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Citation	Diagnostic Microbiology and Infectious Disease, 2012, v. 73 n. 2, p. 182-186
Issued Date	2012
URL	http://hdl.handle.net/10722/157695
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Predominance of pHK01-like incompatibility group FII plasmids encoding CTX-M-14 among extended-spectrum beta-lactamase-producing *Escherichia coli* in Hong Kong, 1996-2008

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Key words: Enterobacteriaceae; CTX-M-14 beta-lactamase; Antimicrobial Drug Resistance; plasmids; Restriction Fragment Length Polymorphism

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Abstract (150 words)

This study assessed temporal changes in the molecular epidemiology of the bacteremic *Escherichia coli* isolates producing CTX-M-14 in Hong Kong. Blood isolates from 1996-1998 (period 1, *n*=50) and 2007-2008 (period 2, *n*=117) were investigated by molecular methods. CTX-M-type ESBL was carried by 98.2% (164/167) of the isolates. In both periods, the CTX-M-9 group and CTX-M-14 allele were the predominant ESBL type. The major clones were found to change from ST68 and ST405 in period 1 to ST131, ST69 and ST12 in period 2. Among 65 CTX-M-14-producing plasmids investigated further, 54 had the FII replicon. Replicon sequence typing and plasmid PCR-restriction fragment length polymorphism showed that 79.6% (43/54) of the FII plasmid subset were similar to the completely sequenced plasmid, pHK01 (human urine, Hong Kong, 2004). These pHK01-like plasmids were found to have spread to the major clones (ST68, ST405 and ST131) and multiple singleton isolates of all four phylogenetic groups.

1. Introduction

In Asia, the burdens of cefotaximase (CTX-M) producing Enterobacteriaceae have increased dramatically in the last decade (Hawkey, 2008; Ho et al., 2005; Ho et al., 2007). Studies from Hong Kong, mainland China, South Korea and Thailand have consistently showed that CTX-M-14 is the most prevalent enzyme (Ho et al., 2007; Lo et al., 2010; Nguyen et al., 2010; Yi et al., 2010). This CTX-M allele was first described in 2001 among *E. coli*, *Klebsiella pneumoniae* and *Shigella* isolates collected from different parts of South Korea in 1995 (Pai et al., 2001). While clonal spread have been reported in Japan and Canada (Pitout et al., 2005; Suzuki et al., 2009), the spread of CTX-M-14 have been found to be mainly caused by conjugative plasmids (Cottell et al., 2011; Ho et al., 2011c; Valverde et al., 2009). In Spain, the spread of CTX-M-14 from 2000 to 2005 was largely due to pRYC105-like plasmids of the IncK incompatibility group disseminated among diverse *E. coli* lineages (Valverde et al., 2009). Recently, it has been showed that pRYC105 is similar to the pCT plasmid that has been found in bacteria from the United Kingdom, mainland China and Australia (Cottell et al., 2011). Recently, we sequenced the IncFII epidemic plasmid, pHK01 and showed that it has disseminated widely among *E. coli* isolates collected from patients with community-acquired, urinary tract infections in 2004 (Ho et al., 2007; Ho et al., 2011c). Variants closely related to pHK01 have been identified among Enterobacteriaceae isolates from mainland China and Vietnam (Ho et al., 2011c; Nguyen et al., 2010; Yi et al., 2010).

In this study, we investigated the clonal structure and relatedness of plasmids encoding CTX-M-14 for a collection of blood culture *E. coli* isolates from patients treated in a healthcare region from 1996 to 2008.

2. Methods

2.1 Bacterial strains and susceptibility testing

The *E. coli* isolates were recovered from blood cultures of patients who were treated in a healthcare region in Hong Kong (Ho et al., 2002; Ho et al., 2005). The healthcare region (QMH) include a network of 5 public hospitals, including 1 acute care, university teaching hospital with 1500 beds and all the clinical disciplines including renal, liver and bone marrow transplantation service and 4 convalescence care hospitals with 110 to 524 beds. (Ho et al., 2005). This study included isolates recovered from two time periods, 1996-1998 (period 1) and 2007-2008 (period 2). The proportion of extended-spectrum β-lactamases (ESBL)-producing *E. coli* among all blood culture *E. coli* isolates for period 1 and period 2 were 6.6% (74/1127) and 26.8% (253/945), respectively (Ho et al., 2002; Ho et al., 2011b). For period 1, 67.6% (50/74) of the ESBL-producing isolates remained viable and all were included. The clinical characteristics for the 50 patients have been described (Ho et al., 2002). For period 2, 46.2% (117/253) of the ESBL-producing isolates from 253 patients (one per patient) were randomly selected for inclusion. Only one ESBL-producing isolate per patient was included in this study. For those patients with more than one isolate, only the first isolate was tested. The VITEK GNI system (bioMerieux, Vitek Inc., Hazelwood, MO, USA) was used for bacterial identification. The disc diffusion method was used for susceptibility testing and results interpreted according to the CLSI (Clinical and Laboratory Standard Institute, 2011). Production of ESBL was determined by the double-disc synergy test (Ho et al., 2000; Ho et al., 2007).

2.2 CTX-M gene detection and epidemiological typing

Genes related to the CTX-M families were sought by PCR and sequencing using primers previously described (Lo et al., 2010; Saladin et al., 2002). Selected isolates were studied by pulsed-field gel electrophoresis (PFGE) of *XbaI*-digested genomic DNA (Amersham Pharmacia Biotech, Little Chalfont, UK) and patterns were analysed with Gelcompar II

software (Applied Maths). A multiplex PCR was used to assign the *E. coli* isolates to one of the four main phylogenetic groups (A, B1, B2 and D) (Ho et al., 2007). Multilocus sequencing was performed as described (Wirth et al., 2006). PCR reactions were used for detection of the 15 major O serotypes (O1, O2, O4, O6, O7, O11, O12, O15, O16, O18, O25, O75, O86, O102, and O157) and the O25b subtype (Clermont et al., 2007; Clermont et al., 2009; Deschamps et al., 2009).

2.3 Conjugation and replicon typing

Conjugation experiments and sizing of plasmids were carried out as described previously (Ho et al., 2011d; Ho et al., 2011c). The replicon (*rep*) content in the plasmids was determined by PCR and sequencing (Carattoli et al., 2005). When a transconjugant cannot be obtained, the replicon type for the plasmid encoding CTX-M-14 was determined in the parent strains. In all the isolates, the replicon location in the plasmids was confirmed by hybridization with probes specific for $bla_{\text{CTX-M-14}}$ and *rep* amplified by PCR from different samples (Ho et al., 2011d; Ho et al., 2011c). The FII plasmids were categorized by the FAB formula using the replicon sequence typing scheme (Villa et al., 2010).

2.4 Analysis of $bla_{CTX\text{-}M\text{-}14}$ genetic support

Based on published studies and sequences deposited in the GenBank database, eight different types of $bla_{\text{CTX-M-14}}$ genetic support have been reported. For purpose of description, they were denoted types I to VIII. Monoplex PCRs using different primer pair combination was used to map the genetic support to one of the recognized type (Table S1).

2.5 PCR-RFLP analysis of FII plasmids

The FII plasmids were analysed further by a PCR-RFLP method previously described by us (Table S2) (Ho et al., 2011c).

2.6 Statistical analysis

The Chi square test was used to compare categorical variables between the two time periods. A P value of <0.05 was considered to indicate statistical significance. All statistic analysis was performed by the Epi Info software (version 3.5.1, Centers for Diseases Control and Prevention). A Dice coefficients similarity threshold of 85% was used to resolve unique PFGE groups (Ho et al., 2007; Lo et al., 2010).

3. Result

3.1 Antimicrobial susceptibilities and ESBL types

The isolates often exhibit co-resistance to the non-β-lactam antibiotics. The overall resistance rates for isolates from period 1 (*n*=50) and period 2 (*n*=117) were: cotrimoxazole, 84% and 54.7%; ciprofloxacin, 78% and 59.8%; and gentamicin, 78% and 48.7%. Most isolates were susceptible to amikacin (98% and 97.4%), and piperacillin-tazobactam (84% and 97.4%). All isolates were susceptible to imipenem at the traditional breakpoint (inhibition zone diameter ≥16 mm, equivalent to MIC ≤4 mg/L). All isolates in period 2 were susceptible to imipenem at the new CLSI breakpoint (inhibition zone diameter ≥23 mm and equivalent to MIC ≤1 mg/L). However, only 90% of the isolates from period 1 were susceptible and 10% (5/50) isolates were imipenem-intermediate (inhibition zone diameters, 20-22 mm, and equivalent to MIC 2 mg/L).

PCR with CTX-M specific primers showed that CTX-M-type ESBL was carried by 98.2% (164/167) of the ESBL-producing isolates (Table 1). The CTX-M gene in 109 isolates was sequenced and a total of five alleles (CTX-M-14, -9, -27, -15 and -79) were found. In

both periods, the CTX-M-9 subgroup and CTX-M-14 allele were the predominant CTX-M-type ESBL.

3.2 Epidemiological typing of strains

Table 2 summarised findings from the epidemiological typing. Phylogenetic PCR showed that 56.3% (94/167) of the isolates were phylogenetic group D, 34.1% (57/167) were group B2, 7.2% (12/167) were group A and 2.4% (4/167) were group B1. The proportion of phylogenetic group D had decreased from 84% (42/50) in period 1 to 44.4% (52/117) in period 2 (P<0.001, chi-square test). Conversely, phylogenetic B2 isolates had increased from 6% (3/50) in period 1 to 46.2% (54/117) in period 2 (P <0.001).

Overall, 41.3% (69/167) of the isolates could be assigned to five clones: ST68, ST405, ST131, ST69 and ST12 (Table 2). All five clones were producers of the CTX-M-14 allele. The remaining 98 isolates were either singleton (n=85) or PFGE nontypeable (n=13). Strains belonging to the ST68 and ST405 clones had decreased from 30% (15/50) and 26% (13/50) in period 1, to 2.6% (3/117) and 0% (0/117) in period 2, respectively (P<0.01). On the contrary, strains related to the ST131 clone increased from 0% in period 1 to 25.6% (30/117). The other two clones (ST69 and ST12) were only found in period 2.

3.3 Replicon types of CTX-M-14 encoding plasmids

A subset of 65 CTX-M-14 isolates, including 15 isolates from period 1 and 50 isolates from period 2 was investigated further. The subset was chosen according to the clones (Table 2). One to seven isolates for each clone and about half of the singleton isolates in each period were chosen. At least one isolate for each unique PFGE within the same MLST was chosen. In conjugation experiments, the plasmids harbouring CTX-M 14 could be transferred in 84.6% (55/65) of the isolates at high frequencies (mean, 10^{-2} per donor cells). In all

transconjugants, the ESBL phenotype was the only resistance detected and there was no co-transfer of resistance to ciprofloxacin, chloramphenicol, cotrimoxazole, gentamicin, nalidixic acid and tetracycline.

Six different replicons (FII, FIA, FIB, I1-Iγ, B/O, K), either alone or in combinations were found among the plasmids encoding CTX-M-14 (Table 3). Replicon FII was equally prevalent among CTX-M-14 plasmids in both periods (80%, 12/15 versus 72%, 36/50; *P*=0.7).

3.4 Genetic environment of the $bla_{CTX\text{-}M\text{-}14}$ gene

PCR mapping and sequencing of representative products categorised the genetic environment of the 65 CTX-M-14 genes into four different types (gene arrays): type II (IS*Ecp1*-$bla_{\text{CTX-M-14}}$-*IS903*, *n*=59), type III (ΔIS*Ecp1*-IS*10*-$bla_{\text{CTX-M-14}}$ -IS*903*, *n*=4), type IV (IS*26*-ΔIS*Ecp1*-$bla_{\text{CTX-M-14}}$-IS*903*, *n*=1) and type VII (Class 1 integron- o*rf1* - IS*Ecp1*- $bla_{\text{CTX-M-14}}$ - IS*903*, *n*=1). The class 1 integron in the isolate with type VII context was found to contain genes encoding integrase (*Δintl1*), aminoglycoside resistance (*aacA4*), chloramphenicol resistance (*cmlA*), quaternary ammonium compound resistance (*qacEΔ1*), and sulphonamide resistance (*sul1*), as in the multi-replicon FII/FIB plasmid pETN48 (GenBank accession FQ482074, originating from a *E. coli* strain TN48 from an adult with urinary tract infection in 2004 in Paris). The proportions of CTX-M-14 genes from period 1 and period 2 with type II environment were 80% (12/15) and 94% (47/50), respectively. Those for type III environment were 20% (3/15) and 2% (1/50), respectively (*P*=0.1). The two isolates with type IV and VII environments were found in period 2.

3.5 Analysis of the FII plasmids

All the 54 plasmids with FII replicon (± additional F1A/F1B replicons) were investigated further by replicon sequence typing and PCR-RFLP analysis (Table 4). Replicon sequence typing revealed that there were eight FII (F2, F11, F18, F29, F31, F35, F47 and F49), two FIA (A1 and A4) and four FIB (B1, B6, B10 and B26) alleles with 11 unique FAB formulas. According to the PCR-RFLP patterns for the seven loci, 29 unique profiles were obtained for the 54 plasmids. These included 20 plasmids (group 1) with all seven PCR-RFLP patterns identical to the reference plasmid (pHK01), 23 plasmids (group 2) with identical restriction patterns at three to six loci, and 11 plasmids (group 3) with identical restriction patterns in less than three loci. Hence, 79.6% (43/54) of the plasmid subset had pRFLP profiles that were identical to that for pHK01 at three or more loci. The presence of the *yac* (plasmid stability system), *malB* (maltoporin), *eitA/eitC* (iron transport system) and *parAB* (plasmid partition) genes were assessed by monoplex PCRs using the purified plasmids as template. The result showed that all group 1 plasmids and most (91.3%, 21/23) group 2 plasmids were positive for all targets. In contrast, these genes were variably found among group 3 plasmids. Group 1 and 2 plasmids were found in isolates from both time periods and harboured by the major clones (ST68, ST405 and ST131) and multiple singleton isolates.

4. Discussion

This study demonstrated the dissemination of pHK01-like plasmids encoding CTX-M-14 among hospital *E. coli* isolates of diverse genetic lineages and their persistence in our locality from more than a decade ago. These pHK01-like plasmids were widespread among the collection of community CTX-M-14-producing *E. coli* strains reported by us earlier (Ho et al., 2007; Ho et al., 2011c). The finding confirms the suspicion that the same epidemic plasmid was involved in the dissemination of CTX-M-14 among bacteria in different ecological

niches. It also established that CTX-M-14 has been endemic in this region since the mid-1990s.

Our data further showed that dissemination of the pHK01-like plasmids was linked to several major *E. coli* clones (ST405, ST69, ST131) that have previously been associated with CTX-M dissemination (Naseer and Sundsfjord, 2011). Of these, ST131 is the most important lineage (Rogers et al., 2011). The pandemic spread of ST131 was identified in 2008 during the investigation of CTX-M-15 producing *E. coli* from three continents (Nicolas-Chanoine et al., 2008). Subsequently, many ST131 variants including both ESBL-producing and non-ESBL-producing strains have been described (Lau et al., 2008; Nicolas-Chanoine et al., 2008; Rogers et al., 2011). In the pHK01-like plasmids, $bla_{\text{CT-M-14}}$ was the only resistance determinant found. Since our ST131 isolates exhibited multidrug resistance involving fluoroquinolone, gentamicin and cotrimoxazole, the finding suggested that these ESBL-producing strains have likely emerged from pre-existing multidrug-resistant strains through the capture of CTX-M-14 harbouring, pHK01-like plasmids. Prior to the CTX-M pandemic, existing data suggest that ST131 is already one of the major *E. coli* lineages in human infections and are often resistant to fluoroquinolone and multiple other antibiotics (Rogers et al., 2011). Our finding that CTX-M-14-producing isolates of ST131 encompassed multiple PFGE groups suggests that diversification has occurred within this lineage. Although in most parts of the world, especially for Europe the ST131 lineage is often associated with CTX-M-15 (Lau et al., 2008; Naseer and Sundsfjord, 2011), our result and other reports showed that the dominant CTX-M allele among ST131 strains in Asia is CTX-M-14 (Shin et al., 2011; Suzuki et al., 2009). Of note, ST131 and ST69 that seems to be emerging in period 2 represent lineages that were usually associated with high virulence gene content (Blanco et al., 2011). This could partially explain reports of CTX-M-14-producing isolates among young and healthy individuals with community-acquired infections (Ho et al., 2007) and the marked

increase in ESBL incidence among *E. coli* isolates in the last few years (Ho et al., 2011a). In our healthcare region, the incidence rate of ESBL-producing *E. coli* bacteremia (as episodes per 10,000 admissions) had increased from 1.9 in 1996-1998 (period 1) to 6.0 in 2003 and 8.5 in 2007-2008 (period 2) (Ho et al., 2002; Ho et al., 2011b).

Multiple PFGE patterns were also found among ST68 and ST405 isolates, indicating that these two clones had also diversified. Interestingly, ST405 which was found in period 1 was not found in period 2. In recent studies, ST405 along with ST131 were important clones associated with the dissemination of CTX-M genes (Naseer and Sundsfjord, 2011; Shin et al., 2011). Besides a change in the major clones, there was an increase in proportion of singletons, a decrease in phylogenetic group D and an increase in phylogenetic group B2 isolates in period 2. In general, phylogenetic group B2 isolates are known to possess more virulence factors (Ferjani et al., 2011). It is possible that the higher virulence of phylogenetic group B2 isolates might give them an advantage for their increased prevalence in period 2. The increased in singletons in period 2 indicated that the pHK01-like plasmids have disseminated more widely in different bacterial populations. Given the expansion small number of successful clones and the horizontal transfer of pHK01-like incompatibility FII plasmids, a change in antibiotic strategies for empirical therapy of presumed *E. coli* infection is necessary. Since pHK01-like plasmids are especially prevalent among isolates causing urinary tract infections, empirical therapy for critically ill patients with presumed *E. coli* bacteremia or serious urosepsis should include a carbapenem while waiting for the culture and sensitivity results (Meier et al., 2011).

In both the FII and non-FII plasmids, the predominant genetic context for $bla_{\text{CTX-M-14}}$ consisted of an upstream IS*Ecp1* and a downstream IS*903*. These mobile elements might be involved in mobilization of $bla_{\text{CTX-M-14}}$ across different plasmid platforms. In the PCR-RFLP scheme, two loci (B1 and B2) were designed to assess genetic organization of the variable

region bearing *bla*$_{CTX-M-14}$, including the regions outside the two *IS* elements. In almost all the isolates, the restriction patterns correlated with a conserved array of genes in the variable region (*yac-malB*-IS*903-bla*$_{CTX-M-14}$-IS*Ecp1-eitABCD*) inserted between the blocks of replication genes and the partition genes. GenBank search revealed that this array of genes is specific to pHK01-like plasmids.

This study characterised the IncFII plasmids by replicon sequence typing and a PCR-RFLP plasmid typing scheme. Although the replicon sequence typing method is easy to use, our finding showed that it cannot accurately reflect similarity or variations in the plasmid scaffold. Plasmids with identical or highly similar pRFLP profiles could exhibit different FAB formulas. On the other hand, plasmids with entirely different pRFLP profiles could have the same FAB formula. The diversity in FAB formula and various plasmid backbone blocks support the notion that this group of pHK01-like plasmids is evolving, and presumably reflecting DNA rearrangements, mutations, gene loss and integration of mobile elements.

In conclusion, this study showed that the spread of CTX-M-14 among bacteremic *E. coli* isolates in this region is associated with the expansion of small number of successful clones and the horizontal transfer of pHK01-like incompatibility group FII plasmids.

Funding

This work was supported by grants from the Research Fund for the Control of Infectious Diseases (RFCID) of the Health, Welfare and Food Bureau of the Government of the HKSAR, and the Consultancy Service for Enhancing Laboratory Surveillance of Emerging Infectious Disease for the HKSAR Department of Health.

Transparency declarations

All authors have no conflicts of interest to declare.

Table 1. Distribution of CTX-M subgroups and alleles among the ESBL-producing *E. coli* isolates

	No. (%)		
	Period 1	Period 2	Total
CTX-M subgroup[a]			
M9	49 (98.0)	101 (86.3)	150 (89.8)
M1	-	12 (10.3)	12 (7.2)
M1+ M9	-	2 (1.7)	2 (1.2)
negative	1 (2.0)	2 (1.7)	3 (1.8)
subtotal	50 (100)	117 (100)	167 (100)
CTX-M allele[b]			
CTX-M-14	47	56	103
CTX-M-9	2	1	3
CTX-M-27	-	1	1
CTX-M-15	-	1	1
CTX-M-79	-	1	1
subtotal	49	60	109

[a] PCR with CTX-M subgroup-specific primers.

[b] CTX-M-14, -9 and -27 are members of the CTX-M-9 subgroup while CTX-M-15 and -79 are members of the CTX-M-1 subgroup. All CTX-M-14 sequences were identical to the CTX-M-14a variant (AF252622).

Table 2. Characteristic of 167 bacteremia ESBL-producing *E. coli* according to time period.

Clone[a]	*n*	No. of PFGE groups[c]	No of isolates, by phylogenetic group				No. of isolates (No. of isolates chosen for plasmid analysis)	
			A	B1	B2	D	Period 1	Period 2
NT-ST68/CTX-M-14	18	3	-	-	-	18	15 (2)	3 (1)
O102-ST405/CTX-M-14	13	2	-	-	-	13	13 (2)	-
O25b-ST131/CTX-M-14	30	3	-	-	30	-	-	30 (7)
O15-ST69/CTX-M-14	5	1	-	-	-	5	-	5 (1)
O12-ST12/CTX-M-14	3	1	-	-	3	-	-	3 (1)
Singletons[b]	98	-	12	4	24	58	22 (11)	76 (40)
Subtotal	167	-	12	4	57	94	50 (15)	117 (50)

[a]The serotype-sequence type and CTX-M allele were shown. NT, non-typeable.

[b]Including 13 isolates that could not be typed because of auto-digestion. These isolates produce various CTX-M alleles (-9, -14, -15, and -27) and one isolate was negative for CTX-M.

[c]Defined at 85% similarity level.

Table 3. Replicon type of plasmids encoding CTX-M-14, Hong Kong

Replicon type[a]	*n*	Plasmid sizes, kb	No. (%)	
			Period 1	Period 2
FII	48	70-150	12 (80.0)	36 (72.0)
FII, FIA, FIB	3	90-100	-	3 (6.0)
FII, FIB	2	70-150	-	2 (4.0)
FII, FIA	1	50	-	1 (2.0)
FIA, FIB	1	50	1 (6.7)	-
I1-Iγ	3	100	2 (13.3)	1 (2.0)
B/O	2	100	-	2 (4.0)
K	1	100	-	1 (2.0)
non-typable	4	70-100	-	4 (8.0)
Total	65		15 (100)	50 (100)

Table 4. Characteristics of IncFII plasmids encoding CTX-M-14

	No of plasmids[c]		
	Group 1	Group 2	Group 3
Number of plasmids	20	23	11
FAB formula			
F2:A-:B-	19	5	1
F35:A-:B-	-	16	3
Others[a]	1	2	7
PCR profile			
malB-yac-eitA-eitC-parAB	20	21	1
Others[b]	-	2	3
None	-	-	7
Time			
Period 1	9	2	1
Period 2	11	21	10
Clonal group			
ST68	2	1	-
ST405	1	1	-
ST131	3	2	2
ST69	-	-	1
Singletons	14	19	8

[a]Including group 1: F47:A-:B- (*n*=1), group 2: F11:A-:B- (*n*=1) and F49:A-:B- (*n*=1), group 3: F18:A-:B- (*n*=1), F2:A1:B-(*n*=1), F29:A-:B10 (*n*=1), F31:A-:B6 (*n*=1), F31:A4:B1 (*n*=2), F31:A4:B26 (*n*=1).

[b]Including the following profiles: *malB-yac-eitA-parAB* (*n*=1), *malB-yac-eitC-parAB* (*n*=1), *eitA-eitC-parAB* (*n*=1), *eitA-eitC* (*n*=1) and *eitA* (*n*=1).

[c]The plasmids were categorised according to their similarity to the reference plasmid: group 1, all seven PCR-RFLP patterns were identical to reference; group 2, three to six restriction patterns were identical; and group 3, less than three restriction patterns were identical.

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