Statistical analyses were performed using Stata 11.2 (StataCorp, College Station, TX, USA).

Results

Quality of whole-genome sequences

Two of the 76 candidate *E. coli* study isolates were excluded because of poor sequence assembly (n50 <1250 and <0.3 Mb assembled into contigs); two *Klebsiella* isolates were excluded because they were non-*pneumoniae Klebsiella* spp. on the basis of mapping

Table 1. Analysis of discordance in phenotypic and/or genotypic resistance predictions for 74 *E. coli* bloodstream isolates

Antibiotic	Discrepancies (*n*; % of total, 74 isolates)			Agreement of gradient diffusion with genotype in all discrepancies (*n*/total discrepancies; %)	Agreement of gradient diffusion with genotype in BD Phoenix-concordant discrepancies (*n*/number of BD Phoenix-concordant discrepancies; %)
	S/R[a] discordant BD Phoenix	BD Phoenix S/S, genotype R	BD Phoenix R/R, genotype S		
Amoxicillin	0 (0)	2 (3)	0 (0)	1/2 (50)	1/2 (50)
Co-amoxiclav	5 (7)	0 (0)	15 (20)	20/20 (100)	15/15 (100)
Gentamicin	1 (1)	0 (0)	0 (0)	1/1 (100)	NA[b]
Ciprofloxacin	0 (0)	0 (0)	0 (0)	NA	NA
Ceftriaxone	0 (0)	1 (1)	1 (1)	0/2 (0)	0/2 (0)
Ceftazidime	1 (1)	11 (15)	1 (1)	1/13 (8)	1/12 (8)
Meropenem	0 (0)	0 (0)	0 (0)	NA	NA
Total	7/518 (1)[c]	14/518 (3)[c]	17/518 (3)[c]	23/38 (61)	17/31 (55)

[a]S/R denotes susceptible/resistant category. Initial BD Phoenix intermediate results were counted as resistant—this occurred in one isolate with an S/R discrepancy for ceftazidime and one isolate with an S/R discrepancy for gentamicin.
[b]NA = not applicable.
[c]*n*/overall total of 518 antimicrobial susceptibility results (%).

Table 2. Analysis of discordance in phenotypic and/or genotypic resistance predictions for 69 *K. pneumoniae* bloodstream isolates

Antibiotic	Discrepancies (*n*; % of total, 69 isolates)			Agreement of gradient diffusion with genotype in all discrepancies (*n*/total discrepancies; %)	Agreement of gradient diffusion with genotype in BD Phoenix-concordant discrepancies (*n*/number of BD Phoenix-concordant discrepancies; %)
	S/R[a] discordant BD Phoenix	BD Phoenix S/S, genotype R	BD Phoenix R/R, genotype S		
Amoxicillin	3 (4)	3[b] (4)	0 (0)	3/6 (50)	1/3 (33)
Co-amoxiclav	2 (3)	0 (0)	6 (9)	7/8 (88)	6/6 (100)
Gentamicin	1 (1)	0 (0)	1 (1)	1/2 (50)	0/1 (0)
Ciprofloxacin	1 (1)	2 (3)	7 (10)	4/10 (40)	3/9 (33)
Ceftriaxone	0 (0)	1 (1)	2 (3)	0/3 (0)	0/3 (0)
Ceftazidime	0 (0)	1 (1)	2 (3)	0/3 (0)	0/3 (0)
Meropenem	1 (1)	0 (0)	0 (0)	0/1 (0)	NA[c]
Total	8/483 (2)[d]	7/483 (1)[d]	18/483 (4)[d]	15/33 (45)	10/25 (40)

[a]S/R denotes susceptible/resistant category. Initial BD Phoenix intermediate results were counted as resistant—this occurred in one isolate with an S/R discrepancy for ciprofloxacin and one isolate with an S/R discrepancy for meropenem.
[b]This applies to an MIC-based assessment of BD Phoenix results, disregarding interpretative guidelines (which would suggest that *K. pneumoniae* be universally reported as amoxicillin resistant for clinical purposes, irrespective of the MIC).
[c]NA = not applicable.
[d]*n*/overall total of 483 antimicrobial susceptibility results (%).

(Figure 1). Assemblies for the 74 remaining *E. coli* isolates had a median of 394 contigs (range: 93–1052) and n50 of 110187 bp (range: 32391–189171 bp). For the 69 *K. pneumoniae* study isolates, the corresponding medians were 255 contigs (range: 171–863) and n50 of 97195 bp (range: 58500–135350 bp).

Investigation of discrepancies

Susceptibility phenotypes for seven antimicrobials were available for 143 study isolates (74 *E. coli* and 69 *K. pneumoniae*), giving 1001 total susceptibility results (518 *E. coli* and 483 *K. pneumoniae*) for comparison with the corresponding genotypic predictions. Gradient diffusion analysis was used to establish the phenotype for 71 antimicrobial–isolate combinations (involving 55 different isolates), including 7 (1%) *E. coli* results and 8 (2%) *K. pneumoniae* results with categorical (S/R) discordance in duplicate BD Phoenix testing and 31 (6%) *E. coli* and 25 (5%) *K. pneumoniae* results with discordance between the predicted genotypic susceptibility and the (concordant) BD Phoenix phenotype [Table 1 (*E. coli*) and Table 2 (*K. pneumoniae*)].

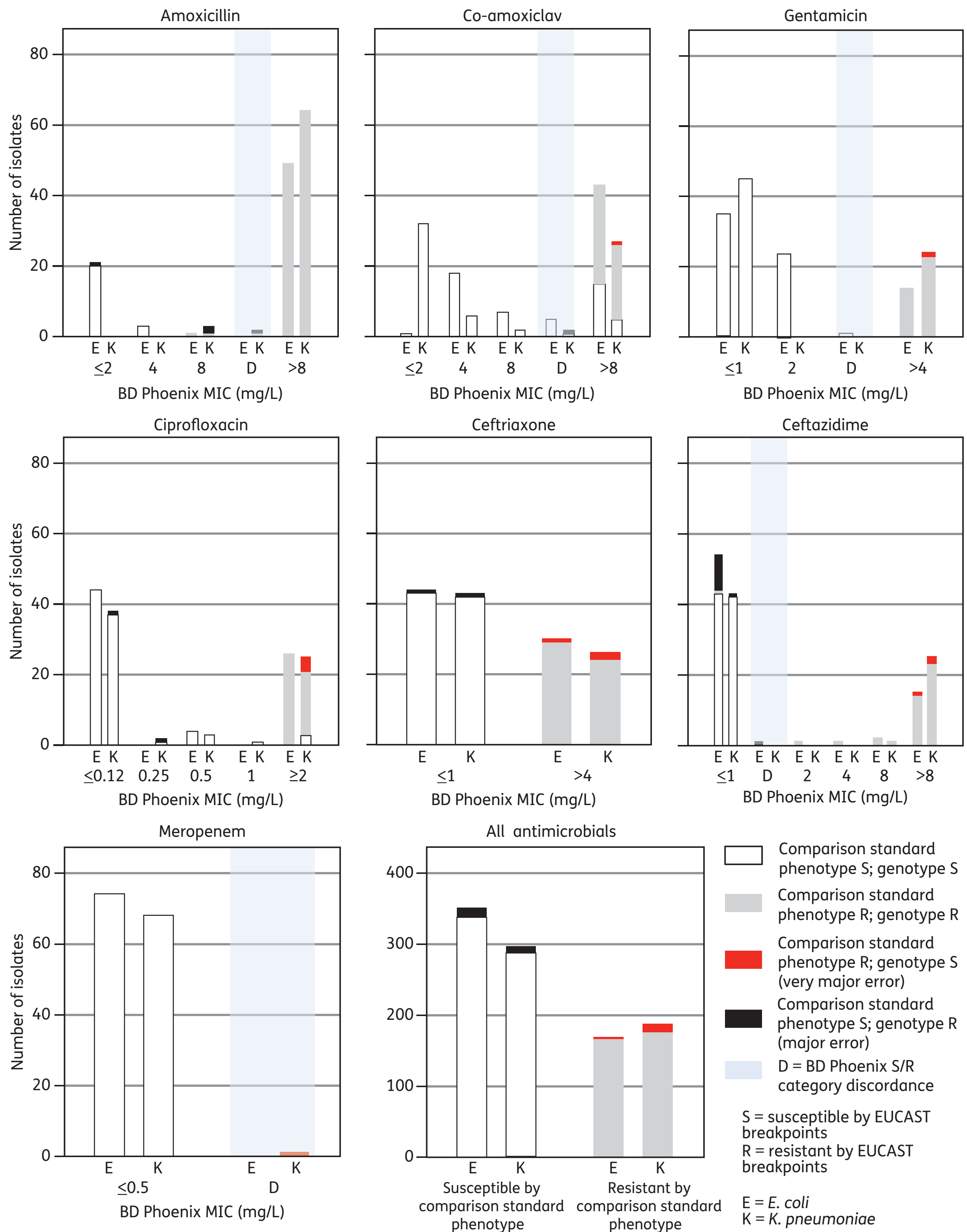

Figure 2. Comparisons of genotypic susceptibility prediction, BD Phoenix phenotype and results of gradient diffusion analyses for discrepancies in either (i) duplicate BD Phoenix testing or (ii) genotypic prediction and concordant BD Phoenix phenotype, for both species across all seven antimicrobials.

Table 3. Sensitivity and specificity of genotypic resistance predictions versus comparison with standard phenotype results for 74 *E. coli* bloodstream isolates.

	Susceptible by comparison standard phenotype		Resistant by comparison standard phenotype			
Antibiotic	susceptible by genotype (row %)	resistant by genotype (row %; major error)	susceptible by genotype (row %; very major error)	resistant by genotype (row %)	Sensitivity (95% CI)	Specificity (95% CI)
Amoxicillin	23 (31)	1 (1)	0 (0)	50 (68)	1.00 (0.91–1.00)	0.96 (0.77–1.00)
Co-amoxiclav	46 (62)	0 (0)	0 (0)	28 (38)	1.00 (0.85–1.00)	1.00 (0.90–1.00)
Gentamicin	60 (81)	0 (0)	0 (0)	14 (19)	1.00 (0.73–1.00)	1.00 (0.93–1.00)
Ciprofloxacin	48 (65)	0 (0)	0 (0)	26 (35)	1.00 (0.84–1.00)	1.00 (0.91–1.00)
Ceftriaxone	43 (58)	1 (1)	1 (1)	29 (39)	0.97 (0.81–1.00)	0.98 (0.87–1.00)
Ceftazidime	43 (58)	11 (15)	1 (1)	19 (26)	0.95 (0.73–1.00)	0.80 (0.66–0.89)
Meropenem	74 (100)	0 (0)	0 (0)	0 (0)	—	1.00 (0.94–1.00)
Total	337 (65)	13 (3)	2 (0.3)	166 (32)	0.99 (0.95–1.00)	0.96 (0.94–0.98)

Table 4. Sensitivity and specificity of genotypic resistance predictions versus comparison standard phenotype results for 69 *K. pneumoniae* bloodstream isolates

	Susceptible by comparison standard phenotype		Resistant by comparison standard phenotype			
Antibiotic	susceptible by genotype (row %)	resistant by genotype (row %; major error)	susceptible by genotype (row %; very major error)	resistant by genotype (row %)	Sensitivity (95% CI)	Specificity (95% CI)
Amoxicillin	0 (0)	3 (4)	0 (0)	66 (96)	1.00 (0.93–1.00)	—
Co-amoxiclav	47 (68)	1 (1)	0 (0)	21 (30)	1.00 (0.81–1.00)	0.98 (0.88–1.00)
Gentamicin	45 (65)	0 (0)	1 (1)	23 (33)	0.96 (0.77–0.98)	1.00 (0.90–1.00)
Ciprofloxacin	45 (65)	2 (3)	4 (6)	18 (26)	0.90 (0.67–0.98)	0.92 (0.80–0.97)
Ceftriaxone	42 (61)	1 (1)	2 (3)	24 (35)	0.92 (0.73–0.99)	0.98 (0.86–1.00)
Ceftazidime	42 (61)	1 (1)	2 (3)	24 (35)	0.92 (0.73–0.99)	0.98 (0.86–1.00)
Meropenem	68 (99)	0 (0)	1 (1)	0 (0)	0 (0–0.95)	1.00 (0.93–1.00)
Total	289 (60)	8 (2)	10 (2)	176 (36)	0.95 (0.90–0.97)	0.97 (0.95–0.99)

Genotypic prediction versus comparison standard phenotype

Overall, the sensitivity of genotype for predicting resistance across all antibiotics for both species was 0.96 (95% CI: 0.94–0.98) and the specificity was 0.97 (95% CI: 0.95–0.98) (Figure 2). Very major and major error rates, at 1.2% and 2.1%, respectively, were within the <1.5% and <3% FDA limits. For *E. coli*, the overall sensitivity was 0.99 (95% CI: 0.95–1.0) and the specificity was 0.96 (95% CI: 0.94–0.98) (Table 3); the major individual drug deficit being suboptimal specificity for ceftazidime (0.80; 95% CI: 0.66–0.89). Very major (0.3%) and major (3%) error rates were again within the FDA limits. For *K. pneumoniae*, the overall sensitivity was 0.95 (95% CI: 0.90–0.97) and the specificity was 0.97 (95% CI: 0.95–0.99), with a very major error rate (2%) just outside the 1.5% FDA limit, but an acceptable major error rate (2%, compared with <3% as per FDA) (Table 4).

In *E. coli*, in 23 (61%) of the 38 isolate–antimicrobial combinations with a phenotype–genotype discrepancy according to BD Phoenix results, gradient diffusion analysis supported the genotypic prediction (Table 1). For the remaining 15 confirmed genotype–phenotype discrepancies, the results are summarized in Table 5. In 13 (87%) of these cases, a clear-cut genetic resistance mechanism was identified despite phenotypic susceptibility, although for 9 (69%) of these the gradient diffusion MIC was at the susceptibility breakpoint. The remaining 2 (13%) of the 15 discrepant genotype–phenotype cases had no identifiable genetic resistance mechanism, despite unequivocal phenotypic resistance.

In *K. pneumoniae*, in 15 (45%) of the 33 isolate–antimicrobial combinations with a phenotype–genotype discrepancy according to the BD Phoenix results, gradient diffusion analysis supported the genotypic prediction (Table 2). For the remaining 18 confirmed genotype–phenotype discrepancies, the results are summarized in Table 5. In 6/18 (33%) instances, a recognized resistance

Table 5. List of relevant genotypic profiles for 13 *E. coli* and 15 *K. pneumoniae* isolates with genotype-gradient diffusion susceptibility discrepancies for one or more antimicrobials

Species	Number of isolates	Antibiotic discrepancy	Genotypic prediction	Genotypic mechanism for resistance prediction	Phenotypic result	MIC (mg/L) on gradient diffusion (EUCAST susceptibility breakpoint)	Supplementary data Table no./ complete genotypic profile number
E. coli	1	amoxicillin	R	P3 TEM-promoter and $bla_{TEM\text{-}1}$	S	6 (8)	S4/3
E. coli	1	ceftriaxone[a]	S	none	R	>32 (1)	S4/3
		ceftazidime[a]	S		R	4 (1)	
E. coli	1	ceftriaxone[b]	R	T-32A *ampC* promoter mutation	S	0.38 (1)	S4/10
		ceftazidime[b]	R		S	1 (1)	
E. coli	1	ceftazidime	R	$bla_{CTX\text{-}M\text{-}15}$	S	0.25 (0.25)	S4/2
E. coli	7	ceftazidime	R	$bla_{CTX\text{-}M\text{-}15}$	S	1 (1)	6×S4/4, 1×S4/2
E. coli	1	ceftazidime	R	$bla_{CTX\text{-}M\text{-}14}$	S	0.5 (1)	S4/14
E. coli	1	ceftazidime	R	$bla_{CTX\text{-}M\text{-}1}$	S	1 (1)	S4/6
K. pneumoniae	2	amoxicillin	R	bla_{LEN}	S	4, 8 (8)	S7/4
K. pneumoniae	1	amoxicillin	R	bla_{SHV}	S	6 (8)	S7/3
K. pneumoniae	1	co-amoxiclav	R	$bla_{OXA\text{-}1}$	S	8 (8)	S7/19
K. pneumoniae	1	ceftriaxone[c]	R	$bla_{SHV\text{-}27}$	S	0.064 (1)	S7/11
		ceftazidime[c]	R		S	0.25 (1)	
K. pneumoniae	1	ceftriaxone[d]	S	none	R	8 (1)	S7/8
		ceftazidime[d]	S		R	64 (1)	
K. pneumoniae	1	ceftriaxone[e]	S	none	R	>32 (1)	S7/1
		ceftazidime[e]	S		R	8 (1)	
K. pneumoniae	1	meropenem	S	none	R	>32 (2)	S7/7
K. pneumoniae	1	ciprofloxacin	R	2 *gyrA* mutations (S83F+D87A)	S	0.064 (0.5)	S8/5
K. pneumoniae	1	ciprofloxacin	R	2 *gyrA* mutations (S83I+D87N)	S	0.047 (0.5)	S8/10
K. pneumoniae	2	ciprofloxacin	S	1 *parC* mutation (S80I)	R	8, >32 (0.5)	S8/4
K. pneumoniae	1	ciprofloxacin	S	1 *parC* mutation (S80I)+ *aac(6′)-Ib-cr*	R	2 (0.5)	S8/12
K. pneumoniae	1	ciprofloxacin	S	1 *parC* mutation (E84K)+ 1 *parE* mutation (S458T)	R	>32 (0.5)	S8/17
K. pneumoniae	1	gentamicin	S	none	R	16 (2)	S9/1

[a–e]Multiple genotype–phenotype discrepancies observed for several antibiotics for the same isolate.

mechanism was identified in phenotypically susceptible isolates, although for two of these the MIC was at the susceptibility breakpoint. This group included two isolates predicted to be ciprofloxacin resistant based on chromosomal mutations (double *gyrA* amino acid replacements) that were phenotypically ciprofloxacin susceptible. In contrast, four isolates predicted to be ciprofloxacin susceptible [based on a single mutation in *parC* (three isolates with S80I) or combined mutations in *parC* (E84K)+*parE* (S458T)] were phenotypically resistant, suggesting the presence of unidentified resistance mechanisms. Similarly, six other cases with unequivocal phenotypic resistance to one or more agents from several antibiotic classes (ceftriaxone, ceftazidime, meropenem and gentamicin; eight total agent–isolate combinations) had no identifiable resistance mechanism.

Resistance gene profiles—E. coli

Genotypic resistance profiles in *E. coli* are summarized in Tables S4 (β-lactam resistance), S5 (fluoroquinolone resistance) and S6 (aminoglycoside resistance). There were 15 distinct profiles for β-lactam resistance mechanisms, 22 for ciprofloxacin-associated resistance mechanisms and 12 for aminoglycoside-associated resistance mechanisms.

β-Lactam resistance

Twelve (16%) isolates had bla_{TEM}, $bla_{OXA\text{-}1}$ and $bla_{CTX\text{-}M}$ conferring β-lactam resistance; 15 (20%) had two of these three mechanisms, 24 (32%) had one and 23 (31%) had none. Most bla_{TEM}-containing isolates had $bla_{TEM\text{-}1}$ (35/36), with five distinct nucleotide sequences (including the reference sequence) observed. In addition to the P3 and Pa/Pb $bla_{TEM\text{-}1}$ promoters,[19] two novel promoter sequences were identified [single nucleotide polymorphisms compared with promoter P3: C→T at position 75 (Sutcliffe numbering);[30] G→A at position 175]. However, co-amoxiclav resistance was identified only in the presence of other explanatory mechanisms with these novel promoter sequences. All bla_{OXA} variants were $bla_{OXA\text{-}1}$ and most $bla_{CTX\text{-}M}$ variants were $bla_{CTX\text{-}M\text{-}15}$ (25/29).

Only one isolate had a chromosomal *ampC* promoter mutation previously associated with significant resistance (T-32A).[22] This

isolate was resistant only to amoxicillin and co-amoxiclav, with no other mechanism identified to explain this, and was phenotypically susceptible to ceftriaxone and ceftazidime (on duplicate BD Phoenix testing and gradient diffusion analysis).

Quinolone resistance

Ciprofloxacin resistance was invariably associated with S83L/D87N mutations in *gyrA*; almost all (23/26; 88%) ciprofloxacin-resistant isolates also had S80I/E84V mutations in *parC*. The presence of *aac-6′-Ib-cr* was also common in ciprofloxacin-resistant isolates, although not universal (23/26; 88%); *aac-6′-Ib-cr* was also identified in one ciprofloxacin-susceptible isolate without any resistance-conferring chromosomal mutations. A single isolate had a *gyrB* quinolone resistance-determining region (QRDR) mutation (S463A) with a *parE* truncation; this isolate was phenotypically susceptible. No *qnr* variants or *qepA* or *oqxAB* loci were found.

Aminoglycoside resistance

Four (5%) isolates had four or five different aminoglycoside resistance-conferring elements; 15 (20%) had three, 12 (16%) had two, 11 (15%) had one and 32 (43%) had none. All gentamicin resistance was associated with the presence of *aac(3′)-II*-like enzymes, mostly *aac(3′)-IIe* variants (13/14), with one isolate containing *aac(3′)-IId*. Other aminoglycoside resistance loci included *aac(6′)-Ib-cr* (24 isolates), *aadA1a* (3), *aadA4* (17), *aadA5* (17), *aph(6′)-Id* (16), *aph(6′)-Id*-like loci (>80% but <95% sequence homology; 3 isolates) and *aph(3′)-Ia* (4).

Resistance gene profiles—K. pneumoniae

Genotypic profiles associated with resistance in *K. pneumoniae* are summarized in Tables S7 (β-lactam resistance, 24 profiles), S8 (fluoroquinolone resistance, 20 profiles) and S9 (aminoglycoside resistance, 17 profiles).

β-Lactam resistance

Twenty-one (30%) isolates had three or four β-lactam resistance-conferring elements; 8 (12%) had two, 29 (42%) had one and 11 (16%) had none. All bla_{TEM} were bla_{TEM-1}, with P3 (n=24) or Pa/Pb (n=3) promoters. All bla_{CTX-M} were $bla_{CTX-M-15}$ and all bla_{OXA} were bla_{OXA-1} (only observed with $bla_{CTX-M-15}$).

Most *K. pneumoniae* isolates (61/69; 88%) contained bla_{SHV} genes encoding β-lactamases. Six contained bla_{LEN} (two bla_{LEN-7}, four novel variants), one $bla_{OKP-B-6}$ and one bla_{LAP-2} in conjunction with bla_{SHV-11}. The most common bla_{SHV} β-lactamase variant was bla_{SHV-1} (n=28), with additional variants in order of frequency as follows: bla_{SHV-11} (19), bla_{SHV-28} (4), bla_{SHV-33} (2), $bla_{SHV-121}$ (2), bla_{SHV-27} (1), bla_{SHV-60} (1) and $bla_{SHV-135}$ (1). Three novel amino acid bla_{SHV} variants were identified (Y7F+S14F, Y7F+M211L and D101H; assigned allele numbers 169, 170 and 171, respectively, in the Lahey database).[17] One of the 69 *Klebsiella* isolates contained none of these resistance loci; its β-lactam resistance was explained by the presence of bla_{TEM-1}, bla_{OXA-1} and $bla_{CTX-M-15}$.

Quinolone resistance

Isolates with wild-type amino acids or only single amino acid mutations in the QRDRs of *gyrA*, *gyrB*, *parC* and *parE*, and no more than one plasmid-mediated resistance mechanism (*aac-6′-Ib-cr*, *qnr* or *qepA*), were all ciprofloxacin susceptible (41 wild-type isolates, four single amino acid mutations). In contrast, isolates with both *aac-6′-Ib-cr* and *qnrB1* (n=15) were invariably resistant, irrespective of underlying chromosomal mutations. Likewise, isolates with single *gyrA* and *parC* mutations and a plasmid-mediated resistance mechanism (n=2: S83I+S80I+*aac-6′-Ib-c*; S83T+S80I+*qnrS1*), or a double mutation in *gyrA*, a single mutation in *parC* and a plasmid-mediated resistance mechanism (n=1: S83F+D87N+S80I+*aac-6′-Ib-cr*), were also resistant.

There were no observed mutations compared with wild-type in the QRDR of *gyrB*. All isolates contained *oqxAB*, which is commonly located chromosomally in *K. pneumoniae*, although its association with ciprofloxacin resistance in this context is unclear.[31]

Aminoglycoside resistance

One (1%) isolate had five different resistance-conferring elements; 16 (23%) had three, 9 (13%) had two, 7 (10%) had one and 36 (52%) had none. As in *E. coli*, gentamicin resistance in *K. pneumoniae* was typically associated with the presence of *aac(3′)-II*-like enzymes, mostly *aac(3′)-II-e* (19/23). Three isolates had an *aac(3′)-IId* enzyme and one an *aac(3′)-Ia* variant. Other aminoglycoside resistance loci included *aph(6′)-Id* (25 isolates), *aph(3′)-Ia* (4), *aadA2* (4), *aadA1* (2) and *aadA16* (1).

Discussion

In this study, we determined the sensitivity and specificity of a genotypic prediction algorithm for the two most commonly isolated Gram-negative species, *E. coli* and *K. pneumoniae*, using whole-genome data from clinical isolates from bacteraemic patients with a wide range of resistance phenotypes. In our centre, the epidemiology of these organisms has been found to be similar to the wider national and European contexts.[12,13] Using publicly available resources, we determined the presence/absence of published variants (including genes and resistance-determining mutations) in >100 resistance-associated gene families, with particular reference to those relevant to commonly used antimicrobials. Relative to a comparison standard phenotype based on BD Phoenix plus gradient diffusion testing, genotype-based resistance prediction yielded overall sensitivity and specificity values of 0.96 and 0.97, respectively, plus rates of very major errors (1.2%) and major errors (2.1%) below the corresponding FDA-specified thresholds of 1.5% and 3%.

Applying genetic 'resistotyping' to Gram-negative species is not new, with PCR-based methods having been widely used in the epidemiological assessment of both *E. coli* and *K. pneumoniae* collections. However, the number of resistance mechanisms involved is extremely large, limiting the use of comprehensive PCR methods in any real-time diagnostic capacity. One response to this challenge has been to develop microarray-based approaches to assess a much larger panel of resistance mechanisms than is feasible with PCR; this method, however, has issues with sensitivity and cannot easily identify numerous mutation-based mechanisms of resistance.[32] In addition, microarrays are expensive to develop and difficult to upgrade flexibly in response to the evolution of resistance mechanisms. We have demonstrated that whole-genome sequencing provides a viable alternative approach.

This study has also demonstrated that novel variants of known resistance-associated loci can be easily identified using our approach. To expand on this, BLASTn-based cut-offs could be made less stringent to facilitate the discovery of putative, distantly related resistance genes or a tBLASTx-based approach could be used to identify protein homologues with different underlying coding sequences. Similar approaches have been used in the past, although in a limited manner.[33]

Our data highlight some known issues with the accuracy of some phenotypic methods commonly used in diagnostic microbiology—particularly with the assessment of β-lactam/β-lactamase inhibitor susceptibilities. Duplicate BD Phoenix tests gave discordant results in 7 (5%) of 143 co-amoxiclav tests performed; on gradient diffusion, 6/7 isolates had MICs more than one dilution away from the breakpoint. In 21 instances where the co-amoxiclav genotypic prediction disagreed with the BD Phoenix results (all involving genotypically susceptible isolates that were resistant by BD Phoenix), all isolates were susceptible according to gradient diffusion, suggesting that in 15% of tests automated phenotyping was overcalling resistance. Problems with the correct assessment of susceptibility by phenotyping for β-lactam/β-lactamase inhibitor combinations have been observed previously,[34] particularly in the context of complex β-lactamase genotypes,[35] which were disproportionately represented in our dataset.

For extended-spectrum cephalosporins and *E. coli*, we found certain genetic mechanisms known to be associated with resistance (such as the CTX-M enzyme family) in isolates considered susceptible. However, this was using the new, lower EUCAST breakpoints in the context of EUCAST's decision (mirrored by the CLSI) to report susceptibilities as observed without interpretative modifications for these drugs. This phenomenon has also been documented in other studies of CTX-M-producing organisms from China and New Zealand[36,37] and highlights the controversy over whether an *in vitro* MIC or the presence of a genetic mechanism is more predictive of clinical outcomes[38]—whether this is a limitation or a strength of genotypic resistance prediction methods is therefore unclear. The large-scale clinical outcome data needed to resolve this quandary are currently lacking, but could be obtained by using integrated routine clinical, phenotyping and antibiotic-prescribing data, combined with whole genome-based, comprehensive assessments of resistance mechanisms.

Overall, among 1001 isolate–antimicrobial combinations tested, we found 12 instances of phenotypic resistance that were supported by gradient diffusion analysis without any resistance mechanism being identified, indicating deficits with our initial gene reference database and/or genotypic prediction algorithm. We have yet to systematically investigate potential contributions made by other known resistance mechanisms, such as porin genes or efflux pumps, in part because associations of the latter with phenotypic resistance are incompletely defined. Assessing the performance of our approach in determining all known mechanisms of resistance, including rare variants, is clearly important future work. For this study, however, we were particularly focused on characterizing the potential of genotypic resistance prediction for organisms typically isolated in our clinical practice. Of interest, given the absence of any initial mechanism identified for carbapenem resistance in the single meropenem-resistant *K. pneumoniae* isolate, we subsequently studied the *ompK35* and *ompK36* loci as possible candidate loci using our BLASTn-based approach and identified a 5 bp deletion in *ompK36* leading to a truncation at position 227. Although we did not measure protein expression, porin deficiencies associated with prematurely truncated *ompK36*, coupled with the presence of $bla_{CTX\text{-}M\text{-}15}$, have been associated previously with carbapenem resistance[39] and could plausibly explain resistance in this isolate. This demonstrates that once an isolate's genome sequence is available, it can be reassessed rapidly for additional resistance gene mechanisms as necessary, without the need for further laboratory work.

There are several limitations to our approach as described. Establishing the sequencing and computational infrastructure required to process large volumes of sequencing data in real time involves a substantial initial investment in terms of time and money. Our study was a retrospective, proof-of-principle experiment and further work would be required to assess its performance and cost-effectiveness in a routine diagnostic setting on a larger dataset. In addition, it remains to be seen whether predictions would be equally successful for all antimicrobials currently incorporated in phenotypic susceptibility testing strategies. The bioinformatic strategy used does not determine plasmid copy number and therefore cannot quantify the possible contribution of multiple gene copies (e.g. of bla_{TEM}), which might lead to hyperproduction of certain enzymes and phenotypic resistance by a gene dosage effect. Another limitation is that the phenotypic manifestations of certain allelic variants and promoter/attenuator mechanisms are not fully determined (e.g. for some of the bla_{SHV} variants), precluding reliable predictions. Importantly, resistance mechanisms evolve; approaches based on genotypic prediction rely on a resistance locus reference database requiring regular updating based on a scheme incorporating ongoing phenotyping, albeit in a more limited number of samples, such as those isolated from treatment failures. Phenotyping would also be needed to validate any novel genetic resistance mutations/mechanisms. Finally, epigenetic and expression-associated mechanisms cannot be determined using our DNA-based analysis, thus highlighting the intrinsic limitation of approaches based on gene/mutation identification with no direct evidence of functional resistance. However, alternative sequencing-based methods could be explored to address this shortcoming, such as RNA-Seq, chromatin immunoprecipitation sequencing or methylation analysis.[40]

Despite these limitations, our approach achieved high sensitivity and specificity in proof-of-principle experiments using typical clinical isolates and its performance was comparable to that of some phenotyping methods currently in routine use. Whole-genome sequencing-based approaches may well become part of routine microbiology workflows in some settings within the next 5 years. This would afford the ability to undertake species identification, strain typing for epidemiological purposes or infection prevention and control, and prediction of antimicrobial susceptibilities reliably and quickly using a single method for ∼£40/isolate.[1]

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Disclaimer

The views expressed in this publication are those of the author(s) and not necessarily those of the National Health Service, the NIHR or the Department of Health.

Supplementary data

Tables S1–S9 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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