Original Article

Antibiotic Resistance in Gram-negative Bacilli: A Singapore Perspective

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Abstract

Introduction: Antibiotic resistance in gram-negative bacilli is an area of increasing importance. This prospective study was performed to survey antibiotic resistance in *Escherichia coli (E. coli)*, *Klebsiella* spp., *Pseudomonas aeruginosa* and *Acinetobacter* spp. over a 1-year period. Materials and Methods: Non-duplicate isolates of *E. coli*, *Klebsiella* spp., *P. aeruginosa* and *Acinetobacter* spp. were collected from participating Singapore hospitals during defined collection periods in 2006 and 2007. Confirmatory identification and antibiotic susceptibility testing were performed at Changi General Hospital. Minimum inhibitory concentrations (MIC) to a defined panel of antibiotics were determined using microbroth dilution methods. The presence of extended-spectrum beta lactamases and AmpC beta-lactamases in *Enterobacteriaceae* was determined by phenotypic methods, and susceptibility results were defined using current breakpoints from the Clinical Laboratory Standards Institute (CLSI). Results: Seven hundred and forty-six gram-negative bacilli were received for testing. Resistance to extended-spectrum cephalosporins was present in a third of *Enterobacteriaceae* isolates, and extended-spectrum beta-lactamases (ESBL) carriage was present in 19.6% and 30.1% of *E. coli* and *Klebsiella pneumoniae*, respectively. AmpC enzymes were also detected in 8.5% and 5.6% of *E. coli* and *K. pneumoniae* isolates respectively. All *Enterobacteriaceae* were susceptible to imipenem and meropenem. The most active antibiotics against *P. aeruginosa* were amikacin, meropenem and piperacillin-tazobactam. A third of *P. aeruginosa* showed reduced susceptibility to polymyxin B. Carbapenem resistance was significantly higher in *Acinetobacter baumannii* (70.5%) than in other *Acinetobacter* species (25.0%). The most active antibiotic against *A. baumannii* was polymyxin B. Conclusion: Antibiotic resistance is prevalent in gram-negative bacilli isolated from Singapore hospitals. The MIC testing surveillance programme complemented susceptibility data from wider laboratory-based surveillance, and has revealed emerging mechanisms of antibiotic resistance.

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Keywords: *Enterobacteriaceae*, ***Acinetobacter baumannii*, *Pseudomonas aeruginosa*, Microbial sensitivity tests**

Introduction

In contrast to recent media reviews on gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci and *Clostridium difficile*, the increasing complexity and multiplicity of antibiotic resistance in gram-negative bacilli has generally gone unnoticed by the general public. Antimicrobial resistance in gram-negative bacilli has increased worldwide over the past decade.[1,2] This problem is compounded by the fact that many antibiotic resistance determinants in gram-negative bacilli confer resistance to multiple classes of antibiotics, and are placed on mobile genetic elements such as plasmids and transposons that may be transferred between and across different bacterial species with relative ease.[1]

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Compared to infections caused by drug-susceptible gram-negative bacteria, infections caused by their drug-resistant counterparts are associated with higher morbidity and mortality, particularly if inappropriate empirical antibiotic therapy is prescribed.[1,3] The socio-economic burden of healthcare costs and resource utilisation is also considerably higher.[4,5] This problem is made even more acute by the lack of effective antibiotics against the most resistant isolates currently and in the foreseeable future – an issue that is of lesser significance now for antibiotic-resistant gram-positive pathogens.[1]

In terms of frequency and clinical impact, the presence of quinolone resistance and beta-lactamases in *Enterobacteriaceae* that mediate resistance to third- and fourth-generation cephalosporins feature highest on the list,[6] because these organisms are the most frequently isolated gram-negative bacilli in most clinical settings. Besides the ever-increasing number of extended-spectrum beta-lactamases (ESBL) charted, emerging resistance mechanisms such as plasmid-mediated AmpC enzymes and metallo-beta-lactamases are also being increasingly reported in common *Enterobacteriaceae* such as *Escherichia coli* and *Klebsiella pneumoniae*.[6]

Antibiotic resistance mechanisms in non-fermentative gram-negative bacilli such as *Pseudomonas aeruginosa* and *Acinetobacter* spp. are far more varied, comprising not just enzymes that inactivate antibiotics, but also a variety of efflux pumps.[7] Although these organisms form a smaller proportion of clinical isolates, they tend to cause clinical infections in intensive care and immunocompromised populations. Therefore, antibiotic resistance in these strains may have a disproportionate impact on medical care.

In the fourth quarter of 2007, the most frequently isolated bacteria in Singapore hospitals were *Escherichia coli* (22%), *Staphylococcus aureus* (16%), *Klebsiella* spp. (12%), *Pseudomonas aeruginosa* (9%), *Enterococcus* spp. (5%) and *Acinetobacter* spp. (3%). Passive laboratory surveillance from all public hospitals in Singapore initiated by the Network for Antimicrobial Resistance Surveillance (Singapore) detected high proportions of third-generation cephalosporin resistance (17.5% and 35.9% respectively) among *E. coli* and *Klebsiella* spp. in 2006, while carbapenem resistance in *Acinetobacter* spp. was reported at 49.6%.[8]

Passive laboratory surveillance does not allow for the detection of specific resistance determinants in bacterial strains. The use of different laboratory standards and methods also adds a degree of inaccuracy to the analyses. This study was conceived to address the above 2 issues, and to provide detailed susceptibility information for a subset of clinical isolates of *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter* spp.

Materials and Methods

Sample Collection

The institutions that participated in isolate collection were Alexandra Hospital, Changi General Hospital, KK Women's and Children's Hospital, National University Hospital, Tan Tock Seng Hospital and Singapore General Hospital. Isolates were collected at defined intervals over a 1-year period, beginning from February 2006. Each institute was assigned a target number of isolates to collect (chosen to be representative of the relative size of the institution). Collection of each target organism began at the same time period, and continued until the assigned number of isolates was achieved. Only unique clinical isolates of each organism were included, and screening isolates were excluded. Test isolates were referred to Changi General Hospital for additional testing.

Bacterial Identification

Bacterial identification was initially performed at each participating laboratory, using the local protocols of each laboratory. Submitted *E. coli* and *P. aeruginosa* isolates were sub-cultured at the central laboratory on to Chromagar Orientation medium (Becton Dickinson, Maryland, USA) and trypticase soy agar with 5% sheep blood (Bloxwich, Malaysia), respectively. Species identification was accepted for isolates with the appropriate colonial morphology when grown on these sub-culture media. Isolates with atypical morphology were identified using Microbact (Oxoid, Basingstoke, UK). All *Klebsiella* spp. were identified using Microbact together with selected supplementary biochemical tests. A PCR-based multiplex assay was used to differentiate *A. baumannii* from other *Acinetobacter* spp.[9]

Minimum Inhibitory Concentration Testing

Minimum inhibitory concentrations (MIC) to a panel of antibiotics were obtained by custom-made dehydrated microbroth dilution panels (Trek Diagnostics, West Sussex, UK) performed according to the manufacturer's recommendations. In brief, turbidity-adjusted bacterial suspensions from fresh overnight cultures were added to Mueller-Hinton broth to conform to an inoculation density of 10^4 cfu/mL. Inoculum size was checked for every study isolate tested. Test values of quality controls strains of *E. coli* ATCC® 25922 and *P. aeruginosa* ATCC® 27853 were in line with published standards.[10]

Phenotypic Testing for Resistance Mechanisms

E. coli and *Klebsiella* spp. were screened for phenotypic production of ESBL using ceftazidime, ceftazidime/clavulanate, cefotaxime and cefotaxime/clavulanate antibiotic discs. Zones of inhibition were measured, and

the presence of ESBL inferred by an increase in zone diameter in the clavulanate-augmented discs of ≥5mm.[10] Both species were also screened for phenotypic evidence of AmpC beta-lactamase activity, using a combination of 30 μg-cefoxitin discs (Becton Dickinson, Maryland, USA), and cefoxitin discs with supplemented cloxacillin (200 μg) or boronic acid (400 μg). A ≥5 mm increase in zone size to either inhibitor-supplemented discs was interpreted as a positive result for AmpC production.[11,12]

Susceptibility

Susceptibility criteria for MIC values were interpreted according to current guidelines from the Clinical Laboratory Standards Institute (CLSI),[10] except for tigecycline. Isolates with phenotypic expression of ESBL or AmpC production were considered intrinsically resistant to all cephalosporins. Multi-drug resistance was defined as isolates that were resistant to gentamicin, third-generation cephalosporins and beta-lactam/beta-lactamase inhibitor combinations.

Analysis of Data

Data were entered into and analysed by Excel (Microsoft, Redmond, USA) and WHONET 5.4 (WHO). Statistical analysis, relative risk ratios and chi-square tables were generated by EpiInfo (CDC, Atlanta, USA). A *P* value of <0.05 was accepted as statistically significant.

Results

Enterobacteriaceae

Three hundred and eight-seven *Enterobacteriaceae* were included in the study. Of which, 48.9% (n = 189) were *E. coli*, 50.1% (n = 194) were *K. pneumoniae* and 0.01% (n = 4) were *K. oxytoca*.

All tested *Enterobacteriaceae* were susceptible to imipenem and meropenem. Resistance to extended-spectrum cephalosporins was detected in 27% (n = 51) of *E. coli* strains, while the corresponding figure for *Klebsiella* spp. was 30.8% (n = 61). Resistance to all tested oral antibiotics was present in 26.3% (n = 52) of *Klebsiella* spp. The antibiotic susceptibilities for *Enterobacteriaceae* are shown in Tables 1 and 2.

There are currently no interpretative breakpoints for tigecycline from the CLSI. Based on either US Food and Drug Administration-approved breakpoints for *Enterobacteriaceae* [susceptible (S) ≤2 μg/mL, resistant (R) ≥8 μg/mL) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (S ≤1 μg/mL, R ≥2 μg/mL)], all isolates of *E. coli* were susceptible to tigecycline. The corresponding tigecycline susceptibility interpretations for *Klebsiella* spp. were 96% (n = 190) susceptible (using FDA-breakpoints) or 90.4% (n = 179) susceptible (using EUCAST breakpoints). MIC distributions for tigecycline against *Enterobacteriaceae* are shown in Figure 1.

The presence of ESBL enzymes was detected in 19.6% (n = 37) of *E. coli*, and a further 8.5% (n = 16) were AmpC producing isolates. AmpC and ESBL enzymes were concurrently present in 2 isolates. The presence of ESBL was significantly associated with resistance to ciprofloxacin (relative risk RR 2.3), trimethoprim-sulphamethoxazole (RR 1.6) and gentamicin (RR 3.1).

ESBL enzymes were detected in 30.3% (n = 60) of *K. pneumoniae* and 5.6% (n = 11) were positive for AmpC, with 5.1% (n = 10) isolates positive for both ESBL and AmpC enzymes. The presence of ESBL was significantly associated with resistance to ciprofloxacin (RR 4.6),

Table 1. Antibiotic Susceptibilies of *Escherichia coli*

Antibiotic name	n	%			μg/mL	
		R	I	S	MIC90	MIC range
Amoxicillin/Clavulanic acid	189	10.1	29.6	60.3	32	1-128
Piperacillin/Tazobactam	189	1.1	3.2	95.8	16	1-128
Ceftriaxone	189	27	0	73	128	0.12-128
Cefepime	189	27	0	73	64	0.25-64
Imipenem	189	0	0	100	0.5	0.06-1
Meropenem	189	0	0	100	0.064	0.06-0.12
Amikacin	189	1.1	0	98.9	8	1-32
Gentamicin	189	18.5	0	81.5	64	0.5-64
Ciprofloxacin	189	41.8	1.6	56.6	16	0.06-16
Trimethoprim/Sulfamethoxazole	189	51.3	0.5	48.1	16	0.06-16
Tigecycline*	189	0	0	100	0.5	0.12-1

MIC: minimum inhibitory concentration
* using breakpoints suggested by the FDA

Table 2. Antibiotic Susceptibilities of *Klebsiella* spp.

Antibiotic name	n	%			µg/mL	
		R	I	S	MIC90	MIC range
Amoxicillin/Clavulanic acid	198	22.7	13.1	64.1	32	0.5-64
Piperacillin/Tazobactam	198	12.6	8.1	79.3	128	1-256
Ceftriaxone	198	30.8	0	69.2	128	0.12-128
Cefepime	198	30.8	0.5	68.7	64	0.25-64
Imipenem	198	0	0	100	1	0.06-4
Meropenem	198	0	0	100	0.125	0.06-4
Amikacin	198	0	0	100	4	1-16
Gentamicin	198	18.2	2	79.8	64	0.25-64
Ciprofloxacin	198	35.9	3.5	60.6	16	0.06-16
Trimethoprim/Sulfamethoxazole	198	35.9	0	64.1	16	0.06-16
Tigecycline*	198	2.5	1.5	96	1	0.25-8

MIC: minimum inhibitory concentration
* using breakpoints suggested by the FDA

Table 3. Antibiotic Susceptibilities of *Pseudomonas aeruginosa*

Antibiotic name	n	%			µg/mL	
		R	I	S	MIC90	MIC range
Piperacillin/Tazobactam	188	11.7	0	88.3	128	1-256
Ceftazidime	188	23.4	3.2	73.4	64	1-128
Cefepime	188	11.2	14.9	73.9	32	1-64
Imipenem	188	12.8	4.8	82.4	16	0.25-64
Meropenem	188	9	2.1	88.8	8	0.25-64
Amikacin	188	9.6	0	90.4	16	1-128
Gentamicin	188	17.6	1.1	81.4	64	0.25-64
Ciprofloxacin	188	22.3	3.2	74.5	16	0.06-16
Polymyxin B	188	0	33	67	4	0.5-4

MIC: minimum inhibitory concentration

trimethoprim-sulphamethoxazole (RR 4.6) and gentamicin (RR 10.8), but not with resistance to tigecycline.

Ceftriaxone MIC values for ESBL-producing *Enterobacteriaceae* were significantly higher (P <0.01) than those for AmpC-producing isolates (Fig. 2). Ceftriaxone MICs for AmpC-producing isolates ranged from 0.125 to 32 µg/mL (Fig. 2), suggesting that current CLSI breakpoints (S ≤8 µg/mL, R ≥64 µg/mL) are not discriminatory for strains possessing AmpC beta-lactamases. The imipenem MIC for 4% (n = 8) isolates of *K. pneumoniae* was >1 µg/mL, and these were strains possessing either ESBL or AmpC enzymes, or both.

Pseudomonas aeruginosa

One hundred and eighty-eight isolates were received for testing. The most active agents against *P. aeruginosa* were amikacin (90.4% susceptible), meropenem (88.8% susceptible) and piperacillin-tazobactam (88.3% susceptible). Ciprofloxacin susceptibility was present in 74.5% (n = 140) of isolates. Ten per cent (n = 19) were susceptible to ≤3 antibiotics on the testing panel, of which 3.2% (n = 6) were susceptible to only 1 antibiotic, namely, polymyxin B. Antibiotic susceptibilities and MIC distributions for *P. aeruginosa* are shown in Table 3.

For polymyxin B, 33% (n = 62) of isolates fell into the intermediate susceptibility category. The MIC90 for polymyxin B was 4 µg/mL, and the modal MIC was 2 µg/mL, suggesting that "polymyxin B susceptible" strains of *P. aeruginosa* may be poorly differentiated by current CLSI breakpoints (Fig. 3).

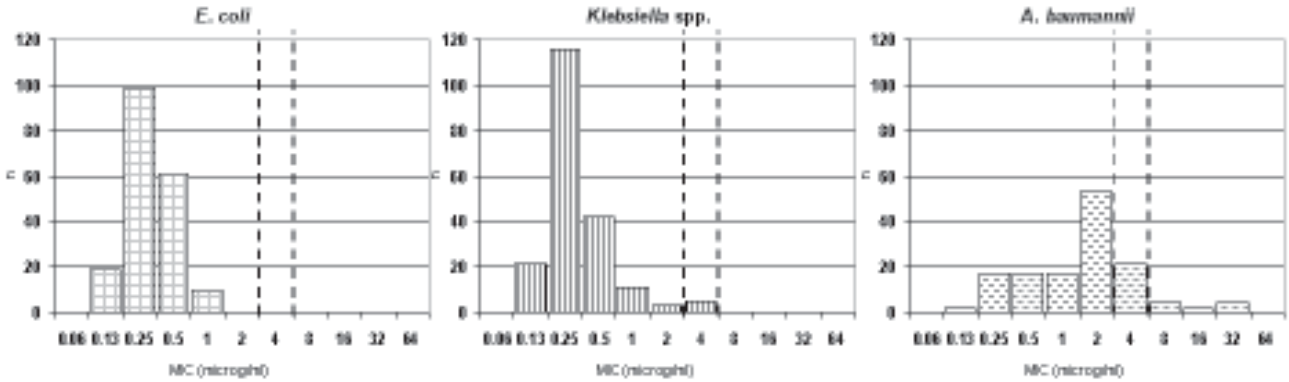

Fig. 1. Tigecycline MIC values for *Enterobacteriaceae* and *Acinetobacter* spp.
FDA-suggested breakpoints for susceptible and resistant categories for *Enterobacteriaceae* indicated as hatched lines

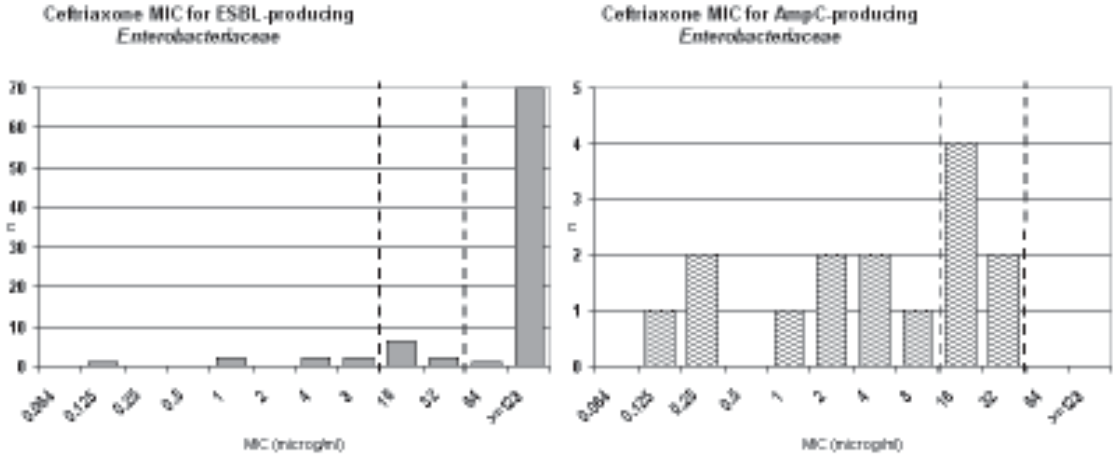

Fig. 2. Ceftriaxone MIC values for ESBL and AmpC producing isolates of *Enterobacteriaceae*.
CLSI breakpoints for susceptible and resistant categories indicated as hatched lines

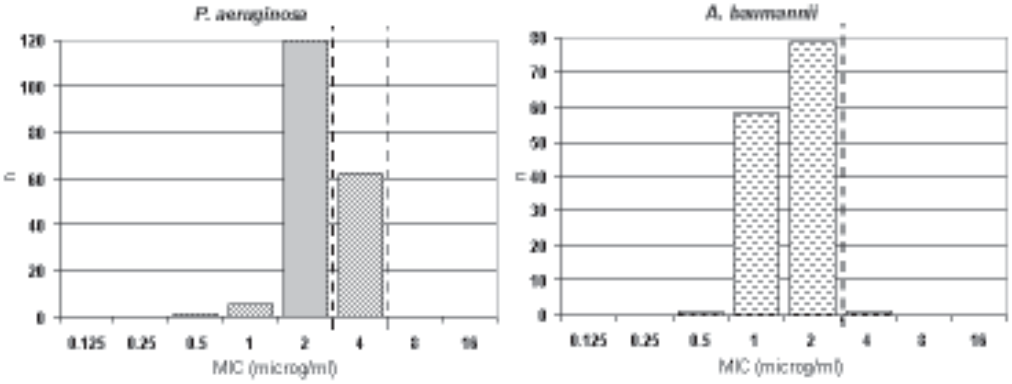

Fig. 3. Polymyxin B MIC distributions for *Acinetobacter* spp. and *P. aeruginosa.*
CLSI breakpoints for susceptible and resistant categories indicated as hatched lines

Acinetobacter species

One hundred and seventy-one isolates were available for testing. Of which, 81.3% (n = 139) were identified as *Acinetobacter baumannii*. 70.5% (n = 98) of isolates of *A. baumannii* were resistant to the carbapenems. Of the carbapenem-resistant strains, the most active remaining antibiotics were polymyxin B (99.0% susceptible, n = 97) and minocycline (53.1% susceptible, n = 52), and 9.4% (n = 13) isolates of *A. baumannii* were susceptible only to polymyxin B. *A. baumannii* were significantly more likely to be resistant to the carbapenems (RR 2.64), ceftazidime (RR 3.28) and ampicillin-sulbactam (RR 6.64) than other

Table 4. Antibiotic Susceptibilities of *Acinetobacter* spp.

Antibiotic name	Species	n	%			μg/mL	
			R	I	S	MIC90	MIC Range
Ampicillin/Sulbactam	*A. baumannii*	139	62.6	15.1	22.3	128	2 - 128
	non-*baumannii*	32	9.4	15.6	75	16	2 - 128
Piperacillin/Tazobactam	*A. baumannii*	139	79.9	7.2	12.9	256	1 - 256
	non-*baumannii*	32	21.9	37.5	40.6	128	1 - 256
Ceftazidime	*A. baumannii*	139	71.9	5	23	128	1 - 128
	non-*baumannii*	32	18.8	18.8	62.5	128	2 - 128
Cefepime	*A. baumannii*	139	79.1	1.4	19.4	64	0.5 - 64
	non-*baumannii*	32	25	15.6	59.4	64	1 - 64
Imipenem	*A. baumannii*	139	70.5	0	29.5	64	0.25 - 64
	non-*baumannii*	32	25	6.2	68.8	64	0.25 - 64
Meropenem	*A. baumannii*	139	70.5	0	29.5	64	0.25 - 64
	non-*baumannii*	32	25	3.1	71.9	64	0.25 - 64
Amikacin	*A. baumannii*	139	61.2	1.4	37.4	128	2 - 128
	non-*baumannii*	32	25	3.1	71.9	128	2 - 128
Gentamicin	*A. baumannii*	139	72.7	0.7	26.6	64	0.5 - 64
	non-*baumannii*	32	43.8	3.1	53.1	64	1 - 64
Ciprofloxacin	*A. baumannii*	139	75.5	1.4	23	16	0.06 - 16
	non-*baumannii*	32	34.4	3.1	62.5	16	0.125 - 16
Polymyxin B	*A. baumannii*	139	0.7	0	99.3	2	0.5 - 4
	non-*baumannii*	32	3.1	0	96.9	2	0.5 - 16
Minocycline	*A. baumannii*	139	5.8	29.5	64.7	8	0.25 - 32
	non-*baumannii*	32	0	0	100	0.5	0.25 - 1
Tigecycline*	*A. baumannii*	139	7.9	15.1	77	4	0.12 - 32
	non-*baumannii*	32	3.1	3.1	93.8	0.5	0.12 - 8

* based on following breakpoints: S ≤2 μg/mL, R ≥8 μg/mL

non-*baumannii* species (*P* <0.01 for all). Similarly, carbapenem resistance in *A. baumannii* was associated with an increased risk of resistance to amikacin (RR 6.86), ampicillin-sulbactam (RR 3.69), ceftazidime (RR 2.57) and ciprofloxacin (RR 3.65). Antibiotic susceptibilities and MIC distributions of *Acinetobacter* spp. are shown in Table 3.

For *A. baumannii*, the MIC90 for tigecycline was 4 μg/mL (modal, MIC 2 μg/mL; range, 0.125-32 μg/mL). There are currently no interpretative susceptibility criteria for tigecycline against *Acinetobacter* spp. If current FDA-approved tigecycline breakpoints for *Enterobacteriaceae* are applied (S ≤2 μg/mL, R ≥8 μg/mL), 77% (n = 107) of the isolates would be considered susceptible. If more conservative breakpoints from EUCAST are applied (S ≤1 μg/mL, R ≥2 μg/mL), then 38% (n = 53) of the isolates would be considered susceptible.

Discussion

This is the first study that has comprehensively examined the antibiotic susceptibilities of common gram-negative bacilli from all public hospitals in Singapore. The strength of this study is that a standardised dilution method was used for testing isolates, and antibiotic susceptibilities were interpreted using a common standard. This method allows standardisation of results across institutions that use different susceptibility testing methods (e.g. disk diffusion, semi-automated testing systems) and varying breakpoints (e.g. CLSI, CDS or EUCAST) for determining susceptibility. MIC testing methodology has also revealed useful susceptibility information for newly introduced antibiotics (e.g. tigecycline) and for antibiotics where there are known limitations with commonly used testing methodologies (e.g. polymyxin B).

Because of the pre-defined isolate collection criteria, the

study strains are likely to be representative of the bacterial population encountered in each laboratory. However, not all isolates are likely to be associated with clinical infection, and isolates from commonly cultured sites would have formed the majority of our study isolates.

The susceptibility testing results confirm that certain mechanisms of antibiotic resistance are prevalent in Singapore. A third of the *Enterobacteriaceae* were resistant to extended-spectrum cephalosporins as a consequence of ESBL and/or AmpC production. The phenotypic methods used in our study are unable to differentiate between chromosomally and plasmid-borne *ampC* genes. However, data from other studies performed in Singapore suggest that plasmid-borne *ampC* is prevalent in both *E. coli* and *Klebsiella pneumoniae*.[13,14] The presence of these broad-spectrum cephalosporinases was also associated with co-resistance to other classes of antibiotics, including the very commonly prescribed ciprofloxacin, for which only 6 of every 10 *Enterobacteriaceae* isolates remain susceptible.

Antibiotic resistance was also noted in the non-fermenting gram-negative bacilli. Among *P. aeruginosa* isolates, 1 in 10 was resistant to multiple antibiotics. At least a third of *P. aeruginosa* isolates also demonstrated low-level resistance to polymyxin B although the clinical significance of such low-level resistance remains to be determined. Carbapenem resistance in *A. baumannii* was high, and for a significant proportion of isolates, the only active antibiotic was polymyxin B. Although tigecycline – a newly introduced glycylcycline antibiotic that has anecdotally been successfully used to treat infections with *Acinetobacter* spp[15] – has barely been used in Singapore, its activity against the multi-drug resistant *Acinetobacter* strains tested in this study was found to be marginal. Further surveillance for the activity of tigecycline against *Acinetobacter* spp. is warranted, especially given early data suggesting that antibiotic resistance may emerge during the course of therapy.[16,17]

The results from this study provide a snapshot of the antibiotic resistance profile of prevalent gram-negative bacilli in Singapore hospitals. Due to the limited sample size, uncommon forms of resistance could not be detected. For example, resistance to the carbapenems (imipenem and meropenem) was not detected in this study in *Enterobacteriaceae*, although such isolates have been reported.[8] For optimal surveillance of antibiotic resistance, detailed MIC testing should be combined with a broader laboratory-based surveillance programme. The MIC-based testing has revealed unusual susceptibility profiles for several antibiotics, such as polymyxin B and tigecycline, which warrant further investigation. The study has also provided a framework for the investigation of emerging resistance mechanisms. Continued surveillance programmes are a priority to track current and emerging antibiotic resistance trends, and to monitor the effectiveness of control measures.

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