J Antimicrob Chemother 2010; **65**: 465–469
doi:10.1093/jac/dkp471 Advance publication 6 January 2010

Journal of Antimicrobial Chemotherapy

Molecular characterization of extended-spectrum β-lactamases in clinical *Escherichia coli* and *Klebsiella pneumoniae* isolates from Surabaya, Indonesia

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Received 28 May 2009; returned 17 June 2009; revised 19 November 2009; accepted 1 December 2009

Background: No detailed reports regarding extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae are currently available from Indonesia, the fourth most populous country in the world.

Methods: A survey was carried out to investigate the molecular epidemiology and genetic characteristics of clinical ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates originating from the Dr. Soetomo Academic Hospital in Surabaya, Indonesia, over a 4 month period (January to April 2005). ESBLs were characterized by isoelectric focusing and PCR assays. Clonality of the isolates was assessed by PFGE and repetitive-sequence-based PCR (rep-PCR). Phylogenetic grouping was performed among CTX-M-15-producing *E. coli*.

Results: In total, 73 consecutive non-duplicate ESBL-positive *E. coli* and 72 *K. pneumoniae* strains were isolated. The $bla_{\text{CTX-M-15}}$ gene was found to be highly prevalent (69/73 strains, 94.5%) among the 73 ESBL-positive *E. coli* isolates. The gene was detected in both clonal and non-clonal isolates, as defined by PFGE and rep-PCR. Sixteen CTX-M-15-positive *E. coli* could be assigned to a single rep-PCR type and phylogenetic group B2 and belonged to the well-known O25b-ST131 clone. Among the 72 ESBL-positive *K. pneumoniae* isolates, $bla_{\text{CTX-M-15}}$ was again the most prevalent ESBL (40/72, 55.6%). Several SHV-type enzymes were also frequently detected: SHV-5 ($n=28$); SHV-12 ($n=13$); and SHV-2 ($n=6$). TEM-type ESBLs were not detected in any of the isolates.

Conclusions: Indonesia is another developing country affected by the emergence and spread of bacterial strains harbouring ESBL genes, including the CTX-M-15-producing B2-*E. coli* O25b-ST131 clone.

Keywords: antibiotic resistance, CTX-M-15, Asia, O25b-ST131, PFGE

Introduction

The number of extended-spectrum β-lactamase (ESBL) variants has increased rapidly since the first description of a plasmid-encoded ESBL was published in 1983, with current evidence now indicating worldwide dissemination. In particular, the spread of the highly virulent CTX-M-15-producing B2-*Escherichia coli* O25b-ST131 clone is of great concern.[1] Unfortunately, limited data are currently available on the prevalence of different ESBL types within the Southeast Asian region of the world,[2] with the molecular characterization of clinical ESBL-producing bacterial isolates having been described in isolates

from Malaysia, Singapore, Thailand and Vietnam.[2,3] However, no detailed reports of ESBL-producing Enterobacteriaceae from Indonesia, the world's fourth most populous country, have been published.

Therefore, the present study was set up to investigate the molecular epidemiology and genetic characteristics of clinical ESBL-positive *E. coli* and *Klebsiella pneumoniae* isolates obtained from the 1432 bed Dr. Soetomo Academic Hospital in Surabaya, Indonesia.

Materials and methods

Bacterial isolates

Between January 2005 and April 2005, 1910 clinical specimens (comprising 767 urine specimens, 548 blood cultures, 355 wound specimens, 137 sputum specimens, 85 stool specimens and 18 CSF specimens) were received and cultured by the microbiology laboratory of the Dr. Soetomo Academic Hospital in Surabaya, Indonesia. All non-duplicate isolates of *E. coli* and *K. pneumoniae* were screened for ESBL production using ceftazidime and cefotaxime disc diffusion testing, as recommended by the CLSI.[4] Phenotypic confirmation of ESBL production was performed using disc diffusion with discs of ceftazidime with and without clavulanic acid (Oxoid, Basingstoke, UK). Preliminary identification was performed using the Microbact™ System (Medvet diagnostics, Thebarton, Adelaide, Australia), whilst the identification of isolates that were characterized by molecular methods was repeated using the VITEK®2 system (bioMérieux, Marcy-l'Étoile, France). In the case of an inconclusive VITEK®2 result, a definitive API 20E test (bioMérieux) was performed.

Antimicrobial susceptibility testing

Antimicrobial susceptibility to additional antibiotics was determined using the VITEK®2 system (card AST-N041).

β-Lactamase characterization

The expression of β-lactamases was detected by isoelectric focusing (IEF) using the PhastSystem apparatus (Pharmacia AB, Uppsala, Sweden).[5] The presence of bla_{TEM} and bla_{SHV} was detected by PCR according to previously published methods.[6,7] A multiplex PCR assay was used to detect $bla_{CTX\text{-}M}$ genes.[8] Products resulting from amplifications were subjected to sequencing using a 3100 ABI Prism Genetic Analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Molecular epidemiology

Clonal relatedness of all ESBL-positive isolates was established using PFGE.[5] Genotypic relatedness was evaluated using Bionumerics software (version 3.0; Applied Maths, Ghent, Belgium), after reference to a dendrogram generated using the Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA) algorithms. Isolates with >80% similarity were considered similar. CTX-M-15-positive *E. coli* were further analysed using the repetitive-sequence-based PCR (rep-PCR) DiversiLab™ Microbial Typing System® (bioMérieux) according to the manufacturer's instructions. Using this technique, isolates with >95% identical profiles were considered to be closely related.

Phylogenetic grouping and detection of O25b-ST131 isolates

CTX-M-15-positive *E. coli* were assigned to one of the four main *E. coli* phylogenetic groups by PCR.[9] Any CTX-M-15-positive *E. coli* that belonged to phylogenetic group B2 was investigated using a multiplex PCR for the presence of the O25b-ST131 clone.[10]

Results

Bacterial isolates

Among the 403 *E. coli* and 291 *K. pneumoniae* that were consecutively isolated, 73 *E. coli* and 69 *K. pneumoniae* were ESBL positive using confirmatory disc diffusion and molecular characterization (Table 1). Three additional *K. pneumoniae* isolates showed a typical ESBL phenotype using disc diffusion, VITEK®2 and additional Etests (bioMérieux), and were thus considered 'truly' ESBL positive. However, we did not succeed in characterizing the genes responsible in these isolates.

The majority of ESBL-positive *E. coli* isolates were recovered from urine (67.1%), with decreasing isolation rates from wound specimens (12.3%), stool samples (12.3%), sputum (5.5%) and blood (2.7%). ESBL-positive *K. pneumoniae* were isolated from urine (62.5%), blood (16.7%), wounds (12.5%), sputum (4.2%), stool samples (2.8%) and CSF (1.4%).

ESBL characterization

CTX-M-15 was the most widespread ESBL found in both *E. coli* (69/73 strains, 94.5%) and *K. pneumoniae* (40/72 strains, 55.6%) (Table 1). A bla_{SHV}-type ESBL gene was detected in seven *E. coli* strains (9.6%), three of which also carried a $bla_{CTX\text{-}M}$ gene. Among *K. pneumoniae*, SHV-type ESBLs were also frequently detected (47/72, 65.3%), comprising SHV-5 ($n=28$), SHV-12 ($n=13$) and SHV-2 ($n=6$). For two of the three isolates with an uncharacterized ESBL, a β-lactamase enzyme in the CTX-M pI range was observed by IEF. The third isolate did not, however, show any banding pattern after IEF and was negative in all other molecular techniques. TEM-type ESBLs were not detected in any of the isolates.

Antimicrobial susceptibility

All of the ESBL-producing isolates were susceptible to imipenem (data not shown), though resistance to trimethoprim/sulfamethoxazole, gentamicin, tetracycline and ciprofloxacin was observed (Table 1).

Molecular epidemiology

PFGE revealed extensive genomic heterogeneity among both ESBL-producing *E. coli* and *K. pneumoniae* isolates [Figures S1 and S2, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. The majority of the 42 *E. coli* PFGE types represented unique isolates (31/42, 73.8%), though PFGE type 22 was associated with seven isolates, and types 17 and 19 with four isolates each. It was therefore concluded that limited clonality existed among the *E. coli* isolates. As 10 of the CTX-M-15-positive isolates revealed band-smearing patterns and were thus non-typeable by PFGE, additional rep-PCR typing

Table 1. Occurrence of *bla* types among, and antimicrobial susceptibility results of, 73 ESBL-producing *E. coli* and 72 ESBL-producing *K. pneumoniae* consecutively isolated from patients admitted to the Dr. Soetomo Academic Hospital in Surabaya, Indonesia

		No. of isolates in combination with		Percentage resistant to[a]				
Type of ESBLs	No. of isolates (%)	TEM-1	SHV-1	GEN	AMK	CIP	SXT	TET
E. coli								
CTX-M-15	66[b] (90.4)	35	0	72.7	7.6	80.3	57.6	84.8
SHV-5	3[c] (4.1)	2	–	100	33.3	0	100	100
SHV-5 + CTX-M-15	2[d] (2.7)	1	–	100	100	50.0	100	100
SHV-12 + CTX-M-15	1 (1.4)	0	–	0	0	100	100	100
SHV-12	1 (1.4)	1	–	100	100	100	100	0
total	73 (100)	39	0	74.0	12.3	76.7	61.6	84.9
K. pneumoniae								
CTX-M-15	21[e] (29.2)	5	18	85.7	9.5	81.0	76.2	76.2
SHV-5	20[f] (27.8)	3	–	75.0	0	20.0	90.0	70.0
CTX-M-15 + SHV type[g]	19[h] (26.4)	4	–	78.9	52.6	68.4	94.7	73.7
SHV-2	4[i] (5.6)	1	–	0	25.0	75.0	75.0	50.0
SHV-12	3[j] (4.2)	1	–	66.7	33.3	66.7	66.7	66.7
miscellaneous[k]	2 (2.8)	0	1	50.0	0	0	100	50.0
uncharacterized[l]	3 (4.2)	1	0	33.3	0	0	0	0
total	72 (100)	15	19	72.2	19.4	54.2	81.9	68.1

AMK, amikacin; CIP, ciprofloxacin; GEN, gentamicin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline.
[a]Rates of resistance include resistant as well as intermediately susceptible isolates.
[b]PFGE: 36 types + 10 non-typeable isolates; rep-PCR: 23 types.
[c]PFGE: 3 types.
[d]PFGE: 1 type + 1 non-typeable isolate; rep-PCR: 2 types.
[e]PFGE: 18 types + 2 non-typeable isolates.
[f]PFGE: 16 types.
[g]bla_{SHV} types found in combination with CTX-M-15 were: SHV-12 ($n=10$); SHV-5 ($n=7$); and SHV-2 ($n=2$).
[h]PFGE: 14 types.
[i]PFGE: 4 types.
[j]PFGE: 3 types.
[k]Other (combinations of) ESBL genes included CTX-M-9 ($n=1$) and CTX-M-9 + SHV-5 ($n=1$). PFGE types: 2 types.
[l]bla_{SHV-11} was detected in two isolates. This is a broad-spectrum β-lactamase, but not an ESBL.

was performed (for all 69 CTX-M-15-positive strains). Using this technique, a total of 25 rep-PCR profiles were observed, designated rep-PCR types A–Y [Figure S3, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. Of these rep-PCR types, type G was the most prevalent ($n=16$), containing all of the seven previous PFGE type 22 isolates, as well as nine additional isolates. rep-PCR type D ($n=7$) contained all PFGE type 17 isolates and three additional isolates. Two CTX-M-15-positive isolates recovered from blood were found to be identical (PFGE type 17; rep-PCR type D). Genotypically similar isolates were not restricted to specific locations within the Dr. Soetomo Academic Hospital (Figure S3). rep-PCR typing and PFGE were concordant, with PFGE genotyping being the method exhibiting the highest resolution.

K. pneumoniae isolates were found to be more genotypically diverse than *E. coli* isolates, with a total of 55 PFGE types being identified, 45 of which (81.8%) represented unique isolates (three isolates were non-typeable) (Figure S2). The largest genotypic cluster comprised only four CTX-M-15-positive isolates (PFGE type 9).

Phylogenetic groups and detection of O25b-ST131

The phylogenetic group of each isolate was determined for all except one CTX-M-15-positive *E. coli*, with isolates belonging to phylogenetic groups B2 (36.8%), A (33.8%), D (27.9%) and B1 (1.5%). All 16 rep-PCR type G isolates could be assigned to phylogenetic group B2 and belonged to the O25b-ST131 clone.

Discussion

Overall, the epidemiology of clinical ESBL-producing *E. coli* in Surabaya in 2005 is consistent with the current worldwide situation, with the CTX-M types being more prevalent than the classic TEM and SHV variants. In this study, the ESBL-positive *E. coli* isolates encoded mainly the CTX-M-15 enzyme (94.5%), with some clonality between isolates as demonstrated by PFGE and rep-PCR. This finding suggests that the clonal spread of epidemic strains as well as the transfer of genetic elements between non-related strains have contributed to the spread of CTX-M-15-positive *E. coli* in Surabaya. On a worldwide basis,

it is not yet known whether the extensive dissemination of CTX-M-positive *E. coli* isolates has occurred via the spread of a highly virulent clone or via antimicrobial resistance gene transfer between non-related isolates. Interestingly, previous reports have shown heterogeneity between nosocomial CTX-M-positive *E. coli* isolates,[3] though more recent publications have described the spread of a highly virulent CTX-M-15-positive, ciprofloxacin-resistant, B2-*E. coli* O25b-ST131 clone.[1,10] Further, Pitout *et al.*[1] recently demonstrated that the DiversiLab™ system could successfully identify this multidrug-resistant *E. coli* ST131 clone. In the present study, 25 out of 68 (36.8%) CTX-M-15-positive *E. coli* from Surabaya belonged to phylogenetic group B2, with 16 of these isolates exhibiting identical rep-PCR profiles (type G) and belonging to the O25b-ST131 clone.

CTX-M-15 was also the most prevalent bla_{ESBL} present in *K. pneumoniae* (55.6%), though, unlike *E. coli*, there appeared to be a lesser amount of genotypic clonality among the CTX-M-positive isolates. These findings suggest that the spread of CTX-M-15 among *K. pneumoniae* is mainly driven by the transfer of genetic elements that may have been acquired from *E. coli* (though verification of this hypothesis requires further research). It has been suggested that the insertion sequence (IS) element IS*Ecp*1 may play a role in the transfer of the CTX-M-1 group genes among Enterobacteriaceae. This element is present in the majority of CTX-M-15-positive isolates from Thailand and may also play a role in Indonesia.[3]

Not only CTX-M genes, but also SHV-type ESBL genes were prevalent (65.3%) among *K. pneumoniae* isolates in this study. Given the extensive genetic heterogeneity observed between isolates, patient to patient transmission of strains is unlikely, meaning that transfer of genetic elements or *de novo* mutation and selection are more probable in the distribution of SHV types. Of note, CTX-M-14, one of the dominant CTX-M types in most Asian countries,[2] was not found in our survey. A limitation of our study, however, is that confirmatory testing was performed using only ceftazidime with and without clavulanic acid. Therefore, we could have missed some ESBL strains, especially those with CTX-M β-lactamases, since these enzymes are much more active against cefotaxime as a substrate than against ceftazidime.

In conclusion, we have shown that Indonesia is another (developing) country situated in the Southeast Asian region that is affected by the spread of bacterial isolates harbouring ESBL genes within the clinical environment.

Acknowledgements

We thank Liset Dullaart, Jolanda Maaskant, Willemien Zandijk, Liesbeth van der Zwaan, Tsz Shan Chan, Maria van Dullemen and Deborah Horst-Kreft for excellent technical assistance. We also thank Prof. Dr E. Denamur for providing a positive control strain for the O25b-ST131 PCR.

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Dr. Soetomo Academic Hospital–School of Medicine, Airlangga University, Surabaya, Indonesia: Prof. Widjoseno Gardjito, MD; Erni P. Kolopaking, MPPM; Prof. Karjadi Wirjoatmodjo, MD; Prof. Djoko Roeshadi, MD PhD; Prof. Eddy Suwandojo, MD; Prof. Eddy Rahardjo, MD PhD; Prof. Ismoedijanto MD PhD; Prof. Paul Tahalele, MD PhD; Prof. Hendromartono, MD PhD; Hari Parathon, MD; Usman Hadi, MD; Nun Zairina, HospPharm; Mariyatul Qibtiyah, HospPharm; Endang Isbandiati, MD PhD; Kartuti Deborah, MD; Prof. K. Kuntaman, MD PhD; Prof. Ni Made Mertaniasih, MD PhD; Marijam Purwanta, MSc; Lindawati Alimsardjono, MD; Maria Inge Lusida, MD PhD.

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Radboud University Medical Center, Nijmegen, The Netherlands: Monique Keuter, MD PhD.

Funding

This work was facilitated by RUTI grant number RUTI II–KMRT 2003 from the Ministry of Research and Technology, Jakarta, Republic of Indonesia, and by grant number 99-MED-3 from the Royal Netherlands Academy of Sciences and Arts in the framework of its Scientific Program Indonesia-Netherlands (SPIN), Amsterdam, The Netherlands.

Transparency declarations

No conflicts of interest to declare.

Supplementary data

Figures S1, S2 and S3 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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