J Microbiol Immunol Infect.
2008;41:428-432

Brief Communication

CTX-M type beta-lactamases among fecal *Escherichia coli* and *Klebsiella pneumoniae* isolates in non-hospitalized children and adults

Pak-Leung Ho[1], River Chun-Wai Wong[2], Kin-Hung Chow[2], Kenneth Yip[1], Samson Sai-Yin Wong[1], Tak-Lun Que[2]

[1]Division of Infectious Diseases, Department of Microbiology and Centre of Infection, University of Hong Kong, Pokfulam; and [2]Department of Clinical Pathology, Tuen Mun Hospital, Tuen Mun, Hong Kong

Received: October 9, 2007 Revised: December 1, 2007 Accepted: January 30, 2008

We investigated the occurrence and diversity of extended-spectrum beta-lactamase (ESBL) enzymes among antibiotic-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates obtained from human feces. All ESBL-positive isolates were characterized at the molecular level by polymerase chain reaction, sequencing and pulsed-field gel electrophoresis (PFGE). Eight of 46 antibiotic-resistant *E. coli* (6 from children and 2 from adults) and 4 of 8 *K. pneumoniae* (all from adults) isolates were found to be ESBL-positive by the double-disk synergy test. Seven isolates were found to have CTX-M-14, 2 each had CTX-M-24 and CTX-M-38, and 1 had CTX-M-9. In addition, 8 isolates were found to carry TEM-1b or TEM-1c. No SHV-type enzyme was found among the *E. coli* strains. In 9 strains, the plasmidic bla_{CTX-M} determinants were transferable to *E. coli* by conjugation. Analysis by PFGE showed evidence of clonal and non-clonal spread. The present study shows fecal carriage of organisms producing bla_{CTX-M} determinants and underscores the role that commensals could play as a reservoir for their dissemination.

Key words: beta-Lactamases; Drug resistance, microbial; Epidemiology; *Escherichia coli*; Feces

Introduction

The CTX-M family is a rapidly growing group of extended-spectrum beta (β)-lactamases (ESBLs) that have disseminated globally. In Asia, it is now the predominant ESBL type among *Enterobacteriaceae*, and similar epidemiological changes are taking place in Europe [1,2]. Most CTX-M β-lactamases confer a higher level of resistance to cefotaxime than ceftazidime. Spread of CTX-Ms is facilitated by their linkage with insertion sequence, integron and plasmid, although multiclonal spread has also been recognized [1,3]. Currently, 5 different clusters of CTX-Ms (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25), comprising more than 60 allelic types, are recognized according to their amino acid sequence [4]. There are geographical variations in the prevalence of CTX-M cluster groups. In Asia, CTX-M-9 cluster is most common, while in Europe the CTX-M-1 cluster, in particular CTX-M-15, predominates [1,3]. Unlike TEM- and SHV-derived ESBLs, the prevalence of CTX-M enzymes has been increasing to a greater extent in the community than in the hospital setting. Recently, it was suggested that the rise of CTX-M in health care settings might be a consequence of the influx of these enzymes from the community [3].

In this study, we describe the bla_{CTX} genes in commensal *Escherichia coli* and *Klebsiella pneumoniae* from non-hospitalized individuals. The findings confirm the community distribution of the CTX-M genes and underscore the potential for commensal organisms to serve as a reservoir of these emerging resistance determinants [5].

Corresponding author: Dr. Pak-Leung Ho, Division of Infectious Diseases, Department of Microbiology and Centre of Infection, University of Hong Kong, Queen Mary Hospital, Pokfulam Road, Pokfulam, Hong Kong.
E-mail: plho@hkucc.hku.hk

Methods

Strains

The isolates included in this study were derived from 2 stool samplings conducted between August and December 2002. The first sample comprised fecal specimens from pediatric outpatients submitted to a hospital microbiology department for investigation of bacterial pathogens and/or parasites. These samples (approximately 0.1 g feces) were seeded in MacConkey agar supplemented with 8 mg/L ciprofloxacin. The second sample comprised adult volunteers who were not ill at the time of sampling. The volunteers were mostly university students (age range, 20 to 35 years) and some were research assistants whose duties did not involve visiting patient areas. Fecal specimens from this sample were inoculated in MacConkey agar supplemented with 2 mg/L cefotaxime. After overnight incubation, colonies suggestive of *Enterobacteriaceae* were investigated further. One colony for each morphotype per plate was selected for bacterial identification by Vitek GNI card (bioMérieux Vitek Inc., Hazelwood, MO, USA). All specimens were obtained in an anonymous manner. The investigators were blinded to the individual identity of the samples. In total, 189 single individual samples from children and 53 from adults were tested. The samples from young children yielded 44 *E. coli* and 4 *K. pneumoniae* isolates in the ciprofloxacin selection agar plates. In the adult volunteer sample, the cefotaxime-containing agar yielded 2 *E. coli* and 4 *K. pneumoniae*. In total, 46 antibiotic-resistant *E. coli* and 8 *K. pneumoniae* isolates were retrieved from the archives for this study.

ESBL detection and antimicrobial susceptibility testing

The double-disk synergy test was used for detecting ESBL production [6]. Susceptibilities of the isolates to antibiotics were determined by the disk diffusion test and/or microbroth dilution test and interpreted according to the Clinical and Laboratory Standard Institute guidelines [7]. Antibiotic susceptibilities to tetracycline, trimethoprim-sulfamethoxazole, ciprofloxacin, gentamicin, amikacin, chloramphenicol, imipenem, meropenem and amoxicillin-clavulanic acid were tested by the disk diffusion method. Microbroth dilution (minimal inhibitory concentration [MIC]) testing was determined using Muller-Hinton broth (Oxoid, Basingstoke, United Kingdom). The following agents were tested: cefotaxime, cefotaxime + clavulanic acid, ceftazidime, ceftazidime + clavulanic acid, aztreonam, cefepime, cefmetazole and meropenem. The antibiotic powders were obtained from the respective manufacturers.

Molecular studies

A polymerase chain reaction (PCR) and sequencing strategy was used to characterize enzymes related to the TEM, SHV and CTX-M families using primers and conditions described previously [2,8,9]. Primers M9U forward 5'-ATG GTG ACA AAG AGA GTG CA-3' (position 112-131, accession D89862) [Invitrogen, Hong Kong] and M9L backward 5'-CCC TTC GGC GAT GAT TCT C-3' (position 957-975, D89862) [Invitrogen] were used in amplification and sequencing of the CTX-M-9 group enzymes. The nomenclature used for the bla_{TEM} variants was as previously proposed [10]. For the CTX-M-positive strains, the region immediately upstream from bla_{CTX-M} was explored by direct sequencing of amplicons obtained with the forward primer IS*Ecp1*U1 (Invitrogen) and the consensus reverse MA2 primer (Invitrogen) [9]. Presence of class I and II integrons and *orf*513 in the strains was evaluated by PCR using specific primers [2]. The epidemiological relationships of the isolates were studied by pulsed-field gel electrophoresis (PFGE) using *Xba*I for DNA digestion, and the results were interpreted according to Tenover et al [11]. Phylogenetic grouping of the *E. coli* isolates was determined by a multiplex PCR-based approach [12].

Conjugation experiments were carried out on sterilized filters (Whatman International Ltd, Maidstone, UK) with *E. coli* J53Az^r as the recipient [2]. Donor and recipient cells were mixed at a ratio of 1:10. Transconjugants were selected on Mueller-Hinton agar plates containing cefotaxime (0.5 mg/L) together with sodium azide (150 mg/L; Sigma Chemical Co., St. Louis, MO, USA). Frequency of transfer was calculated by dividing the number of transconjugants by the number of donors.

Small plasmids (less than 20 kb) were extracted by the High Pure Plasmid Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) and analyzed by conventional gel electrophoresis. Large plasmids were sized by the S1 nuclease (Sigma Chemical Co., St. Louis, MO, USA) and PFGE technique, as described previously [8].

Results

ESBL detection and antimicrobial susceptibilities

Eight of the 46 *E. coli* and 4 of the 8 *K. pneumoniae* were found to be ESBL-positive by the double-disk

synergy test. Six of the ESBL-positive *E. coli* isolates were obtained from children and 2 were obtained from adults. All ESBL-positive *K. pneumoniae* isolates were obtained from adults. The ESBL-positive isolates were studied further.

Analysis of the antimicrobial susceptibilities revealed multi-resistant profiles among all 8 ESBL-positive *E. coli* isolates, involving 3 or more of tetracycline, trimethoprim-sulfamethoxazole, ciprofloxacin, gentamicin and chloramphenicol. In contrast, the ESBL-positive *K. pneumoniae* strains were generally susceptible to the non-β-lactam antibiotics. All 4 *Klebsiella* isolates were susceptible to tetracycline, trimethoprim-sulfamethoxazole and gentamicin. Only 2 strains showed coresistance to ciprofloxacin and chloramphenicol. All *E. coli* and *K. pneumoniae* strains were susceptible to amikacin, imipenem and meropenem. The ceftazidime MICs for the strains were always at least 4-fold lower than those of cefotaxime (Table 1). The activities of cephalosporins could be restored by clavulanic acid.

Characteristics of the ESBL-producing strains

Findings from the molecular analysis of the 12 strains are summarized in Table 2. All CTX-M enzymes identified belonged to the CTX-M-9 group family: 7 isolates had CTX-M-14, 2 each had CTX-M-24 and CTX-M-38, and 1 had CTX-M-9. All except 1 isolate (EC-N15X) could be amplified with the forward primer IS*Ecp*U1 and the reverse CTX-M consensus primer MA2. In all strains with an upstream IS*Ecp1*U1 element, sequencing of the PCR products revealed a right inverted repeat (IRR) and a –10 putative promoter region and a 42 bp interval between the IRR and the start codon of $bla_{\text{CTX-M}}$. All *E. coli* strains gave a negative result in PCR for bla_{SHV}. Conjugal transfers of cephalosporin resistance were observed in 9 isolates and were associated with mobile plasmids with sizes of 45 to 180 kb. A double-disk diffusion test revealed synergistic activity between clavulanic acid and cefotaxime against all transconjugants. The presence of $bla_{\text{CTX-M}}$ in the transconjugants was confirmed by PCR. Analysis by PFGE showed that 2 *E. coli* isolates (B6 and

Table 1. Antimicrobial susceptibility profiles of donors, recipients and transconjugants in the conjugation experiments

Group and strain	MIC (μg/mL)[a]							
	CTX	CTX + CLA	CAZ	CAZ + CLA	ATM	FEP	CMZ	MEM
Strain (donor)[b]								
EC-N15X	16	2	1	0.25	4	4	4	0.032
EC-N52X-2	256	2	2	1	8	8	16	0.032
EC-A2	16	0.12	1	0.25	4	4	2	0.016
EC-B4	32	0.12	1	0.25	4	4	2	0.016
EC-B6	16	0.25	4	0.5	16	2	2	0.016
EC-D5	16	0.25	1	0.25	8	4	1	0.016
EC-D9	8	0.25	0.5	0.12	16	2	1	0.016
EC-G1	32	1	2	1	8	2	16	0.016
KP-N07X	16	2	4	1	16	4	4	0.032
KP-N22X	16	1	4	1	8	2	4	0.032
KP-N31X	128	2	1	0.125	16	2	4	0.032
KP-N38X	256	2	1	0.25	16	2	8	0.032
Recipient, J53Az^r	0.06	0.06	0.5	0.5	0.25	0.25	2	0.016
Transconjugant								
15X_T1	4	0.12	0.5	0.06	8	0.5	1	0.016
52X_T1	64	0.06	1	0.012	4	2	1	0.016
A2_T1	16	2	2	1	8	4	4	0.016
B6_T1	32	4	1	0.5	8	4	4	0.016
G1_T1	16	2	2	1	8	2	2	0.016
N07X_T1	4	0.03	2	0.25	4	1	2	0.016
N22X_T1	4	0.03	0.5	0.12	8	4	1	0.016
N31X_T1	256	1	1	0.25	16	2	4	0.032
N38X_T1	64	1	1	0.25	16	1	4	0.032

Abbreviations: MIC = minimal inhibitory concentration; CTX = cefotaxime; CLA = clavulanic acid; CAZ = ceftazidime; ATM = aztreonam; FEP = cefepime; CMZ = cefmetazole; MEM = meropenem; EC = *Escherichia coli*; KP = *Klebsiella pneumoniae*

[a]CLA at a fixed concentration of 4 mg/L.

[b]Resistance was transferred for the underlined strains.

Table 2. Characteristics of CTX-M beta (β)-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*

Organism and strain	Genetic elements[a]	β-Lactamase content	Conjugation frequency	Plasmid profile (kb)	Transferred plasmid (kb)	Resistance cotransferred
EC-N15X	*intI* 1, *orf*513	CTX-M-9, TEM-1b	10–5	45, 180	45, 180	SXT
EC-N52X-2	*intI* 1	CTX-M-14, TEM-1b	10–5	2, 4, 100	100	None
EC-A2	-	CTX-M-14	10–4	2, 3, 30, 60, 90, 130	60	None
EC-B4	-	CTX-M-14, TEM-1b	-	1, 3, 6, 12, 60, 100	-	-
EC-B6	*intI* 1	CTX-M-14, TEM-1b	10–4	3, 6, 18, 100, 150	150	None
EC-D5	*intI* 1	CTX-M-14, TEM-1c	-	6, 18, 60, 90, 120	-	-
EC-D9	-	CTX-M-14	-	90	-	-
EC-G1	*intI* 1	CTX-M-14, TEM-1b	10–4	60, 130	60	None
KP-N07X	*intI* 1	CTX-M-38, TEM-1b	10–3	2, 4, 5, 50, 60, 180	60	CHL
KP-N22X	*intI* 1	CTX-M-38, TEM-1b	10–3	2, 4, 5, 50, 60, 180	60	CHL
KP-N31X	-	CTX-M-24	10–3	4, 5, 100, 160, 240	100	None
KP-N38X	-	CTX-M-24	10–4	4, 5, 100, 160, 240	100	None

Abbreviations: EC = *Escherichia coli*; KP = *Klebsiella pneumoniae*; SXT = trimethoprim-sulfamethoxazole; CHL = chloramphenicol
[a]No class II integron was found.

D5) had a possibly related pulsotype, while those for the other *E. coli* isolates were different (Fig. 1). PFGE divided the 4 *Klebsiella* strains into 2 groups: patterns for strains N07X and N22X were closely related, and patterns for strains N31X and N38X were closely related. Phylogenetic group analysis showed that 6 *E. coli* were group D strains and 2 (strains B6 and D5) were group A strains.

Discussion

This study documents the occurrence of CTX-M determinants among antibiotic-resistant commensals

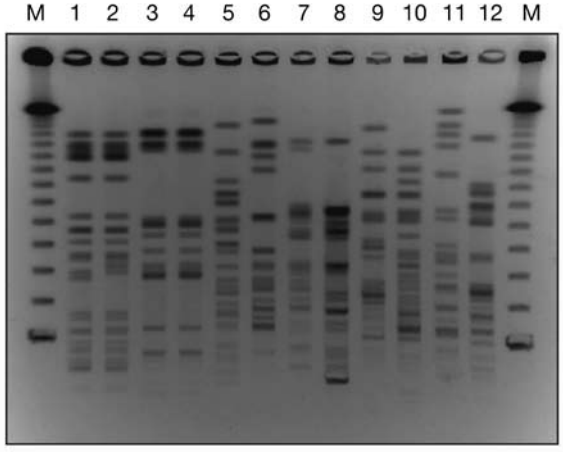

Fig. 1. Pulsed-field gel electrophoresis profiles of *Xba*I macrorestriction fragments of CTX-M-positive strains. Lane M, λ DNA pulsed-field gel eletrophoresis markers; lanes 1-4, *Klebsiella pneumoniae* strains (lane 1, N38X; lane 2, N31X; lane 3, N22X; lane 4, N07X); lanes 5-12, *Escherichia coli* strains (lane 5, G1; lane 6, D9; lane 7, D5; lane 8, B6; lane 9, B4; lane 10, A2; lane 11, N52X-2; lane 12, N15X).

carried by non-hospitalized adults and children. Among young children, the stool sampling showed that carriage of fluoroquinolone-resistant *E. coli* was common (44/189, 23.3%) and that a significant proportion (6/44, 13.6%) of these isolates were ESBL producers. The latter figure is in line with resistance data among our clinical isolates. In Hong Kong, data from hospital laboratories showed that 10% to 20% of all fluoroquinolone-resistant *E. coli* isolates were ESBL-positive. Among ESBL-producing *E. coli* isolates in clinical specimens, approximately two-thirds were fluoroquinolone-resistant. Since fluoroquinolones are not used in young children, the source of the fluoroquinolone-resistant and CTX-M-positive isolates is unclear. Acquisition from the food chain and adult carriers are some plausible modes of transmission [5,13]. Since ciprofloxacin-containing media was used, ESBL-producers that were fluoroquinolone-sensitive would not have been recovered. Therefore, the data do not allow us to define clearly how often ESBL producers were carried by children.

Our findings emphasize the endemicity of alleles of the CTX-M-9 cluster in this region. In our previous studies, alleles in the same cluster were found to predominate among isolates from clinical specimens and food animal carriers [2,5,8,13]. This study showed that most of the CTX-M determinants were located in transferable plasmids and some strains also carry other mobile resistance elements, including insertion sequence (IS*Ecp1*U1), transposase (*orf*513) and integron. As suggested previously, these elements could be playing important roles in the translocation and dissemination of CTX-Ms [1]. Despite the limited

number of commensal isolates tested, it is interesting that the most frequent allele was CTX-M-14, which was similar to the situation among ESBL-positive clinical isolates [5].

The present study is limited by the relatively small sample size. Since some of the adult volunteers were classmates and friends, the possibility of person-to-person transmission of commensal bacteria or the resistant determinant could not be excluded. More detailed epidemiological studies to address transmission of CTX-M in different school and social settings are necessary. In conclusion, this study documents fecal carriage of ESBL producers among non-hospitalized individuals and the CTX-M-9 group as the predominant ESBL type in this region.

Acknowledgments

The work is supported by research grants from the University Development Fund Project-Research Centre of Emerging Infectious Diseases of University of Hong Kong, and the Research Grants Council HKU 7513/06M. We thank RS Duan for technical assistance.

References

1. Cantón R, Coque TM. The CTX-M beta-lactamase pandemic. Curr Opin Microbiol. 2006;9:466-75.
2. Ho PL, Shek RH, Chow KH, Duan RS, Mak GC, Lai EL, et al. Detection and characterization of extended-spectrum beta-lactamases among bloodstream isolates of *Enterobacter* spp. in Hong Kong, 2000-2002. J Antimicrob Chemother. 2005;55:326-32.
3. Woodford N, Ward ME, Kaufmann ME, Turton J, Fagan EJ, James D, et al. Community and hospital spread of *Escherichia coli* producing CTX-M extended-spectrum beta-lactamases in the UK. J Antimicrob Chemother. 2004;54: 735-43.
4. Jacoby G, Bush K. Amino acid sequences for TEM, SHV and OXA extended-spectrum and inhibitor resistant β-lactamases. Available at: http://www.lahey.org/studies
5. Ho PL, Poon WW, Loke SL, Leung MS, Chow KH, Wong RC, et al; COMBAT study group. Community emergence of CTX-M type extended-spectrum beta-lactamases among urinary *Escherichia coli* from women. J Antimicrob Chemother. 2007;60:140-4.
6. Ho PL, Chow KH, Yuen KY, Ng WS, Chau PY. Comparison of a novel, inhibitor-potentiated disc-diffusion test with other methods for the detection of extended-spectrum beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae*. J Antimicrob Chemother. 1998;42:49-54.
7. Clinical and Laboratory Standard Institute. Performance standards for antimicrobial susceptibility testing. 17th informational supplement. Wayne, PA: National Committee for Clinical Laboratory Standards; 2005.
8. Ho PL, Ho AY, Chow KH, Wong RC, Duan RS, Ho WL, et al. Occurrence and molecular analysis of extended-spectrum (beta)-lactamase-producing *Proteus mirabilis* in Hong Kong, 1999-2002. J Antimicrob Chemother. 2005;55: 840-5.
9. Saladin M, Cao VT, Lambert T, Donay JL, Herrmann JL, Ould-Hocine Z, et al. Diversity of CTX-M beta-lactamases and their promoter regions from *Enterobacteriaceae* isolated in three Parisian hospitals. FEMS Microbiol Lett. 2002;209: 161-8.
10. Leflon-Guibout V, Heym B, Nicolas-Chanoine M. Updated sequence information and proposed nomenclature for bla(TEM) genes and their promoters. Antimicrob Agents Chemother. 2000;44:3232-4.
11. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol. 1995;33:2233-9.
12. Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol. 2000;66:4555-8.
13. Duan RS, Sit TH, Wong SS, Wong RC, Chow KH, Mak GC, et al. *Escherichia coli* producing CTX-M beta-lactamases in food animals in Hong Kong. Microb Drug Resist. 2006;12: 145-8.