

Antimicrobial Susceptibility of *Klebsiella pneumoniae* and *Escherichia coli* with Extended-Spectrum β -lactamase associated Genes in Hospital Tengku Ampuan Afzan, Kuantan, Pahang

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Abstract

Background: To assess antimicrobial susceptibility of extended-spectrum β -lactamase (ESBL-) producing *Klebsiella pneumoniae* and *Escherichia coli* isolates from Hospital Tengku Ampuan Afzan (HTAA), as well as to identify ESBL genes.

Methods: Non-duplicate *K. pneumoniae* and *E. coli* isolates were recovered from various clinical samples. Isolates were screened for antimicrobial resistance by disc diffusion method. Isolates resistant to oxyimino-cephalosporins were subjected to phenotypic ESBL production. Detection of resistance genes was then performed using primers specific for ESBL genes (*bla*_{CTX-M}, *bla*_{SHV} and *bla*_{TEM}).

Results: Piperacillin/tazobactam and carbapenems remained the active β -lactam antibiotic against *K. pneumoniae* and *E. coli*. ESBLs were detected among 35.5% (39/110) of *K. pneumoniae* and 18.8% (28/149) of *E. coli* isolates. CTX-M β -lactamase was detected in 90% of all ESBL-positive isolates, whereas *bla*_{SHV} and *bla*_{TEM} genes were found among 56% and 52% of them, respectively. Twenty-eight percent (28%) of the total ESBL-positive isolates harboured the three ESBL genes, while 50% carried two of the tested ESBL genes.

Conclusion: ESBLs encoded by at least one ESBL genes are frequently isolated among *K. pneumoniae* and *E. coli* in HTAA. The significant proportion rate of these resistant determinants is alarming, thus monitoring their transmission and dissemination is essential to control it at an early phase.

Keywords: beta-Lactamases, *Escherichia coli*, *Klebsiella pneumoniae*, antimicrobial resistance, hospital

Introduction

Over the past several decades, antibiotic-resistant *Enterobacteriaceae* have increased significantly, being reported worldwide and causing a real health issue that is expensive to treat. *Escherichia coli* and *Klebsiella pneumoniae* in particular are the most common pathogens associated with drug resistance and can exhibit resistance to multiple antibiotics and even to all currently known antibiotics (1,2). These pathogens, whose normal habitats are the intestinal tract of humans and animals, are frequently associated with serious nosocomial as well as community-acquired infections such as pneumonia, sepsis, urinary tract infections,

and several intra-abdominal infections (1–4). Genes encoding extended spectrum β -lactamases (ESBLs) are currently one of the most common drug resistance determinants (1,3).

ESBLs are heterogeneous enzymes characterised by their ability to hydrolyse almost all β -lactam antibiotics except carbapenems and cephamycins. They can be inhibited by β -lactamase inhibitors such as clavulanic acid and tazobactam (5,6). According to Bush and Jacoby's (7) classification system of β -lactamase, most ESBLs are assigned to subgroup 2be. ESBL resistance genes are plasmid-encoded genes generated by genetic mutations and modifications

of native *bla*_{SHV-1} and/or *bla*_{TEM-1} genes (8,9). These genes are commonly found with other resistance genes, causing a co-resistance profile inclusive of other non- β -lactam antibiotics such as aminoglycosides and tetracycline (10). More than 150 different variants of ESBLs among *Enterobacteriaceae* and other genera are the cause of several outbreaks worldwide (5,6,11). ESBL was initially detected in the 1980s among *K. pneumoniae* isolates in Europe and subsequently spread widely elsewhere. TEM-1, TEM-2 and SHV-1 derivatives were the most common ESBL types until the 1990s and have been associated with several outbreaks throughout the world. However, since the past decade the scenario has changed and CTX-M-type ESBL has increasingly been reported and is becoming the dominant ESBL-encoding gene, especially among *E. coli* isolates (12). In this cross-sectional study, we evaluated the antimicrobial susceptibility pattern of ESBL-producing *K. pneumoniae* and *E. coli* bacilli isolated from patients admitted to Hospital Tengku Ampuan Afzan (HTAA) and determined the genes that were associated with ESBL phenotype among the isolates.

Materials and Methods

Bacterial strains

Over a period from May to September, 2014, a total of 259 non-duplicate *E. coli* and *K. pneumoniae* isolates were serially included in this study. These isolates were recovered from various types of clinical specimens sent to the bacteriology laboratory in HTAA. HTAA is a tertiary hospital in Pahang and a referral for many district hospitals within Pahang as well as certain regions of southern Terengganu. The specimens were collected from both outpatients and inpatients. The isolates were derived from blood (n = 101), urine (n = 135), and swabs (n = 23), respectively. API20E (bioMérieux, France) along with conventional differential culture media were used to identify the isolates to the species level.

Antimicrobial susceptibility testing

Susceptibility testing was performed for all the identified clinical isolates by the standard Kirby-Bauer disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (13). ATCC 25922 *E. coli* was used as control strain and was run simultaneously with the test organisms. The antibiotics used included: amoxicillin/clavulanic acid (20/10 μ g), gentamycin (10 μ g), amikacin (30 μ g), ampicillin (10 μ g), ciprofloxacin (5 μ g), piperacillin (100

μ g), piperacillin/tazobactam (100/10 μ g), sulbactam/ampicillin (10/10 μ g), ceftazidime (30 μ g), cefotaxime (30 μ g), cefuroxime (30 μ g), cefepime (30 μ g), chloramphenicol (30 μ g), trimethoprim/sulfamethoxazole (25 μ g), imipenem (10 μ g), meropenem (10 μ g), ertapenem (10 μ g), and polymyxin B (300 μ g). All of the antibiotic discs were manufactured by Becton Dickinson (USA).

Phenotypic Detection of ESBL

Isolates that showed resistance to oxyiminocephalosporins (ceftazidime and/or cefotaxime) were considered as putative ESBL producers and were further subjected to ESBL phenotypic tests using combination disc diffusion as recommended by Clinical and Laboratory Standards Institute (CLSI) document M100-S21 (13). In this phenotypic test, discs (Becton Dickinson, USA) of ceftazidime (30 μ g) and cefotaxime (30 μ g) alone and with clavulanic acid (30/10 μ g) were used. An increase of >5 mm in the zone of inhibition for the combination of ceftazidime or cefotaxime/clavulanic acid disc versus the zone for the disc containing the drug alone was considered a confirmed ESBL producer. ATCC 25922 *E. coli* was used as non-ESBL-producing strain.

Molecular characterization of ESBL

DNA was obtained using a boiling method similar to that described by Mathers et al., (14) with some modifications. 1 mL of a fresh overnight bacterial broth was transferred to 1.5 mL Eppendorf tube and centrifuged for 3 minutes at 8000x g. The supernatant was removed and the cell pellet re-suspended in 300 μ L of sterile distilled water. The tubes were placed at 95 °C for 20 minutes and then centrifuged at 10000x g for 3 minutes. Finally, the supernatants were used as DNA template for polymerase chain reaction (PCR) amplification. Biophotometer (Eppendorf, Germany) was used to determine the purity and quality of extracted DNA.

PCR amplification and detection

Phenotypic ESBL-positive isolates were screened for SHV, TEM, CTX-M β -lactamases using conventional PCR amplification assay, based on published primer sequences by Wiegand et al. (15) (Table 1). PCR reactions were conducted using Taq polymerase (Top Taq® DNA polymerase, Qiagen, Germany) under conditions recommended by the manufacturer and 10 μ L of the boiled bacteria was used as template. *E. coli* ATCC 25922 was used as negative control. PCR cycling protocols were 5 minutes at 95 °C

followed by 35 cycles at 94 °C for 60 s, annealing temperature specific for each primer (Table 1) for 1 minute, and extension for 7 minutes at 72 °C. 10 µL of each PCR product was subsequently separated by electrophoresis in 1.5% agarose at 100 volts for 45 minutes. A 100 base pairs (bp) DNA ladder was used as molecular weight marker. Ethidium bromide (Merck Milipore, Germany) was used for DNA staining and finally visualised under ultraviolet light (Gel Doc XR System, Bio-Rad, California, USA).

Results

Isolates identification and antimicrobial susceptibility patterns

Standard identification methods using API 20E and conventional culturing methods revealed that 149 of the total isolates were *E. coli* and 110 *K. pneumoniae*. *E. coli* was commonly derived from urine (30.5%), followed by blood (22.7%), and swabs (4.2%). A similar pattern of the specimens' frequency for *K. pneumoniae* was observed, where 21.6%, 16.2%, and 4.6% were recovered from urine, blood and swabs, respectively. High resistance rates were found against ampicillin, piperacillin and trimethoprim-sulfamethoxazole, whereas carbapenems, amikacin and polymyxin B were demonstrated to be very effective antibiotics by in vitro tests, showing less than 10% susceptibility rates (Table 2). Overall, *K. pneumoniae* isolates were observed to have higher resistance rates than *E. coli* strains. Less than 5% of the *K. pneumoniae* isolates were resistant to carbapenems. Meanwhile, 100% of the *E. coli* isolates were susceptible to meropenam and imipenem, and only one isolate (0.7%) was resistant to ertapenem.

ESBL detection and characterization

ESBL was detected among 35.5% (39/110) *K. pneumoniae* and 18.8% (28/149) of *E.*

coli isolates. The majority 41/67 (61.2%) were recovered from urine followed by 26/67 (38.8%) from blood. Distribution of ESBL genes among isolates are shown in Table 3. Molecular characterisation revealed that 90.0% (45/50) of the total ESBL-producing isolates were blaCTX-M-positive (Table 3). The bla_{SHV} and bla_{TEM} genes were found in 56% and 52%, respectively, of the total ESBL-positive isolates. Twenty-eight percent (28%) (14/50) of the ESBL-positive strains were found carrying the three genes in the same isolate. Moreover, 50% of ESBL-positive strains carried two of the ESBL genes, bla_{CTX-M}+bla_{SHV} or bla_{CTX-M}+bla_{TEM} combination.

Discussion

This is a study on antibiotic resistance and ESBL genotyping of *K. pneumoniae* and *E. coli*, which are nosocomially and community-associated bacteria commonly harbouring ESBLs. Such study is lacking in the Eastern region of Malaysia. We found that carbapenems were still the most active antibiotics against *K. pneumoniae* and *E. coli*. Careful usage of this drug as the last-line antibiotic to cure infection caused by EBLs-producing Enterobacteriaceae is crucial in order to curb incidence of carbapenem resistance. However, low rates of carbapenem resistance were detected from this study, which makes it worthy of further investigation in the future. Other than carbapenems, piperacillin/tazobactam, amikacin, and polymyxin B also generally have high susceptibility rates in both species, even though *K. pneumoniae* showed increased resistance towards these drugs. A new class of antibiotic, glycyglycine, has been designed to reduce the development of antibiotic-resistant bacteria. Thus far, the only drug approved by the Food and Drug Administration under this class is tigecycline. Even though this drug is active against multidrug-resistant bacteria, it has not yet been introduced

Table 1: Base sequences of primers (Paterson, 2006) used for detection of bla genes.

Target gene	Sequence (5' → 3')	Amplicon size (bp)	Annealing Temperature used (°C)
bla _{CTX-M}	F-GCTTTGCGATGTGCAG	550	53
	R-ACCGCGATATCGTTGGT		
bla _{TEM}	F-ATTCTTGAAGACGAAAGGGCCTC	1050	55
	R-TTGGTCTGACAGTTACCAATGC		
bla _{SHV}	F-ATGCGTTATATTCGCCTGTG	400	54
	R-GTTAGCGTTGCCAGTGCTCG		

in HTAA due to the low rate of resistance towards carbapenems, amikacin and polymyxin B. Accordingly, no record on susceptibility testing against tigecycline was available.

ESBLs among *K. pneumoniae* and *E. coli* have been reported in almost every region throughout the world. In this study, *E. coli* were the major isolates collected from HTAA, and also

Table 2: Distribution of antimicrobial susceptibility of the *E. coli* and *K. pneumoniae* isolates to and non β -lactam antibiotics

Antimicrobial agents	<i>E. coli</i>			<i>K. pneumoniae</i>		
	n (%)					
β -lactams	S	I	R	S	I	R
<i>Ampicillin</i>	44 (29.5)	1 (0.7)	104 (69.8)	3 (2.7)	2 (1.8)	105 (95.5)
<i>Amoxicillin/Clavulanic Acid</i>	104 (69.8)	21 (14.1)	24 (16.1)	67 (60.9)	9 (8.2)	34 (30.9)
<i>Piperacillin</i>	86 (57.7)	2 (1.3)	61 (41.0)	50 (45.5)	12 (10.9)	48 (43.6)
<i>Piperacillin/Tazobactam</i>	134 (90.0)	2 (1.3)	13 (8.7)	76 (69.1)	5 (4.5)	29 (26.4)
<i>Sulbactam/Ampicillin</i>	106 (71.1)	8 (5.4)	35 (23.5)	72 (65.5)	2 (1.8)	36 (32.7)
<i>Cefotaxime</i>	116 (77.9)	0 (0.0)	33 (22.1)	70 (63.6)	1 (0.9)	39 (35.5)
<i>Ceftazidime</i>	126 (84.6)	0 (0.0)	23 (15.4)	71 (64.5)	1 (1.0)	38 (34.5)
<i>Cefepime</i>	128 (85.9)	5 (3.4)	16 (10.7)	77 (70.0)	2 (1.8)	31(28.2)
<i>Cefuroxime</i>	114 (76.5)	1 (0.7)	34 (22.8)	67 (60.9)	4 (3.6)	39 (35.5)
<i>Meropenem</i>	149 (100)	0 (0.0)	0 (0.0)	106 (96.4)	0 (0.0)	4 (3.6)
<i>Imipenem</i>	149 (100)	0 (0.0)	0 (0.0)	106 (96.4)	0 (0.0)	4 (3.6)
<i>Ertapenem</i>	148 (99.3)	0 (0.0)	1 (0.7)	105 (95.5)	0 (0.0)	5 (4.5)
Non β -lactams	S	I	R	S	I	R
<i>Amikacin</i>	149 (100)	0 (0.0)	0 (0.0)	106 (96.4)	0 (0.0)	4 (3.6)
<i>Ciprofloxacin</i>	123 (82.5)	4 (2.7)	22 (14.8)	78 (70.9)	10 (9.1)	22 (20.0)
<i>Gentamicin</i>	131 (87.9)	0 (0.0)	18 (12.1)	81 (73.6)	1 (0.9)	28 (25.5)
<i>Chloramphenicol</i>	122 (81.9)	1 (0.7)	26 (17.4)	86 (78.2)	0 (0.0)	24 (21.8)
<i>polymyxin B</i>	144 (96.6)	0 (0.0)	5 (3.4)	95 (86.4)	0 (0.0)	15 (13.6)
<i>Trimethoprim-Sulfamethoxazole</i>	79 (53.0)	1(0.7)	69 (46.3)	66 (60.0)	2 (1.8)	42 (38.2)

Table 3: The frequency of single and multiple ESBL-encoding genes among *K. pneumoniae* and *E. coli* isolates

Organism	Total ESBL genotype, n (%)	One ESBL gene			Two ESBL genes		Three ESBL gene
		<i>bla</i> _{CTX-M} , n (%)	<i>bla</i> _{TEM} , n (%)	<i>bla</i> _{SHV} , n (%)	<i>bla</i> _{CTX-M} + <i>bla</i> _{TEM} , n (%)	<i>bla</i> _{CTX-M} + <i>bla</i> _{SHV} , n (%)	<i>bla</i> _{CTX-M} + <i>bla</i> _{TEM} + <i>bla</i> _{SHV} , n (%)
<i>K. pneumoniae</i>	32 (29.0)	28 (87.5)	17 (53.1)	16 (50.0)	16 (50.0)	14 (43.8)	8 (25.0)
<i>E.coli</i>	18 (12.0)	17 (94.4)	9 (50.0)	12 (66.7)	9 (50.0)	11 (61.1)	6 (33.3)
Total	50 (19.3)	45 (90.0)	26 (52.0)	28 (56.0)	25 (50.0)	25 (50.0)	14 (28.0)

the most common aetiological agent for urinary tract infection. However, the frequency of ESBL-production by *K. pneumoniae* (35.5%; 39/110) was higher than that by *E. coli* (18.8%; 28/149). Most of the ESBL-producing *K. pneumoniae* were also derived from urine specimens. The high occurrence of ESBL among *K. pneumoniae* is perhaps due to the nature of its nosocomial presence and the survival of diverse replicons carried by the multidrug-resistant *K. pneumoniae* isolates (16). The mechanism of drug resistance has been associated with several chromosomal and plasmid-encoded genes (1,3). The ESBL prevalence shown in this study was relatively similar when compared to that reported in the National Surveillance of Antibiotic Resistance (NSAR) report for 2014 (17), which was lower than that documented in India, China, Thailand and Pakistan, in which the prevalence of ESBLs ranged between 47% and 70% for *K. pneumoniae* and 37% and 67% for *E. coli* (2)(18,19).

Since the past decade CTX-M-positive *K. pneumoniae* and *E. coli* have been reported as significant and the most prevalent ESBL producers throughout the world and particularly in several Asian countries (2) (18,19). In the present study, *bla*_{CTX-M} was the dominant gene detected among 90% of the total ESBL-positive isolates (Table 3). This finding is consistent with the known geographic distribution of CTX-M-type ESBL in the Asia-Pacific region, 97% in China, 95.4% in India, 93.1% in Malaysia, 87.1% in Singapore, and 85.4% in South Korea (16,19–21). The CTX-M-positive strain is not only found in a clinical setting but is also dominant among *Klebsiella* sp. and *Escherichia* sp. in the environment, such as on the urban water surface in Malaysia (22). Transmission of resistant organisms between environments is possible (1). The high rates of CTX-M β-lactamase production might be due to the high selective pressure imposed by widely used cephalosporins, particularly cefotaxime and ceftriaxone.

Our finding was consistent with other reports on ESBL genotype prevalence from Malaysian hospitals (16,23); however, it was in contrast to the previous report by Lim et al., (24) whereby they found that the *bla*_{SHV} gene was the predominant ESBL genotype among *K. pneumoniae* isolates. Changes in distribution of ESBL genes may vary, as differences in genotype prevalence have been noted not only between different countries but even between different institutions in the same region (18).

The high rates of ESBL-positive strains of *K. pneumoniae* and *E. coli* were also associated with

the presence of *bla*_{SHV} (56%) and *bla*_{TEM} (52%) genes (Table 3). This is in line with a study conducted in an Indian tertiary hospital, which revealed that *bla*_{SHV} and *bla*_{TEM} genes were detected among 60% and 56% of the above ESBL-positive isolates, respectively (25). Several ESBL-positive strains were detected carrying more than one ESBL-encoding gene. Three categories of isolates were noted; the first carried only one ESBL-encoding gene, the second carried two different genes and the third harboured three ESBL-encoding genes as summarised in Table 3. The presence of two ESBL gene types, *bla*_{CTX-M} + *bla*_{SHV} or *bla*_{CTX-M} + *bla*_{TEM} combination, was found among 50% of the total ESBL-positive isolates. The presence of a three-ESBL-gene combination in the same strain was found in 28% (14/50) of the total ESBL-positive strains. Similar findings were reported in a study conducted in Thailand (26). These enzymes are plasmid-encoded β-lactamases, which can be easily transferred among isolates and between species in nature. Further study on the plasmids characteristic of isolates carrying multiple *bla* genes would be worthwhile to understand their mechanism of resistance and dissemination.

Conclusion

In conclusion, several antimicrobial agents recommended for treatment of *K. pneumoniae* and *E. coli* infections were found to be effective in-vitro. Approximate prevalence and molecular characterisation of ESBL-producing *K. pneumoniae* and *E. coli* isolates from HTAA demonstrated a high rate of CTX-M-positive strains. The same strains may also harbour multiple ESBL genes. The significant proportion rate of these resistant determinants is alarming. Thus, early and regular surveillance of these determinants and their genotypic characterization is essential for monitoring their transmission and dissemination in order to halt it or control it at an early phase.

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Conflict of Interests:

None.

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Authors' Contributions

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Drafting of the article: SMYM, MMIA

Critical revision of the article for important intellectual content: HAH, MMIA

Final approval of the article: SMYM, HAH, MMIA, RB

Provision of study materials or patients, obtaining of funding: HAH

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