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Faecal carriage of extended-spectrum β-lactamase-producing Enterobacteriaceae among humans in Java, Indonesia, in 2001–2002

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Abstract

OBJECTIVE To characterise commensal *Escherichia coli* and other Enterobacteriaceae with reduced susceptibility to cefotaxime that were collected in a large survey carried out among 3995 patients and healthy persons in two urban regions on Java, Indonesia, in 2001–2002.

METHODS The putative extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae were analysed using double-disk synergy tests, isoelectric focusing, PCR assays, DNA sequencing, and pulsed-field gel electrophoresis (PFGE).

RESULTS On the day of discharge after five or more days of hospitalisation, at least 95 of 999 (9.5%) patients carried ESBL-positive Enterobacteriaceae as dominant faecal flora. Six patients were simultaneously colonised with *E. coli* and *Klebsiella pneumoniae* isolates with ESBL activity. On admission, only 6 of 998 (0.6%) patients were colonised. Faecal carriage of ESBL-producing Enterobacteriaceae among healthy persons or persons visiting a public health centre was not detected. The 107 ESBL-positive strains included 68 *E. coli*, 35 *K. pneumoniae*, and four other Enterobacteriaceae. $bla_{\text{CTX-M-15}}$ was the most prevalent ESBL in both *E. coli* (47.1%) and *K. pneumoniae* (45.7%), but the *E. coli* O25b-ST131 clone was virtually absent. Other ESBL types found were: SHV-2, -2a, -5, -12, CTX-M-3, -9, -14, and TEM-19. PFGE revealed extensive genetic diversity among the isolates.

CONCLUSIONS In 2001–2002, faecal carriage of ESBL-producing Enterobacteriaceae as dominant flora in Indonesia was almost exclusively hospital-associated. The presence of various bla_{ESBL} genes and the extensive genetic diversity among isolates argue against a single/dominant strain outbreak.

keywords Asia, antibiotic resistance, colonisation, ESBL, CTX-M-15

Introduction

In the past two decades, extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae have increasingly been reported worldwide, causing outbreaks as well as sporadic infections (Paterson & Bonomo 2005). The human intestinal tract provides an important reservoir for ESBL-producing bacteria and colonised persons are at risk

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for subsequent infection (Peña *et al.* 1998; Ben-Ami *et al.* 2006; Mesa *et al.* 2006). In the hospital, patient-to-patient transmission of these bacteria may occur (Ben-Ami *et al.* 2006; Harris *et al.* 2007; Kola *et al.* 2007). Therefore, it is necessary to know the epidemiology of ESBLs among patients on admission, during hospitalisation and at discharge, in order to be able to promote effective control practices.

Hospitals in Indonesia are also affected by the spread of ESBL-genes. In a survey at the Dr. Soetomo Academic Hospital in Surabaya, Indonesia, in 2005, $bla_{\text{CTX-M-15}}$ was present in clonal and non-clonal clinical *Escherichia coli* strains, while different SHV- and CTX-M-type ESBLs co-resided in *Klebsiella pneumoniae* strains (Severin *et al.* 2010). In 2001–2002, the 'Antimicrobial Resistance in Indonesia: Prevalence and Prevention' (AMRIN) study group investigated rectal carriage of resistant bacteria among 3995 people in two cities on the island of Java (Surabaya and Semarang) (Lestari *et al.* 2008). *E. coli* strains resistant to third-generation cephalosporins were found to be highly prevalent in the faecal flora of patients at discharge after a hospital stay of five or more days (12.5%), but not among healthy relatives or patients visiting primary health centres (0.7% and 0.9%, respectively). However, the presence, type, and expression of ESBLs were not studied. The aim of the present analysis was to further characterise these commensal cefotaxime-resistant *E. coli* and also other Enterobacteriaceae with reduced susceptibility to cefotaxime that were collected during the AMRIN study.

Materials and methods

Study setting

The AMRIN study was carried out in two governmental teaching hospitals (Dr. Soetomo Academic Hospital in Surabaya, Dr. Kariadi Academic Hospital in Semarang) and three primary health centres (two in Surabaya and one in Semarang). A total of 3995 individuals were studied for intestinal carriage of resistant microorganisms using rectal swab cultures (Kuntaman *et al.* 2005; Lestari *et al.* 2008). These individuals were patients on the day of admission to the hospital (admission group, n = 998), patients on the day of discharge after at least 5 days of hospitalisation (discharge group, n = 999), patients visiting a primary health centre (PHC group, n = 1000), and healthy relatives or household members of admission group patients (relatives group, n = 998). Individuals could be included only once in the study. Informed consent was obtained from all enrolled adults and carers of children. The departments involved were: Internal Medicine, Surgery, Gynaecology/Obstetrics, and Paediatrics. Individuals were excluded from the study if they were transferred from another hospital, or if they had been admitted to a hospital within the previous three months (admission, PHC, and relatives group). The specimens were collected from July to October 2001 in Surabaya and from January to May 2002 in Semarang. The medical ethics committees of the hospitals approved the study protocol.

Bacterial isolates, antimicrobial susceptibility testing, and detection of ESBL

Rectal swabs were cultured on CHROMagar Orientation (Becton Dickinson, Heidelberg, Germany) for *E. coli* (pink colonies) and other Enterobacteriaceae (blue colonies) (Filius *et al.* 2003). From each swab, two colonies representing the dominant growth in the faecal flora were collected. Susceptibility to cefotaxime was determined using disk diffusion according to the guidelines by the Clinical and Laboratory Standard Institution (CLSI, formerly NCCLS) (National Committee for Clinical Laboratory Standards 2001). All non-susceptible (i.e. resistant and intermediately susceptible) isolates were further analysed. Phenotypic confirmation of ESBL-production was performed by the double-disk synergy test (DDST) using four indicator antibiotics (cefotaxime, ceftazidime, cefepime, and aztreonam) which were placed 20 mm (edge to edge) away from an amoxicillin/clavulanic disk (Oxoid, Basingstoke, UK) (Jarlier *et al.* 1988; Drieux *et al.* 2008). Additional antibiotic susceptibility testing was carried out by the VITEK®2 system using AST-N041 cards (bioMérieux, Marcy-l'Étoile, France). This card also contains a specific test for ESBL-detection. ESBL-positive isolates, either confirmed by DDST or as indicated by the VITEK®2 system, were identified to the species using the VITEK®2 system and further genetically characterised.

β-Lactamase characterisation

The expression of β-lactamases was detected by isoelectric focusing (IEF) using the PhastSystem apparatus (Pharmacia AB, Uppsala, Sweden) as described before (Gruteke *et al.* 2003). PCR assays to determine the presence of bla_{TEM}, bla_{SHV}, and $bla_{\text{OXA-1-like}}$ genes were carried out using primers and conditions described previously (Mabilat & Goussard 1993; Nüesch-Inderbinen *et al.* 1997; Karisik *et al.* 2006). A multiplex PCR assay was used to detect $bla_{\text{CTX-M}}$ genes (Woodford *et al.* 2006). Isolates that tested positive for the CTX-M-1 group or CTX-M-9 group β-lactamase underwent amplification with separate sets

of primers (Villegas *et al.* 2004). Products resulting from amplifications were subjected to sequencing using a 3100 ABI Prism genetic analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The nucleotide and deduced amino acid sequences were analysed using MegAlign software (DNAStar Inc., Madison, USA) and programs available at the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Discrepancy analysis

Isolates that were phenotypically confirmed as ESBL-producers by DDST, but which were negative for bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ ESBL-genes, were further analysed by detailed review of the VITEK®2 report and were subjected to additional ESBL E-tests containing ceftazidime, cefotaxime, and cefepime, all with and without clavulanate (AB Biodisk, Solna, Sweden).

Pulsed-field gel electrophoresis (PFGE)

PFGE of *Xba*I digests of chromosomal DNA from all ESBL-positive Enterobacteriaceae isolates was performed as described previously (Gruteke *et al.* 2003). Relatedness among the PFGE profiles was evaluated with Bionumerics software (version 3.0; Applied Maths, Ghent, Belgium). Isolates with PFGE profiles showing ≥85% similarity were considered to be clonally related.

Detection of O25b-ST131 isolates

CTX-M-15-positive *E. coli* were investigated using both a multiplex PCR and Diversilab for the identification of members of the O25b-ST131 clone (Clermont *et al.* 2009; Pitout *et al.* 2009).

Results

Bacterial isolates

Screening of the faecal flora of 3995 individuals resulted in 5535 *E. coli* isolates from 3284 individuals and 1637 other Enterobacteriaceae from 1422 subjects. Susceptibility to cefotaxime by disk diffusion was determined for one *E. coli* per person and 482 other Enterobacteriaceae (most dominant growth). In total, 264 *E. coli* and 83 other Enterobacteriaceae were non-susceptible to cefotaxime. ESBL-production was phenotypically confirmed in 68 *E. coli* and 39 other Enterobacteriaceae. Phenotypic confirmatory testing was negative for 130 *E. coli* and 33 other Enterobacteriaceae. The remaining isolates were lost during storage (n = 71, mainly from the discharge group) or did not belong to the family of Enterobacteriaceae (n = 6).

ESBL-producing *E. coli*

A variety of CTX-M- and SHV-type enzymes were present among the ESBL-positive *E. coli* strains (Table 1). TEM-type ESBLs were not found. Sixty-five *E. coli* isolates carried a single bla_{ESBL} and one isolate carried multiple bla_{ESBL} genes. Two additional isolates showed a typical ESBL phenotype in the discrepancy analysis and were thus considered 'truly' ESBL-positive, but did not contain a TEM-, SHV-, or CTX-M-type ESBL-enzyme by neither PCR nor IEF. The majority of these 68 ESBL-positive *E. coli* were isolated from patients at discharge (63/68 strains; 92.6%). Five ESBL-positive strains were isolated from patients at hospital admission. No confirmed

Table 1 Occurrence of *bla* types among 68 ESBL-producing *E. coli* collected as dominant faecal flora from Indonesian people, 2001–2002

Type of ESBLs	No. of isolates (%)	No. of isolates with additional $bla_{NON\text{-}ESBL}$		City		Study group		Department			
		TEM-1	OXA-1	Sem	Sby	Admission	Discharge	Int	Surg	Gyn	Ped
CTX-M-15	32 (47.1)	19	22	25	7	1	31	9	16	3	4
SHV-5	13 (19.1)	8	4	9	4	1	12	1	2	1	9
CTX-M-14	7 (10.3)	6	4	1	6	1	6	3	3	1	
CTX-M-9	5 (7.4)				5		5	1	2	2	
SHV-2	4 (5.9)	2		1	3	1	3		1	1	2
SHV-2a	3 (4.4)	1	1	2	1	1	2	1		1	1
SHV-12	1 (1.5)	1		1			1				1
CTX-M-9+SHV-2	1 (1.5)				1		1		1		
Uncharacterised	2 (2.9)	2			2		2		1		1
Total	68 (100)	39	31	39	29	5	63	15	26	9	18

Sem, Semarang; Sby, Surabaya; Int, Internal Medicine; Surg, Surgery; Gyn, Gynaecology/Obstetrics; Ped, Paediatrics.

ESBL-positive *E. coli* were cultured as dominant faecal flora from individuals in the PHC or relatives group.

The bla_{CTX-M}-type ESBL enzymes were the most prevalent (66.2%). These were CTX-M-15 (n = 32), CTX-M-14 (n = 7), and CTX-M-9 (n = 6). A bla_{SHV}-type ESBL gene was detected in 22 *E. coli* strains (32.4%). Of these, SHV-5 was the most frequently found enzyme (13 strains). PFGE identified 51 different types, of which 42 were represented by a single isolate and 9 included more than one isolate. Five isolates were untypeable due to band-smearing patterns. Among 14 CTX-M-15-positive isolates from patients on the day of discharge from the Department of Surgery in Semarang 12 PFGE types were found. None of the CTX-M-15-positive isolates belonged to the O25b-ST131 clone according to the method by Clermont *et al.* (2009). Using Diversilab (Pitout *et al.* 2009), a single isolate could be assigned to this clone.

The frequencies of resistance among the ESBL-positive *E. coli* isolates were: tetracycline, 85.3%; gentamicin, 73.5%; trimethoprim-sulfamethoxazole, 63.2%; ciprofloxacin, 45.6%; nitrofurantoin, 16.2%; and amikacin, 11.8%. All isolates were susceptible to imipenem (with MIC ≤1 μg/ml).

ESBL-producing Enterobacteriaceae other than *E. coli*

The 39 ESBL-producing Enterobacteriaceae other than *E. coli* included 35 *K. pneumoniae*, 3 *Enterobacter cloacae*, and 1 *Citrobacter freundii*. Most of these were from Semarang (76.9%). All except one of the 39 isolates had been isolated from patients at discharge from the hospital. Among *K. pneumoniae* isolates, the bla_{SHV}-type was the most prevalent ESBL (54.3%): SHV-2a (n = 6), SHV-12 (n = 5), SHV-5 (n = 5), and SHV-2 (n = 3) (Table 2). bla_{CTX-M} type enzymes were detected in 18 *K. pneumoniae* isolates (51.4%), and were predominantly CTX-M-15 (n = 16). All CTX-M-positive *K. pneumoniae* originated from Semarang. ESBL-positive *K. pneumoniae* isolates from Surabaya contained exclusively SHV-type ESBL genes. One strain from Semarang carried bla_{TEM-19}. Another strain from Semarang exhibited a classical ESBL phenotype, but we were unable to identify the responsible ESBL gene. Two *E. cloacae* isolates carried $bla_{CTX-M-9}$ and one carried $bla_{CTX-M-15}$ in combination with bla_{OXA-1}. The *C. freundii* isolate produced SHV-12 in combination with TEM-1.

PFGE analysis revealed extensive genetic heterogeneity. Among *K. pneumoniae* 27 different profiles were observed, of which 23 were unique, one PFGE type was represented by four isolates, one by three isolates, and two by two isolates each. The 11 clonal isolates originated from discharge group patients from Semarang and were all CTX-M-15-positive except for one. One *K. pneumoniae* was untypeable. The *E. cloacae* isolates revealed three unique banding patterns.

Resistance rates to non-related classes of antibiotics were as follows: nitrofurantoin (87.2%), gentamicin (74.4%), trimethoprim-sulfamethoxazole (71.8%), tetracycline

Table 2 Occurrence of *bla* types among 39 ESBL-producing Enterobacteriaceae other than *E. coli* collected as dominant faecal flora from Indonesian people, 2001–2002

Type of ESBLs	No. of isolates (%)	No. of isolates with additional $bla_{NON-ESBL}$			City		Study group		Department			
		TEM-1	SHV-1	OXA-1	Sem	Sby	Admission	Discharge	Int	Surg	Gyn	Ped
CTX-M-15*	14 (35.9)	1	7	13	14			14	5	5	1	3
SHV-2a	5 (12.8)	1			3	2		5	3	1	1	
SHV-5	5 (12.8)				3	2	1	4	3	2		
SHV-2	3 (7.7)	2			1	2		3		2		1
SHV-12†	3 (7.7)	2			1	2		3		1		2
CTX-M-9‡	2 (5.1)				2			2		1	1	
CTX-M-15+SHV-12	2 (5.1)	1		2	2			2		1		1
CTX-M-15+SHV-2a	1 (2.6)			1	1			1		1		
CTX-M-9+SHV-12	1 (2.6)	1			1			1	1			
CTX-M-3	1 (2.6)				1			1		1		
TEM-19	1 (2.6)				1			1				1
Uncharacterised	1 (2.6)	1				1		1		1		
Total	39 (100)	9	7	16	30	9	1	38	12	16	3	8

Sem, Semarang; Sby, Surabaya; Int, Internal Medicine; Surg, Surgery; Gyn, Gynaecology/Obstetrics; Ped, Paediatrics.
*These included 13 *K. pneumoniae* and 1 *E. cloacae*.
†Two *K. pneumoniae* and 1 *C. freundii*.
‡Both strains were *E. cloacae*.

Table 3 Combinations of *E. coli* and *K. pneumoniae* with ESBL activity simultaneously found in one patient

Patient number	bla_{ESBL} in *E. coli*	bla_{ESBL} in *K. pneumoniae*
61049	CTX-M-15	CTX-M-15
62104	CTX-M-15	CTX-M-3
22033	CTX-M-9+SHV-2	SHV-2
64086	SHV-12	CTX-M-15+SHV-12
61079	CTX-M-15	CTX-M-9+SHV-12
22076	Uncharacterised	Uncharacterised

(66.7%), ciprofloxacin (38.5%), and amikacin (7.7%). All isolates had MICs of imipenem of ≤1 μg/ml.

Six patients from the discharge group were simultaneously colonised with *E. coli* and *K. pneumoniae* strains with ESBL activity (Table 3).

Discussion

This study shows that faecal carriage of ESBL-producing Enterobacteriaceae in Java, Indonesia, in 2001–2002 was most prevalent among patients on the day of discharge after ≥5 days of hospitalisation. At least 95 of the 999 patients cultured at discharge (9.5%) carried one or more ESBL-positive microorganisms as dominant faecal flora at that specific moment compared to 0.6% of patients on admission. Among healthy persons in Indonesia, we did not find any ESBL-producing Enterobacteriaceae. Thus, ESBLs as dominant faecal flora of humans in Indonesia in 2001–2002 were almost exclusively hospital-associated.

The key question is whether the ESBL-producing Enterobacteriaceae that appear as dominant intestinal flora during hospitalisation are nosocomially acquired or not. Strong evidence for nosocomial acquisition would be provided when isolates carry the same bla_{ESBL} and are genetically indistinguishable by a typing method. The isolates in the present study carried various ESBL genes and were genetically highly diverse, indicating unrelatedness, and, thus, another mode of acquisition must be considered. One of the possibilities is dissemination of certain mobile genetic elements in the hospital, which could even take place between different species of Enterobacteriaceae (Marchandin *et al.* 1999). We found six patients to be simultaneously colonised with *E. coli* and *K. pneumoniae* strains with ESBL activity and in three of these identical ESBL genes were found, which could suggest transfer of genetic elements within a patient. Plasmid analysis should confirm this hypothesis. However, dissemination of certain plasmids would only partly explain the epidemiology given the diversity of ESBL genes found. In case of SHV and TEM, de novo mutation could be an alternative mode of acquisition. Another possibility could be that ESBL-positive isolates were already present in low numbers in the intestinal flora of patients when admitted to the hospital. Susceptible microorganisms in their intestines would be killed upon exposure to third-generation cephalosporins which would be followed by selection and subsequent enrichment of the resistant Enterobacteriaceae. Previous studies demonstrated the dramatic effect of parenteral extended-spectrum cephalosporins on the human intestinal flora (Sullivan *et al.* 2001). The rectal cultures from patients on admission in our study, however, only rarely revealed ESBL-producing Enterobacteriaceae, but this omission could be due to the applied culture method, i.e. rectal swabs were directly plated on the CHROMagar Orientation and only the dominant flora was stored and analysed. A culture method using a selective enrichment broth would have a higher sensitivity for detecting ESBL-positive Enterobacteriaceae in rectal cultures when only a small subpopulation of the intestinal flora is ESBL-positive (Murk *et al.* 2009).

Most of the 107 ESBL-positive microorganisms had been isolated from patients that were discharged from the departments of Surgery of both hospitals (n = 42). In the two hospitals where our study was carried out, the third-generation cephalosporins were the second most prescribed antibiotics in the hospitals, and most of these were administered in the departments of Surgery (Hadi *et al.* 2008). It is thus reasonable to assume that ESBL-producing microorganisms as dominant intestinal flora in the discharged patients reflects nosocomial exposure to expanded-spectrum cephalosporins and subsequent selection of these putative community-derived strains.

Characterisation of the ESBL-genes revealed a variety of ESBL types. CTX-M-15, however, was the most frequently found ESBL in both *E. coli* and *K. pneumoniae* isolates. Faecal carriage of ESBL-producing organisms confers an increased risk for subsequent invasive infection with the same organism. However, the virulence of *E. coli* is dependent on the phylogenetic group and numerous virulence factors (Kuntaman *et al.* 2005; Ben-Ami *et al.* 2006). The ESBL-positive *E. coli* from our discharged patients belonged to the less virulent phylogenetic groups A, B1, and D, which was determined in a previous study (Kuntaman *et al.* 2005). Nevertheless, among clinical *E. coli* isolates collected in the Dr. Soetomo Academic Hospital in Surabaya in 2005, CTX-M-15 was the most prevalent ESBL (94.5%), and many of those isolates belonged to non-B2 groups (63.2%). Among clinical *K. pneumoniae* bla_{SHV}-type ESBLs were most prevalent. Indeed, commensal *K. pneumoniae* isolates from Surabaya contained exclusively SHV-type ESBL genes. Interestingly, CTX-M-14 and CTX-M-9 were found among the

commensal isolates in the present study, but not among clinical isolates (Severin *et al.* 2010).

Our study has some limitations. First, the estimates of the ESBL prevalence may be too low as the use of cefotaxime for screening might have biased the selection against ESBLs that preferably hydrolyse other oxyiminocephalosporins. Therefore, our prevalence estimation cannot be compared to results from other studies, especially not to studies using more sensitive methods for detection, such as enrichment broths. Also, some strains were lost during storage and could not be analysed. Second, our study was performed in a specific geographical location, and the results may not be taken to reflect the distribution of ESBLs throughout Indonesia. Third, these data were collected in 2001–2002, and colonisation rates may have changed in the population since that time. It is likely that ESBL-positive bacteria are now widespread in the Indonesian community as well, since travellers to Asian countries, including Indonesia, become colonised with these bacteria during their journey (Kennedy & Collignon 2010). However, our data provide insight in the presence and transmission of ESBL-producing bacteria in Indonesian hospitals in an early stage of the ESBL pandemic.

In summary, we have shown that ESBL-producing Enterobacteriaceae were already present as intestinal flora of inhabitants of Java, Indonesia in 2001–2002. They were found in almost 10% of patients at the time of discharge from the hospital, but not among healthy individuals. The presence of various bla_{ESBL} genes and the extensive genetic diversity among isolates argue against a single/dominant strain outbreak. Targeted surveillance of clinical and non-clinical Enterobacteriaceae should be continued in order to monitor the evolving epidemiology of ESBLs in this part of the world.

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