PREVALENCE OF EXTENDED-SPECTRUM BETA-LACTAMASES (ESBLs) PRODUCED IN BLOOD ISOLATES OF GRAM-NEGATIVE BACTERIA IN A TEACHING HOSPITAL IN SOUTHERN THAILAND

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Abstract. One hundred and eighty-three strains of gram-negative bacteria were isolated from 177 bacteremic patients during a 6-month period in 2004 at a teaching hospital in southern Thailand. Extended-spectrum beta-lactamases (ESBL) production was detected in *K. pneumoniae*, *E. coli*, and *E. cloacae*, at rates of 16/36 (44.4%), 3/59 (5.1%), and 2/13 (15.4%), respectively. All but one of the screened positive strains were also positive by both the combination disk method and the E-test. All of the *K. pneumoniae* strains that were resistant to ceftazidime by disk diffusion were demonstrated to be positive for ESBL production by both the combination disk and E-test methods. Most of the ESBL positive strains had a high MIC (more than 32 μg/ml) to ceftazidime. However, all the ESBL positive strains were sensitive to imipenem.

INTRODUCTION

Extended-spectrum beta-lactamases (ESBL) are a variant of the beta-lactamase enzyme. The enzyme has one position mutation in the gene at the active site that is believed to be the cause of high beta-lactamase activity. ESBL mediate resistance to all three generations of cephalosporins, including monobactams (*eg*, aztreonam) (Bradford, 2001; Sturenburg and Mack, 2003). ESBL are mostly reported in *Klebsiella pneumoniae* and *Escherichia coli*. However, they have also been found in other species of enterobacteriaceae, including other genera of gram-negative bacteria (Sturenburg and Mack, 2003). Most ESBL are encoded on a large plasmid that can be horizontally transferred to different genera of bacteria, which may be involved with both prevention and treatment aspects of nosocomial infections, particularly with septicemic patients (Bradford, 2001; Sturenburg and Mack, 2003). In addition, ESBL-producing *Escherichia coli* has been reported in a community-acquired bacteremic infection (Sorlozano *et al*, 2004). There is an increased need to detect ESBL-producing gram-negative bacteria in routine microbiological work. Rapid detection of ESBL is important, not only for treatment guidelines but also to facilitate improved prevention of nosocomial infections (Shah *et al*, 2004). ESBL can be detected using a standard screening test showing reduced susceptibility to five antibiotics, such as ceftazidime, ceftriazone, cefotaxime, aztreonam and cefpodoxime, as detected by standard disk diffusion and minimal inhibition concentration (MIC)(NCCLS,2000). Positive screening test strains are then further subjected to a phenotype confirmatory test that relies on the synergistic effect between an indicator cephalosporin and a β-lactamase inhibitor (usually clavulanic acid). Numerous confirmatory tests have been reported, however only a limit number are convenient for routine use, such as combined disk method, double disk (DD) approximation test, minimum inhibitory concentration (MIC) and E-test ESBL strip (Bradford,2001; Sturenburg and Mack, 2003). There is still some controversy concerning the appropriate ESBL detection method (Essack, 2000). The objective of this investigation was to compare the routine disk diffusion screening test and the confirmation test for the detection of ESBL production among blood isolates of gram-negative bacteria. The prevalence rates of ESBL production in bacteremic patients are also reported.

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MATERIALS AND METHODS

Baterial isolates

From January to June 2004, 183 gram-negative bacteria were isolated from the blood samples of 177 patients at Hat Yai Hospital, Songkhla Province, southern Thailand. Hat Yai Hospital is a 562 bed government hospital and Medical Education Center (MEC). The blood specimens were processed using an automatic machine (BacT-ALERT 3D). The positive samples were subcultured and identified using a standard protocol (Murray *et al*, 1999). The isolated gram-negative strains were kept on nutrient agar slant at 4ºC. *K. pneumoniae* American Type Culture Collection (ATCC) 700603 and *E.coli* ATCC 35218 were used as reference strains for positive and negative ESBL productions, respectively.

Screening by disc diffusion

The disc diffusion test was performed using the Kirby-Bauer disc diffusion method (Murray *et al*, 1999). The zone size was interpreted as ESBL positive according to the National Committee for Clinical Laboratory Standard guidelines (NCCLS, 2000). The type and potency of antimicrobial discs used were: ampicillin (AM,10 μg), amikacin (AN, 30 μg), amoxicillin/culvulanic acid (AMC, 20/10 μg), ceftazidime (CAZ, 30 μg), ciprofloxacin (CIP, 5 μg), ceftriaxone (CRO, 30 μg), cefepime (FEP, 30 μg), gentamicin (GM, 10 μg), imipenem (IPM, 10 μg), netilmicin (NET, 30 μg), cefoperazone/sulbactam (SCF, 30 μg), trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 μg), cefotaxime (CTX , 30 μg), aztreonam (ATM , 30 μg) and cefpodoxime (CPD, 10 μg). The discs were obtained from BBL.

Combination disc diffusion for ESBL production

A phenotype confirmation test of ESBl production was done with all of the isolated gram-negative bacteria according to NCCLS recommendations (NCCLS, 2000). Briefly, the overnight growth in BHI broth of gram-negative bacteria was adjusted to 0.5 Mcfarland standard. The bacterial suspension was spreaded on Mueller-Hinton agar (Difco) using a sterilized cotton swab. After incubation at room temperature for 15 minutes, 6 discs were put on the plates: ceftazidime (30 μg), ceftazidime/clavulanic acid (30/10 μg), cefotaxime (30 μg), cefotaxime/clavulanic acid (30/10), cefpodoxime (10 μg), and cefpodoxime/clavulanic acid (10/1 μg). An increase in zone size ≥5 mm in the disc containing clavulanic acid versus its zone size when tested alone was accepted as confirmation of ESBL production.

E-test

E-test ESBL screen was obtained from AB biodisk (Solna, Sweden). (TZ/TZL). The strip contains a ceftazidime (TZ) gradient (0.5-32 μg/ml) at one end and a ceftazidime plus clavulanate gradient (TZL)(0.064-4 μg/ml plus 4 μg/ml of clavulanic acid) on the other end (Cormican *et al*, 1996). Susceptibility testing was performed on Mueller-Hinton agar by the E-test in accordance with the manufacturer's instructions. Briefly, after overnight growth on brain hearth infusion agar, the organisms were resuspended in saline solution to a turbidity of 0.5 Mcfarland standard. The suspension was then used to inoculate a Mueller-Hinton agar plate by swabbing it with a cotton swab. After the plates dried for 15 minutes, the E-test strips were placed on them and they were incubated for 18 hours at 35ºC. The MIC was interpreted as the point of intersection of the inhibition ellipse with the edge of the test strip. The results were interpreted as ESBL positive if the MIC ratio for TZ/TZL was ≥8 and non-determinable if the TZ MIC was <32 μg/ml and TZL was >4 μg/ml.

RESULTS

A total of 183 strains of gram-negative bacilli were isolated from 177 bacteremic patients. The gram-negative bacterium most commonly isolated was *Escherichia coli* (59), followed by *K. pneumoniae* (36), *Acinetobacter* spp (25), *Salmonella enterica* (18), *E.cloacae* (13), *Pseudomonas aeruginosa* (10), *Burkholderia pseudomallei* (6), *Moraxella* spp (3), *Pseudomonas* spp (3) *E.aerogenes* (2), *E. agglomerans* (1) *Proteus mirabilis* (1), *P. vulgaris* (1), *K. ozaenae* (1), *V. cholerae* non-O1, non O139 (1) , *V. minicus* (1), *Aeromonas* spp (1) and *P. stuzeri* (1). Only *K. pneumoniae*, *E. coli* and *E. cloacae* strains were demonstrated to be ESBL positive by screening disk diffusion (Table 1). ESBL was detected in *K. pneumoniae*, *E. coli* and *E. cloacae* as follows: 16/36 (44.4%), 3/59 (5.1%), and

Table 1
Results of the screening disk diffusion, combination disk and E-test of ESBL positive strains.

ESBL positive strains	Screening disk diffusion					Combination disk			E-test			
	CAZ	CRO	CTX	ATM	CPD	CAZ/CL	CTX/CL	CPD/CL	MIC (μ/ml) of CAZ	MIC (μg/ml) of CAZ/CL	MIC ratio	Test score
1. *K. pneumoniae*	$(12^{+})^{a}$	(15^{+})	(17^{+})	(17^{+})	(12^{+})	+	+	+	>32	0.38	>8	+
2. *K. pneunoniae*	(6^{+})	(6^{+})	(6^{+})	(6^{+})	(6^{+})	+	+	+	>32	1.5	>8	+
3. *K. pneumoniae*	(6^{+})	(13^{+})	(6^{+})	(6^{+})	(6^{+})	+	+	+	>32	2	>8	+
4. *K. pneumoniae*	(6^{+})	(18^{+})	(18^{+})	(6^{+})	(6^{+})	+	+	+	>32	1.5	>8	+
5. *E. coli*	(22^{+})	(6^{+})	(6^{+})	(20^{+})	(6^{+})	+	+	+	6	0.38	>8	+
6. *E. cloacae*	(6^{+})	(11^{+})	(13^{+})	(6^{+})	(6^{+})	+	+	+	>32	0.38	>8	+
7. *K. pneumoniae*	(6^{+})	(11^{+})	(18^{+})	(6^{+})	(6^{+})	+	+	+	>32	0.75	>8	+
8. *K. pneumoniae*	(6^{+})	(6^{+})	(6^{+})	(6^{+})	(6^{+})	+	+	+	>32	1.5	>8	+
9. *K. pneumoniae*	(6^{+})	(6^{+})	(6^{+})	(6^{+})	(6^{+})	+	+	+	>32	1.5	>8	+
10. *E. coli*	(6^{+})	(17^{+})	(6^{+})	(6^{+})	(6^{+})	+	+	+	>32	0.38	>8	+
11. *K. pneumoniae*	(12^{+})	(6^{+})	(6^{+})	(6^{+})	(6^{+})	+	+	+	>32	0.5	>8	+
12. *E. coli*	(12^{+})	(20^{+})	(18^{+})	(18^{+})	(6^{+})	+	+	+	>32	0.38	>8	+
13. *K. pneumoniae*	(6^{+})	(13^{+})	(14^{+})	(6^{+})	(6^{+})	+	+	+	>32	1.5	>8	+
14. *K. pneumoniae*	(6^{+})	(6^{+})	(6^{+})	(6^{+})	(6^{+})	+	+	+	>32	0.75	>8	+
15. *K. pneumoniae*	(13^{+})	(6^{+})	(6^{+})	(6^{+})	(6^{+})	+	+	+	>32	1.5	>8	+
16. *K. pneumoniae*	(6^{+})	(6^{+})	(6^{+})	(6^{+})	(6^{+})	+	+	+	>32	1.5	>8	+
17. *K. pneumoniae*	(6^{+})	(6^{+})	(6^{+})	(6^{+})	(6^{+})	+	+	+	>32	1.5	>8	+
18. *K. pneumoniae*	(6^{+})	(6^{+})	(6^{+})	(6^{+})	(6^{+})	+	+	+	>32	1.5	>8	+
19. *E. cloacae*	(6^{+})	(19^{+})	(6^{+})	(6^{+})	(6^{+})	+	+	+	>32	0.38	>8	+
20. *K. pneumoniae*	(6^{+})	(6^{+})	(6^{+})	(6^{+})	(6^{+})	+	+	+	>32	1.5	>8	+
21. *K. pneumoniae*	(6^{+})	(6^{+})	(6^{+})	(6^{+})	(6^{+})	+	+	+	>32	1.5	>8	+
E. coli ATCC 35218	(28^{-})	(28^{-})	(29^{-})	(30^{-})	(26^{-})	-	-	-	<1	0.25	<8	-
K. pneumoniae ATCC 700603	(12^{+})	(19^{+})	(19^{+})	(19^{+})	(10^{+})	+	+	+	>32	0.75	>8	+

a: zone size diameter in mm. ATM,aztreonam; CAZ, ceftazidime; CPD, cefpodoxime; CRO, ceftriaxone; CTX, cefotaxime; CAZ/CL, ceftazidime/clavulanic; CTX/CL, cefotaxime/clavulanic; CPD/CL, cefpodoxime/clavulanic

2/13 (15.4%), respectively. All *K. pneumoniae* strains that were resistant to ceftazidime were demonstrated to be positive for ESBL production by both the combination test and the E-test. All of the combination disk methods gave the same results as the E-test. There was one strain of *E. cloacae* that was positive with the disk screening method but negative with the combination disk method and the E-test. All but one of the ESBL positive strains had a high MIC (>32 μg/ml) to ceftazidime (Table 1). The antimicrobial resistance patterns of all blood isolates of *K. pneumoniae*, *E. coli* and *E. cloacae* are shown in Fig 1. These ESBL positive strains showed a high degree of resistance to ampicillin (85.7%), cefotaxime (66.7%), gentamicin (71.4%), and cotrimoxazole (85.7%). All of the ESBL producers were sensitive to imipenem.

DISCUSSION

Nosocomial septicemic infection continues to be a public health problem. ESBL production by *K. pneumoniae* was reported in a bacteremic patient (Kim *et al*, 2002). Although *E. coli* strains have been isolated in the highest numbers in bacteremic patients, the highest percentage of ESBL production was found in *K. pneumoniae* (Xiong *et al*, 2002; Jain *et al*, 2003; Chiew, 2004; Rahman *et al*, 2004). Screening disk diffusion has proven to be a useful method for the detection of ESBL production, particularly in *K.*

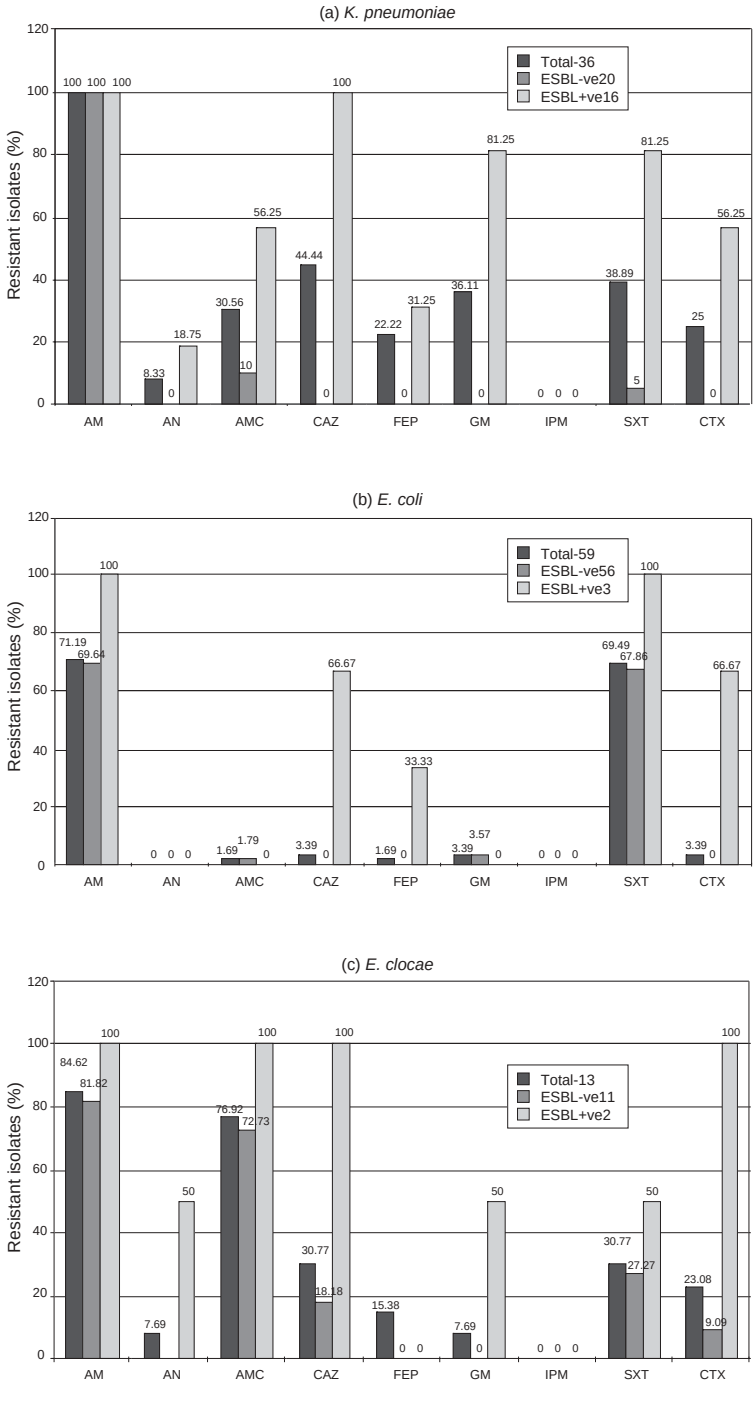

Fig 1–Antimicrobial-resistance patterns of blood isolates of : (a) *Klebsiella pneumoniae*; (b) *Escherichia coli*; (c) *Enterobacter cloacae*. AM, ampicillin; AN, amikacin; AMC, amoxicillin/clavulanic acid; CAZ, ceftazidime; FEP, cefepime; GM, gentamicin; IPM, imipenem; SXT, trimethroprim/sulfamethoxazole; CTX, cefotaxime.

pneumoniae. Cefodoxime has been reported to be the most sensitive screening indicator for ESBL production (Shah *et al*, 2004). In our study, all five screening disks resulted in the same number. All the cases of ceftazidime resistance in *K. pneumoniae* isolated from blood cultures were confirmed to be due to ESBL production. The results alert the microbiologist to perform the confirmation test with the suspected organism. However, there are a wide range of substrates for ESBL. It is important that all five screening disks are included in the screening program. The NCCLS guideline has been shown to work very well (Tenover *et al*, 2003). In our study, there was one strain of *E. coli* that had an inhibition zone of 22 mm (Table 1). This strain could be misread as sensitive to ceftazidime if the investigating microbiologist did not follow carefully the NCCLS guidelines with the confirmation tests to validate ESBL production. However, some false negatives have also been reported, particularly with strains that produce AmpC-like β-lactamase (Coudron *et al*, 2003). The loss of an outer membrane protein combined with co-existing TEM-1 and SHV-1 β-lactamases has been reported to give a false identification of ESBL-producing *K. pneumoniae* (Wu *et al*, 2001). In our study, there was one isolate of *Enterobacter cloacae* that was positive with the screening disk but negative with both the combination disk and E-test phenotype tests. The new cefepime-clavulanate ESBL or combination disk may be included in the confirmatory test to solve this problem (Sturenburg *et al*, 2003). Some researchers now

feel that the tests for ESBL production in gram-negative bacteria other than *K. pneumoniae* and *E. coli* should be revised in the NCCLS guidelines, as the organisms in this group usually produce more than one type of β-lactamase. Molecular techniques, such as PCR and isoelectric focusing, should be used to confirm ESBL production in this group of bacteria (Schwaber *et al*, 2004)

In conclusion, our investigation has demonstrated how important it is for clinical microbiology laboratories to have the ability to detect and report on ESBL production in clinical isolates of gram-negative bacteria. Routine disk diffusion should be carefully monitored for ESBL production, particulary with *K. pneumoniae* and *E. coli* blood isolates.

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