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Molecular Epidemiology of CTX-M-Producing *Escherichia coli* Isolates at a Tertiary Medical Center in Western Pennsylvania[▽]

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A combination of phenotypic and genotypic methods was used to investigate 70 unique *Escherichia coli* clinical isolates identified as producing extended-spectrum β-lactamases (ESBLs) at a medical center in Pittsburgh, PA, between 2007 and 2008. Fifty-seven isolates (81%) produced CTX-M-type ESBLs, among which CTX-M-15 was predominant ($n = 46$). Isolates producing CTX-M-2, -9, -14, and -65 were also identified. One CTX-M-producing isolate coproduced CMY-2 cephalosporinase. Ten isolates (14%) produced SHV-type ESBLs, either SHV-5 or SHV-7. Two isolates produced only CMY-2 or -32. Pulsed-field gel electrophoresis revealed the presence of two major clusters of CTX-M-15-producing *E. coli* isolates, one in phylotype B2 ($n = 15$) and the other in phylotype A ($n = 19$). Of four phylotype B2 isolates that were able to transfer the $bla_{CTX\text{-}M\text{-}15}$-carrying plasmids, three showed fingerprints related (>60%) to those of plasmids from phylotype A isolates. In phylotype B2, all CTX-M-15-producing isolates, as well as three isolates producing CTX-M-14, two producing SHV-5, and one producing SHV-7, belonged to the international epidemic clone defined by serotype O25:H4 and sequence type 131. The plasmids from eight of nine CTX-M-15-producing *E. coli* isolates of phylotype A that were examined were highly related to each other and were also found in two isolates belonging to phylotype D, suggesting horizontal transfer of this $bla_{CTX\text{-}M\text{-}15}$-carrying plasmid between phylotypes. Our findings underscore the need to further investigate the epidemiology and virulence of CTX-M-producing *E. coli* in the United States.

The most common cause of resistance to expanded-spectrum cephalosporins in *Escherichia coli* is the production of extended-spectrum β-lactamases (ESBLs) (29). In the past decade, CTX-M-type ESBLs have replaced TEM- and SHV-type ESBLs in Europe, Canada, and Asia as the most common ESBL type in this species (1, 4, 19, 22, 25, 30). The United States initially appeared immune to this epidemic of CTX-M-type ESBLs. However, an increasing number of reports now indicate that CTX-M-type ESBLs have emerged in *E. coli* and other species of *Enterobacteriaceae* in the United States as well (7, 17, 24). A study performed in Texas revealed that CTX-M-producing *E. coli* strains were already present in small numbers in 2000 and became predominant by 2004 (22).

CTX-M-type ESBLs can be grouped into five clusters according to genetic relatedness: the CTX-M-1, -2, -8, -9, and -25 groups (5a). Of the different CTX-M-type ESBLs, CTX-M-15, which belongs to the CTX-M-1 group, is now the most widely distributed enzyme worldwide. CTX-M-15 was first identified in an isolate from India in 1999 (18) and then became prevalent worldwide (5, 6, 11, 23). CTX-M-15 differs from CTX-M-3 by one amino acid substitution at position 240 (Asp240Gly). This substitution enhances hydrolytic activity against ceftazidime, which otherwise is not a preferred substrate for CTX-M-type ESBLs (31). Of note, a particular clone of CTX-M-15-producing *E. coli*, characterized by phylogenetic type (phylotype) B2, serotype O25:H4, and sequence type 131 (ST131), appears to be responsible for a large part of the international epidemic of CTX-M-producing *E. coli* (11, 27). Here we describe detailed molecular-epidemiology and plasmid analyses of CTX-M-producing *E. coli* isolates identified at our medical center in Western Pennsylvania.

MATERIALS AND METHODS

Bacterial strains. A total of 70 consecutive and unique *E. coli* isolates identified from patient specimens between May 2007 and April 2008 at the clinical microbiology laboratory of the University of Pittsburgh Medical Center were included. These isolates had positive phenotypic confirmatory test results for ESBL production based on ≥5-mm increases in zone diameters when a ceftazidime or cefotaxime disk was combined with clavulanic acid, as described in the Clinical and Laboratory Standards Institute (CLSI) guidelines (10). The sources of isolates and patient locations were recorded, and the isolates were kept at −80°C until use.

Susceptibility testing. The antimicrobial susceptibilities of the study strains to ceftazidime, ceftazidime–clavulanic acid, cefotaxime, cefotaxime–clavulanic acid, cefoxitin, cefepime, imipenem, ciprofloxacin, gentamicin, tetracycline, chloramphenicol, sulfamethoxazole–trimethoprim, and amikacin were determined by using the disk diffusion assay as defined by the CLSI (10). ESBL production was phenotypically reconfirmed by using the same disk method in the research laboratory.

PCR analysis and nucleotide sequencing. (i) PCR for detection of resistance genes and determination of phylotypes. Crude genomic DNA was extracted from the isolates by heat lysis. The DNA was then subjected to PCR using specific primer pairs to screen for β-lactamase genes (consensus $bla_{CTX\text{-}M}$, $bla_{CTX\text{-}M\text{-}2}$,

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TABLE 1. Primers used for amplification of resistance genes and replicon typing

Primer	Target gene/site	Sequence (5′ to 3′)	Annealing temp (°C)	Reference
TEMF	bla_{TEM}	ATGAGTATTCAACATTTCCGTG	55	12
TEMR		TTACCAATGCTTAATCAGTGAG		
SHV S1	bla_{SHV}	ATTTGTCGCTTCTTTACTCGC	55	12
SHV S2		TTTATGGCGTTACCTTTGACC		
CTX-M/F	bla_{CTX-M}	TTTGCGATGTGCAGTACCAGTAA	51	14
CTX-M/R		CGATATCGTTGGTGGTGCCATA		
CTX-M-2 group F	$bla_{CTX-M-2}$	AAATGTGCTGCTCCTTTCGTGAGC	60	12
CTX-M-2 group R		AGGGTTCGTTGCAAGACAAGACTG		
CTX-M-9 group F	$bla_{CTX-M-9}$	GTGACAAAGAGAGTGCAACGC	60	34
CTX-M-9 group R		ATGATTCTCGCCGCTGAAGCC		
CTX-M-15-SF	$bla_{CTX-M-15}$	CACACGTGGAATTTAGGGACT	55	26
CTX-M-15-SR		GCCGTCTAAGGCGATAAACA		
CMY-F	bla_{CMY}	CCGGACACCTTTTTGCTTTT	60	35
CMY-R		TATCCTGGGCCTCATCGTCAGTTA		
OXA-1 F	bla_{OXA-1}	TTTTCTGTTTGGGTTTT	52	2
OXA-2 R		TTTCTTGGCTTTTGTGCTTG		
aac(6′)-1b f	*aac(6′)-Ib*	TTGCGATGCTCTATGAGTGGCTA	55	28
aac(6′)-1b r		CTCGAATGCCTGGCGTGTTT		
rfb.1bis	O typing for O25	ATACCGACGACGCCGATCTG	60	9
rfb25.r		TGCTATTCATTATGCGCAGC		

$bla_{CTX-M-9}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}, bla_{CMY}, and bla_{OXA-1}) (2, 12, 14, 26, 34, 35) and phylotypes (Table 1) (8). PCR analysis was performed using a GeneAmp thermal cycler, model 9700 (Applied Biosystems, Foster City, CA). DNA sequencing of resistance genes was performed using an ABI 3730 instrument with primers that generated the amplified products. Sequences were then analyzed using the BLAST software available from the National Library of Medicine (http://www.ncbi.nlm.nih.gov/blast).

(ii) O25 typing, plasmid incompatibility grouping, and MLST. PCR-based O25 typing was employed for all *E. coli* isolates using the primers listed in Table 1. Ten CTX-M-15-producing *E. coli* isolates were O and H typed at the *E. coli* Reference Center, Pennsylvania State University. Further, to identify the pandemic clone characterized by serotype O25:H4, STs were determined by multilocus sequence typing (MLST) for O25 PCR-positive isolates as well as for CTX-M-15-producing *E. coli* isolates belonging to phylotypes D ($n = 2$) and A ($n = 5$) (37). PCR products from MLST were also sequenced as described above. Allelic profiling and ST determinations were performed through the *E. coli* MLST website maintained at the Max-Planck Institut fuer Infektionsbiologie (now at the ERI, University College Cork [http://mlst.ucc.ie/mlst/dbs/Ecoli]).

PFGE. All isolates were evaluated for genomic clonality by pulsed-field gel electrophoresis (PFGE). Genomic DNAs of 70 isolates were prepared as described previously (32). Fingerprints were generated by restriction endonuclease XbaI (New England Biolabs, Ipswich, MA) and subjected to electrophoresis using a CHEF-DR III system (Bio-Rad, Hercules, CA) at 6 V with pulse times of 2.2 to 54.2 s and linear ramping at 14°C for 22 h. A lambda ladder (New England Biolabs) was used as the DNA size marker. The relatedness of PFGE patterns was determined by the unweighted-pair group method using average linkages and cluster analysis with the Dice setting on Bionumerics software, version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium).

Plasmid extraction, transfer, and Southern hybridization. Plasmids of 16 isolates carrying $bla_{CTX-M-15}$ were analyzed in detail, as described below, in order to compare them within the collection and also with $bla_{CTX-M-15}$-carrying plasmids characterized in non-U.S. isolates. In addition, four isolates producing $bla_{CTX-M-2}$, $bla_{CTX-M-14}$, or $bla_{CTX-M-65}$ were also included in order to compare the relatedness among the plasmids. The transferability of $bla_{CTX-M-15}$-carrying plasmids was assayed by both transformation and conjugation. The transferability of plasmids by conjugation was analyzed by liquid mating in Luria-Bertani (LB) broth with the sodium azide-resistant *E. coli* strain J53Azi^R as the recipient. Transconjugants were selected on LB agar containing sodium azide at 100 μg/ml and ampicillin at 50 μg/ml. The transfer of a bla_{CTX-M}-carrying plasmid was confirmed by the resistance profiles of the transconjugants and positive PCR results for bla_{CTX-M}. For the examination of plasmid transferability by transformation and for use in fingerprinting, plasmids were extracted using the alkaline lysis method and were transformed into *E. coli* DH10B by electroporation as previously described (35). Transformants were selected on LB agar containing 2 μg/ml of cefotaxime (Sigma-Aldrich, St. Louis, MO). The fingerprints of the bla_{CTX-M}-carrying plasmids were generated by digesting plasmids with restriction enzyme HpaI (New England Biolabs). After electrophoresis, plasmid fingerprints were visualized and then transferred to a positively charged nylon membrane (Roche Diagnostics, Indianapolis, IN). DNA probes were prepared from PCR amplicons of $bla_{CTX-M-15}$ using a digoxigenin nucleic acid labeling and detection system (Roche Diagnostics). The clonality of the plasmid fingerprints was assessed using Bionumerics software. The presence of bla_{TEM}, bla_{OXA-1}, and *aac-(6′)-Ib* on these plasmids was determined as well. The plasmid profiles were compared to the published characteristics of $bla_{CTX-M-15}$-carrying plasmids (5, 11).

RESULTS

Antimicrobial susceptibilities of *E. coli* isolates. The sources of the 70 study isolates included urine (48 isolates), superficial wounds (9 isolates), bronchoalveolar lavage fluid (7 isolates), sputum (3 isolates), and blood (3 isolates). No cluster of cases according to patient location was observed. Two isolates showed a phenotype consistent with AmpC production and only minimal inhibition by clavulanic acid. They did not carry ESBL genes but instead carried CMY-type cephalosporinase genes. The remaining 68 ESBL producers were resistant to cefotaxime or ceftazidime or to both and showed inhibition by clavulanic acid (zone diameter increase of ≥5 mm with combination disks containing ceftazidime or cefotaxime plus clavulanic acid compared with disks containing ceftazidime or cefotaxime alone). All but six isolates were resistant to ciprofloxacin (92%). All isolates in this study were susceptible to imipenem.

Resistance genes and phylotypes. Table 2 shows the distribution of ESBL and CMY-type cephalosporinase genes and phylotypes. CTX-M was the predominant ESBL type, produced by 81% of all isolates ($n = 57$). All isolates carrying bla_{CTX-M} showed resistance to cefotaxime. Additionally, 46 CTX-M-producing *E. coli* isolates were also resistant or intermediately resistant to ceftazidime. These 46 isolates carried $bla_{CTX-M-15}$. The other CTX-M-type ESBL genes identified were in the $bla_{CTX-M-2}$ and $bla_{CTX-M-9}$ groups (Table 2). The

TABLE 2. Resistance genes and phylotypes of ESBL-producing *E. coli* isolates

β-Lactamase gene	No. (%) of isolates	No. of isolates of phylotype:			
		A	B1	B2	D
CTX-M	57 (81)	26	0	18	13
CTX-M-15	46 (66)	25[a]	0	15[b]	6
CTX-M-2	1 (1)	0	0	0	1
CTX-M-9 group	10 (14)	1	0	3	6
CTX-M-9	1 (1)	0	0	0	1
CTX-M-14	8 (11)	1	0	3[c]	4
CTX-M-65	1 (1)	0	0	0	1[d]
SHV-5 or -7	10 (14)	1	1	5[e]	3
TEM-10	1 (1)	0	0	1	0
CMY-2 or -32	2 (3)	0	0	0	2
Total	70	27	1	24	18

[a] All isolates possessed bla_{OXA-1} and *aac-6′-Ib*, and 19 isolates shared ≥75% similarities based on PFGE.
[b] All 15 isolates were O25-ST131. The presence of bla_{TEM}, bla_{OXA-1}, and *aac-6′-Ib* differed among the isolates.
[c] All isolates were O25-ST131.
[d] This isolate also possessed bla_{CMY-2}.
[e] Three isolates were O25-ST131.

majority of the 10 isolates in the $bla_{CTX-M-9}$ group possessed $bla_{CTX-M-14}$ ($n = 8$). A single isolate possessed both $bla_{CTX-M-65}$ ($bla_{CTX-M-9}$ group) and bla_{CMY-2}, which were carried on two separate transferable plasmids. SHV-producing *E. coli* isolates were detected less frequently ($n = 10$ [14%]). These isolates carried bla_{SHV-5} or bla_{SHV-7} and were resistant to ceftazidime and intermediately resistant to cefotaxime. The two isolates possessing bla_{CMY-2} or bla_{CMY-32} but no ESBL gene were considered false-positive ESBL producers. This rare phenomenon has been reported previously for some isolates possessing plasmid-mediated cephalosporinase (33, 35). Only a single isolate had a TEM-type ESBL gene, which was identified as bla_{TEM-10}. This isolate was resistant to ceftazidime and had reduced susceptibility to cefotaxime. The bla_{TEM-10}-carrying plasmid was also transferable, and the transformant showed the same resistance profile as the parent isolate.

Overall, there was no predominance of a particular phylotype in this study. A total of 27, 1, 24, and 18 isolates belonged to phylotypes A, B1, B2, and D, respectively (Table 2). Of the 24 isolates in phylotype B2, 15 produced CTX-M-15. Most of the phylotype A isolates (25 of 27) were CTX-M-15 producers as well. ESBLs produced by phylotype D isolates were more variable (Table 2).

O25 typing and MLST. Twenty-one isolates belonging to phylotype B2 (15 producing CTX-M-15, 3 producing CTX-M-14, 2 producing SHV-5, and 1 producing SHV-7) were determined to be serotype O25:H4 and ST131. All six CTX-M-15-producing *E. coli* isolates belonging to phylotype A that were tested for ST had ST410, which belongs to the ST23 complex. These strains were found to have serotype O20:H9. The two CTX-M-15-producing *E. coli* isolates belonging to phylotype D were nonserotypeable and were determined to be ST648.

Molecular typing for clonal detection. DNA fingerprints resulting from PFGE were assigned to pulsotypes. Each pulsotype comprised genetically related or indistinguishable isolates. PFGE revealed the presence of two predominant pulsotypes, designated pulsotype A ($n = 19$), with ≥75% similarities, and pulsotype B ($n = 18$), with ≥50% similarities (Fig. 1). All 19 isolates in pulsotype A had phylotype A and produced CTX-M-15. All 15 CTX-M-15-producing isolates in pulsotype B had phylotype B2. Additionally, three phylotype B2 isolates producing CTX-M-14 and three producing SHV (two SHV-5 isolates and one SHV-7 isolate) belonged to pulsotype B. The rest of the isolates (including 12 CTX-M-15 producers) were clonally diverse, with each pulsotype containing three isolates or fewer.

Plasmid analysis. The antimicrobial resistance genes bla_{OXA-1} and *aac-(6′)-Ib* were consistently present on the $bla_{CTX-M-15}$-carrying plasmids of all phylotype A isolates tested. Unlike the international epidemic clone, however, our $bla_{CTX-M-15}$-carrying plasmids in this clonal group did not possess bla_{TEM} (5, 11). For CTX-M-15-producing *E. coli* isolates in phylotype B2, after multiple attempts at conjugation and transformation from all parent strains, plasmids from only four isolates were transferable by conjugation or transformation. The other antimicrobial resistance genes located on this $bla_{CTX-M-15}$-carrying plasmid were variable, with plasmids from two isolates carrying bla_{OXA-1}, a plasmid from one isolate carrying bla_{TEM}, and a plasmid from one isolate carrying both bla_{TEM} and bla_{OXA-1}. The restriction profiles of three of these four plasmids showed 60% to 85% similarities to each other and up to 60% similarity to those of phylotype A isolates (Fig. 2 and 3). However, Southern hybridization analysis showed that the plasmid fragments containing $bla_{CTX-M-15}$ were all different in size from each other and from those of phylotype A isolates. The profile of a single $bla_{CTX-M-15}$-carrying plasmid from a phylotype B2 isolate (Ec9) was very similar to that of pC15-1a, previously reported from Canada (5). However, the $bla_{CTX-M-15}$ probe hybridized to ca. 23-kb and 4.5-kb plasmid fragments, whereas the Canadian plasmids hybridized to ca. 23-kb and 12-kb plasmid fragments (Fig. 3). The other three plasmids in phylotype B2 isolates (Ec15, Ec23, and Ec27) had profiles similar to those of "cluster I" plasmids, reported in isolates from multiple countries (Fig. 2) (11). Plasmid profiles of phylotype A did not match with published plasmid profiles.

DISCUSSION

Prior to 2007, CTX-M-producing *E. coli* isolates were rarely reported in the United States (7, 22). At our institution, TEM- and SHV-type ESBLs were still predominant until late 2006, when CTX-M-15-producing *E. coli* isolates were identified (13). The number of clinical cases caused by CTX-M-producing *E. coli* then increased steadily after around August 2007. The proportion of CTX-M production among ESBL-producing *E. coli* isolates in our study was high (57 of 70 isolates [81%]). This result was consistent with the findings of a nationwide survey, which detected CTX-M-type ESBLs in 83% of ESBL-producing *E. coli* isolates (7). A recent study conducted in two hospitals in Philadelphia also reported that a significant portion (48%) of their cephalosporin-resistant *E. coli* isolates produced CTX-M-type ESBLs (24). Our findings add to the growing evidence that CTX-M-type ESBLs, in particular CTX-M-15, are becoming the predominant ESBLs in *E. coli* strains in the United States.

Our study identified several noteworthy features of the molecular epidemiology of CTX-M-producing *E. coli* isolates at

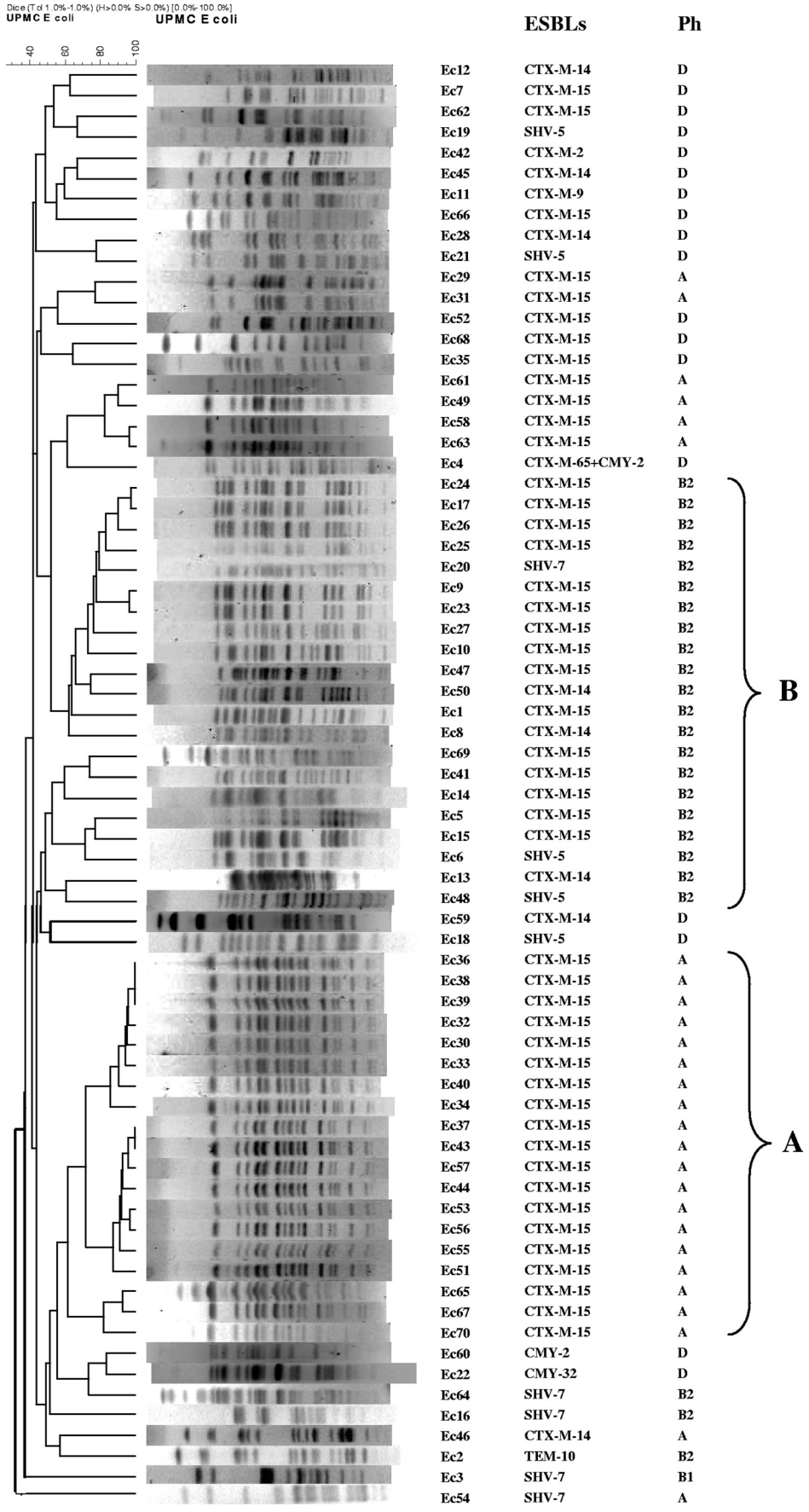

FIG. 1. Dendrogram based on PFGE profiles of 70 *E. coli* isolates. Ph, phylotype; A, pulsotype A; B, pulsotype B.

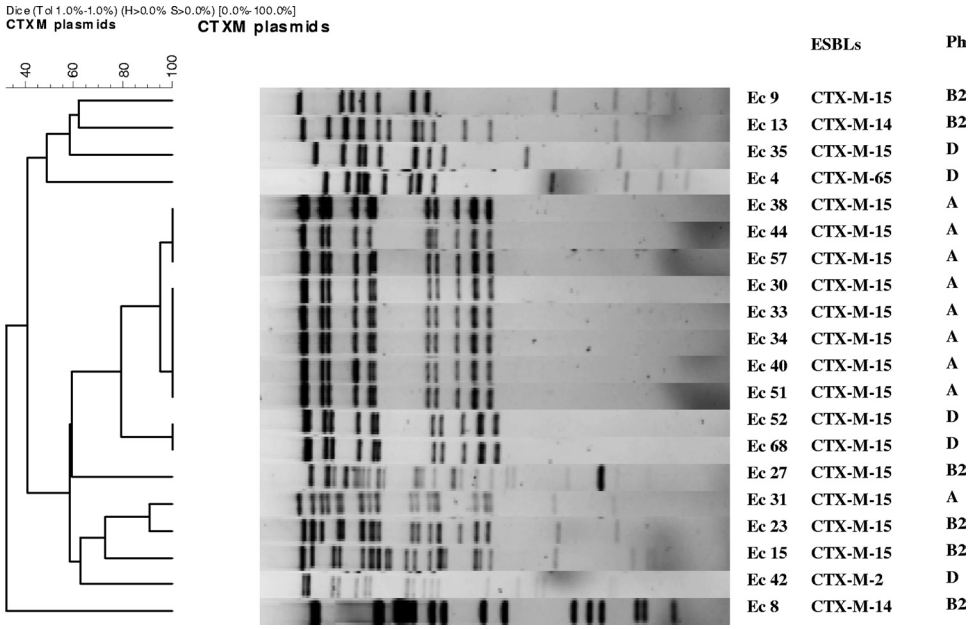

FIG. 2. Dendrogram of plasmids carrying $bla_{\text{CTX-M}}$. Ph, phylotypes.

our institution. First, two major clones of CTX-M-15-producing *E. coli* were identified. The first was a CTX-M-15-producing *E. coli* clone with the characteristics of the international epidemic clone, i.e., phylotype B2, serotype O25:H4, and ST131 (9, 11, 27, 38). Some variation in PFGE profiles was observed for isolates within the ST131 clonal group. This phenomenon has been reported previously (20, 27). The second clone, which is unique to this study, was a CTX-M-15-producing *E. coli* clone belonging to phylotype A and ST410. A recent study of CTX-M-15-producing *E. coli* isolates in the United States reported that 45% of such isolates were ST131 (17). Our study found a lower percentage of ST131 among our CTX-M-15-producing *E. coli* isolates (33%). The predominant CTX-M-15-producing clone at our institution was that in phylotype B2 until late 2007. However, the phylotype A clone replaced the phylotype B2 clone thereafter and continued to predominate until the end of the study period. Isolates in phylotype A, along with those in phylotype B1, are conventionally considered commensals, less virulent than isolates in phylotypes B2 and D. The tendency of the CTX-M-15-producing phylotype A clone to spread therefore mandates further investigation, including detailed clinical analysis and determination of virulence factors.

Second, in addition to the clonal spread of phylotype A CTX-M-15-producing *E. coli*, which was demonstrated by both PFGE and plasmid analysis, horizontal transfer of the $bla_{\text{CTX-M-15}}$-carrying plasmid appears to have occurred between phylotypes A and D. While we cannot determine which phylotype acquired this plasmid first, the fact that this plasmid is able to maintain its stability regardless of different phylogenetic backgrounds is intriguing. On the other hand, plasmid profiles of phylotype B2 isolates were more variable. This is consistent with the findings from Europe, Canada, and Asia documenting the genetic variability of $bla_{\text{CTX-M-15}}$-carrying plasmids in this phylotype (11). It is worth noting that the fingerprints of the $bla_{\text{CTX-M-15}}$-carrying plasmids from phylotype B2 were clonally related (>60% similarity) to that of the plasmid representing the CTX-M-15-producing phylotype A clone, suggesting the possibility of common ancestry of these plasmids across the phylotypes.

We tried to identify the location of $bla_{\text{CTX-M-15}}$ in the rest of the phylotype B2 isolates, whose plasmids were not transferred by conjugation and transformation using several approaches. Southern hybridization analysis using a $bla_{\text{CTX-M-15}}$ probe and a PFGE gel showed that $bla_{\text{CTX-M-15}}$ was located on fragments larger than 200 kb, indicating that these fragments were either on very large plasmids or on the chromosome. Southern hybridization analysis of plasmids from these isolates was negative for $bla_{\text{CTX-M-15}}$ (data not shown). Finally, S1 nuclease analysis revealed the presence of large plasmids (>50 kb) in these isolates, none of which hybridized with the $bla_{\text{CTX-M-15}}$ probe (data not shown) (16). These results suggested that $bla_{\text{CTX-M-15}}$ was likely located on the chromosome for these isolates (11, 15).

Third, while the majority of ST131 isolates in our study produced CTX-M-15 ($n = 15$), we also observed ST131 for *E. coli* isolates producing CTX-M-14 ($n = 3$) and SHV-5 or -7 ($n = 3$). A recent study from Japan showed an association of serotype O25 with ST73 in addition to ST131 (36). In contrast to other studies, all of these ST131 isolates in this Japanese study produced enzymes of the CTX-M-2 and CTX-M-9 groups. ST131 isolates without $bla_{\text{CTX-M-15}}$ have also been isolated from healthy humans in France and have been found among virulent *E. coli* isolates in the United States (3, 21). Thus, it is possible that ST131 strains are particularly adept at acquiring plasmids that carry various resistance determinants, not limited to $bla_{\text{CTX-M-15}}$.

In summary, we characterized the emergence of two predominant clones of *E. coli* producing CTX-M-15 at our institution: one that constitutes part of the worldwide epidemic and

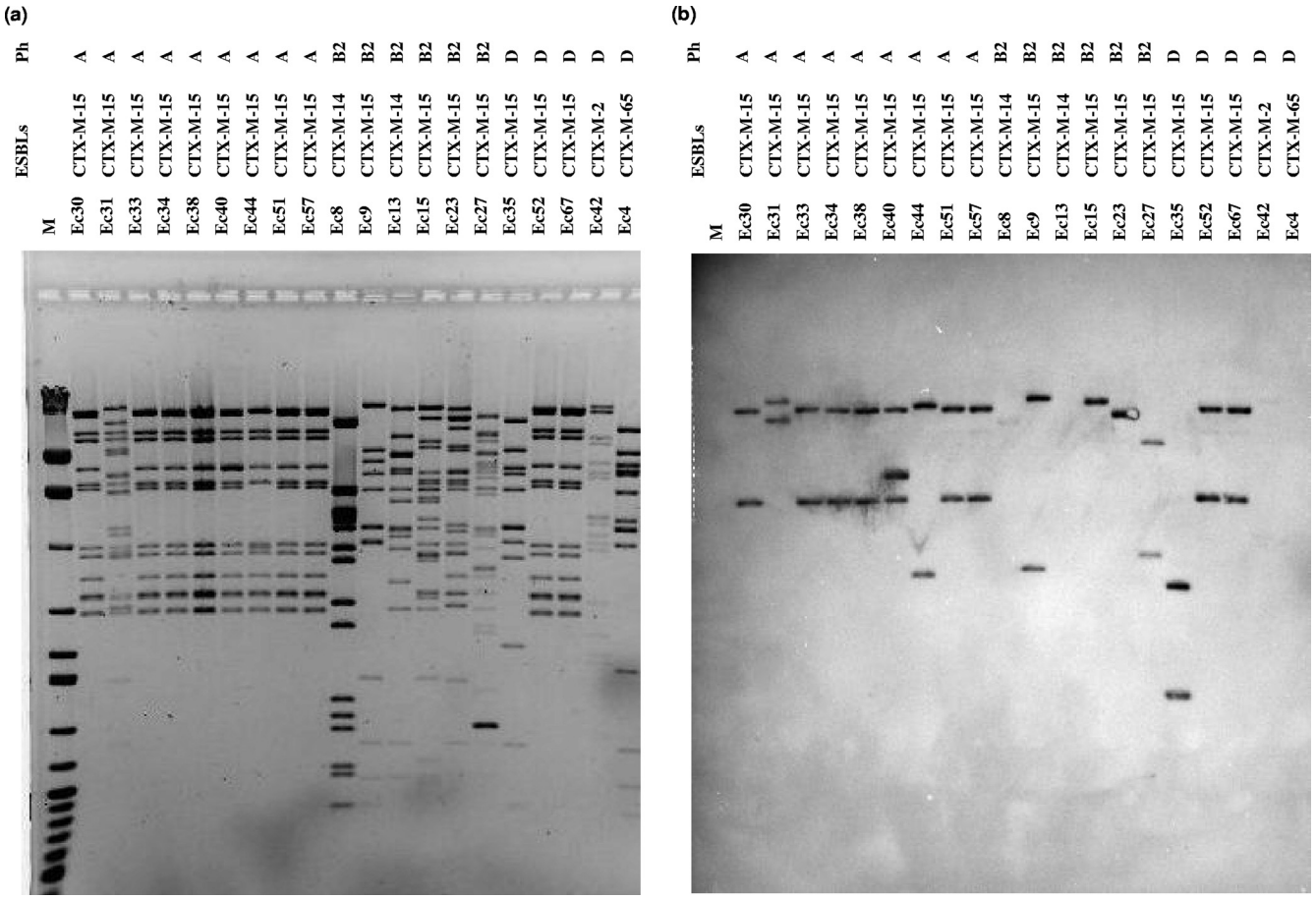

FIG. 3. Profiles of HpaI-digested plasmids from CTX-M-producing *E. coli* isolates and Southern hybridization. (a) Plasmid profiles of transformants; (b) Southern hybridization of plasmids using a digoxigenin-labeled $bla_{\text{CTX-M-15}}$ probe. M, λ/HindIII and 100-bp ladder (MBI Fermentas); Ph, phylotypes.

one that has not been reported previously and thus appears to have emerged locally. Our findings further demonstrate the versatility of CTX-M-15 and the plasmids encoding this ESBL. Detailed molecular typing of plasmids and genomes of isolates from various locations will help better define the epidemiology of CTX-M-15-producing *E. coli* in the Unites States. In addition, further work is required to examine the virulence of CTX-M-15-producing isolates in various phylotypes and to evaluate the clinical impact of acquisition of these isolates.

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