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Prevalence of CTX-M β-Lactamases in Philadelphia, Pennsylvania▽

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CTX-M β-lactamases were thought to be rare in the United States, but a recent study in Texas showed that up to 70% of extended-spectrum β-lactamase (ESBL)-containing members of the *Enterobacteriaceae* family were CTX-M positive (J. S. Lewis, M. Herrera, B. Wickes, J. E. Patterson, and J. H. Jorgensen, Antimicrob. Agents Chemother. 51:4015–4021, 2007). We used PCR to detect CTX-M in all 291 extended-spectrum cephalosporin-resistant gram-negative bacteria isolated in our laboratory during 2007. Thirty (48%) *Escherichia coli* isolates, 6 (3%) *Klebsiella* sp. isolates, and 7 (100%) *Proteus mirabilis* isolates tested were CTX-M positive, with 15% of all *Enterobacteriaceae* tested being positive. The *E. coli* CTX-M groups were I (57%), IV (37%), II (3%), and not groupable (3%); three of the group IV isolates were positive for CTX-M-18, and three of the group I isolates were positive for CTX-M-15. One of seven positive *P. mirabilis* isolates was in group II, with the remainder being positive for a CTX-M-25-like β-lactamase; and 33% of the *Klebsiella* sp. isolates were in group I or IV, with the remainder not being in groups I to IV. CTX-M-producing bacteria were isolated from urine ($n = 13$), blood ($n = 13$), wounds ($n = 12$), and the respiratory tract ($n = 4$). All 31 CTX-M-positive isolates tested for the presence of ESBL were confirmed to produce ESBLs by the use of tests recommended by the CLSI. Pulsed-field gel electrophoresis of the CTX-M-positive isolates showed that six *P. mirabilis* isolates were clonal and that there were seven different *E. coli* clusters. Five of seven *P. mirabilis* isolates were from blood cultures. The CLSI tests for the confirmation of ESBL production reliably detect these isolates if both cefotaxime and ceftazidime are tested, but only about half would be classified as a possible CTX-M producers on the basis of the antibiogram alone. A new panprimer set increases the ability to detect CTX-M-producing strains. CTX-M-positive bacteria are common in our geographic region, are often invasive, and, with the exception of *P. mirabilis*, are multiclonal.

CTX-M β-lactamases were thought to be uncommon in North America, although they are common on many other continents (1, 8, 10). CTX-M β-lactamases are extended-spectrum β-lactamases (ESBLs) that mainly inactivate cefotaxime and ceftriaxone and that have less activity against ceftazidime (8). As opposed to the usual epidemiology of other ESBL-positive members of the *Enterobacteriaceae* family, which are mainly nosocomial, CTX-M β-lactamase producers often appear to be community acquired, albeit in debilitated people with prior antibiotic exposure (8). The recognition of this group of resistant bacteria could have importance for decisions on empirical antibiotic therapy and could influence the accuracy of current procedures for screening for ESBLs. On the basis of the findings of a recent study from Texas showing that 70% of recent ESBL-producing isolates contained CTX-M β-lactamases (7), we tested all extended-spectrum cephalosporin-resistant *Enterobacteriaceae* isolated in the Clinical Microbiology Laboratory at the Hospital of the University of Pennsylvania (HUP) in 2007, as well as all multidrug-resistant *Acinetobacter baumannii* isolates recovered during the same time period. We show that CTX-M β-lactamases are common among extended-spectrum cephalosporin-resistant *Escherichia coli* and *Proteus mirabilis* isolates recovered from patients in hospitals in Philadelphia, PA.

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MATERIALS AND METHODS

All 274 *Enterobacteriaceae* isolates meeting CLSI screening guidelines for confirmatory testing for ESBL production (3) that were isolated from clinical samples submitted to the Clinical Microbiology Laboratory at HUP in 2007 were analyzed for the presence of CTX-M. Only one isolate from each patient was saved and tested. In addition, all 17 single patient *A. baumannii* isolates that were resistant to all extended-spectrum cephalosporins tested and that were collected during the same period were tested for CTX-M; no phenotypic tests for ESBLs were performed with these isolates. In addition to cephalosporin resistance, 16 of the *A. baumannii* isolates were also carbapenem resistant. The laboratory provides diagnostic services to two hospitals: HUP and the Pennsylvania Presbyterian Medical Center (PMC). HUP is a 625-bed academic tertiary- and quaternary-care medical center with approximately 32,000 patient admissions annually. PMC is a 324-bed urban community hospital that is located in West Philadelphia and that has approximately 12,000 patient admissions annually. The primary service area and patient population of PMC are similar to those of HUP.

The bacteria were identified and susceptibility testing was performed with the Vitek 2 system (bioMerieux, Durham, NC). Vitek susceptibility panel GN-5 was used for urine isolates; the other isolates were tested with panel GN-9 for the first half of the year and then with panel GN-20 thereafter. Confirmatory testing of ESBL production was done by the CLSI double-disk method with both cefotaxime and ceftazidime (3).

The bacteria were frozen (−70°C) in tryptone broth containing 20% glycerol, and fresh subcultures were used for testing. The bacteria were grown on Trypticase soy agar with 5% sheep blood (TSA II; BBL). PCR templates were prepared by boiling a dense suspension (an approximately no. 2 McFarland standard) in sterile distilled water; 1 μl of the supernatant of the boiled cell suspension was used as the template in the PCRs.

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CTX-M was detected by PCR with a pan-CTX-M primer set, as described previously (7). Determination of whether a PCR result was positive was based on the presence of the correct band size, as determined by agarose gel electrophoresis of the PCR products. The additional grouping of CTX-M was performed with the primers and by the methods described previously (7). The typing of six CTX-M-positive *E. coli* isolates selected from different clonal groups on the basis of the results of pulsed-field gel electrophoresis (PFGE) was done by sequencing of the PCR products, as described previously for CTX-M group I (9). A representative of the clonal *P. mirabilis* isolates was also selected for CTX-M type determination. The same PCR conditions used for the typing of CTX-M group I were also used for the typing of CTX-M group IV and V isolates. PCR and sequencing primers CTX-M-grp5-F (CGCCGATAACACGCAGAC) and CTX-M-grp5-R (CGGCTCCGACTGGGTGAAGTA) were used for CTX-M group V. PCR and sequencing primers CTX-M-grp4-F (ATGGTGACAAAGAGAG TGCAACG) and CTX-M-grp4-R (TTACAGCCCTTCGGCGATGAT) were used for CTX-M group IV; the internal sequencing primers were CTX-M-grp4-seq-F (GGCGACCCGAGAGACACC) and CTX-M-grp4-seq-R (ACCGAGCT GGGCAATCAATTT).

A second pan-CTX-M primer set was designed after the discovery that the primer set used initially did not amplify a known CTX-M-positive control strain. The modified primer set was composed of pan-CTX-M F modified (CRATRT CRTTSGTSGTRCCRTA) and pan CTX-M R modified (TTTSCVATGTGCA GYACCAGTAA) and was used to test all isolates by real-time PCR. About half (49%) of all isolates tested were randomly selected to be tested with both the original and the modified pan-CTX-M primer sets to determine the relative performance. Real-time PCR was also performed with all CTX-M-positive isolates for the presence of TEM, OXA-1, and SHV with previously described primer sets (7). Real-time PCR was performed with a DNA Engine Opticon 2 system (Bio-Rad, Hercules, CA) and 2× IQ SYBR green supermix (Bio-Rad) in a 25-μl volume. The cycling conditions were 94°C for 2 min and then 30 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s; a final extension of 72°C for 3 min was then performed. Melting curve analysis was used to confirm the identities of the products.

All isolates with positive screening reactions were classified into CTX-M groups with primers specific for groups I to IV and on the basis of the presence of the expected PCR product (7). CTX-M groups I, II, III, IV, V, and Toho-2 correspond to CTX-M groups M-1, M-2, M-8, M-9, M-25, and M-45, respectively (11). Partial DNA sequencing of the CTX-M region of seven CTX-M-positive *E. coli* isolates was performed in addition to more complete sequencing to confirm the specificities of the pan-CTX-M and group CTX-M reactions. Three other isolates found not to be in groups I to IV were partially sequenced to confirm that they were CTX-M positive.

PFGE analysis was performed as described previously (6) The enzymes used to prepare chromosomal digests were XbaI (*E. coli*, *Klebsiella pneumoniae*) and NotI (*P. mirabilis*) (12). All enzymes were obtained from New England BioLabs (Ipswich, MA). Cluster analysis was performed by using the unweighted pair group method with arithmetic averages algorithm, and the similarity of bands was determined by using the Dice coefficient (1.00% band tolerance). Isolates showing approximately 80% or greater relatedness were designated a cluster.

Nucleotide sequence accession number. The sequence of the novel CTX-M-25-like gene has been deposited in GenBank as accession number FJ971899.

RESULTS

Only *E. coli*, *Klebsiella* sp., and *P. mirabilis* isolates were CTX-M positive (Table 1), with a total of 42 CTX-M-positive isolates being detected. *E. coli* isolates constituted the bulk of positive isolates; 48% of all extended-spectrum cephalosporin-resistant *E. coli* isolates were CTX-M positive, and 70% of all CTX-M-positive isolates were *E. coli*. Of the 33 CTX-M-negative *E. coli* isolates, 8 were ESBL positive by the CLSI confirmatory test, 3 were negative, 2 were indeterminate, and 21 were not tested for ESBL production since they were urine isolates with extended-spectrum cephalosporin MICs of >16 μg/ml. The CTX-M groups of *E. coli* detected were group I (57%), group IV (37%), group II (3%), and not groupable in groups I to IV (3%). One of seven positive *P. mirabilis* isolates was in group II, with the remainder being not groupable in groups I to IV; and two of the six positive *Klebsiella* sp. isolates (five *K. pneumoniae* isolates, one *Klebsiella oxytoca* isolate) were in group I or IV, with the remainder being not groupable in groups I to IV. Partial CTX-M sequencing of 10 CTX-M-positive isolates confirmed the CTX-M group assignments for seven isolates, and for those three isolates unable to be grouped in groups I to IV, the PCR product was in a CTX-M group V gene. All *P. mirabilis* isolates tested were CTX-M positive. Of the 182 CTX-M-negative *Klebsiella* sp. isolates, 79 were ESBL positive, 6 were ESBL negative, 8 were ESBL indeterminate, and confirmatory testing for ESBL production was not performed for 89, 91% of which were urine isolates with extended-spectrum cephalosporin MICs of >16 μg/ml.

TABLE 1. CTX-M-positive isolates

Organism	No. of isolates positive/ total no. tested	% Positive	No. of CTX-M-positive isolates by body site			
			Blood	Urine	Wound	Respiratory tract
A. baumannii	0/17	0	0	0	0	0
Enterobacter spp.	0/16	0	0	0	0	0
E. coli	30/63	46	8	9	10	2
Klebsiella spp.	6/188[a]	3	0	3	1	2
P. mirabilis	7/7	100	5	1	1	0
Total	43/291	14	13	13	13	4

[a] Five of the 6 CTX-M-positive isolates were *K. pneumoniae* and 1 was *K. oxytoca*; all but 1 of the 188 isolates screened were *K. pneumoniae*.

All three CTX-M group IV isolates sequenced (all *E. coli* isolates and isolates 73, 115, and 149) were determined to be CTX-M-18 producers. All three CTX-M group I isolates sequenced (all *E. coli* isolates and isolates 91, 161, and 265) were CTX-M-15 producers. The one CTX-M group V isolate (*P. mirabilis* isolate 77) selected for sequencing contained an apparently novel CTX-M type that differed by one amino acid each from CTX-M-25 and CTX-M-39, but it was otherwise identical to these two types in the variable region of CTX-M group V (nucleotides 178 to 800). Since complete sequencing of this novel CTX-M gene is not finished, this gene is referred to as "CTX-M-25-like."

Both the original and the modified pan-CTX-M primers were tested in parallel with 149 bacterial isolates. Sixteen isolates were PCR positive for CTX-M and 123 isolates were PCR negative by the use of both primer sets. Six isolates, all *P. mirabilis*, were positive only when they were tested with the modified primer set ($P = 0.04$, McNemar two-tailed test). All six of these *P. mirabilis* isolates presumably contained CTX-M-18 (CTX-M group V) on the basis of their clonality by PFGE analysis (see below).

The CLSI confirmatory test for ESBL production detected all 32 CTX-M-positive bacteria for which confirmatory testing was performed (Table 2). Confirmatory testing with cefotaxime alone detected only 42% of the CTX-M-positive isolates, whereas testing with both cefotaxime and ceftazidime was required to detect all CTX-M-positive isolates. Ceftazidime by itself was unreliable in detecting these ESBLs, detecting only one CTX-M-positive isolate.

The presence of CTX-M in 15 of the 42 (36%) isolates could not be inferred by inspection of the ceftazidime and ceftriaxone MICs, as both the ceftazidime and the ceftriaxone MICs were >64 μg/ml for these isolates (Fig. 1). The ceftazidime MICs were less than 2 μg/ml for 2 of 29 (6.9%), 4 of 7 (57.1%),

TABLE 2. Results of confirmatory tests for ESBL production for 31 CTX-M-positive isolates

Organism	No. of isolates tested	No. (%) of isolates positive for ESBL production by CLSI confirmatory tests with[a]:			
		CTX only	CAZ only	Both drugs	Total
E. coli	24	7	1	16	24
K. pneumoniae	2	0	0	2	2
P. mirabilis	6	6	0	0	6
Total	32	13 (42)	1	18 (55)	32

[a] CTX, cefotaxime; CAZ, ceftazidime.

TABLE 3. Susceptibilities of CTX-M-positive and -negative bacteria to antimicrobial agents

Antimicrobial	% of isolates				
	E. coli		*Klebsiella* spp.		*P. mirabilis*, CTX-M positive (n = 7)
	CTX-M positive (n = 30)	CTX-M negative (n = 34)	CTX-M positive (n = 6)	CTX-M negative (n = 78)	
Gentamicin	43	68	50	64	100
Amikacin	93	82	83	12	100
Levofloxacin	3	35	33	10	14
Meropenem	100	100	100	65	100

and 0 of 6 CTX-M-positive *E. coli*, *P. mirabilis*, and *Klebsiella* sp. isolates, respectively. Susceptibility to a cephamycin was also insensitive as an indicator of the presence of CTX-M, as 21% and 33% of the CTX-M-positive *E. coli* and *Klebsiella* sp. isolates, respectively, were cephamycin resistant. The antimicrobial resistance phenotypes of the CTX-M-positive *P. mirabilis* isolates were most typical of the classical phenotypes of CTX-M-positive isolates, with a high fraction of isolates being susceptible to ceftazidime and a cephamycin. Of interest, both the ceftriaxone and ceftazidime MICs were ≤1 μg/ml for one CTX-M-positive *P. mirabilis* isolate which had been tested by mistake for the presence of an ESBL.

Multidrug resistance was common in both the CTX-M-positive and -negative bacteria (Table 3). *Klebsiella pneumoniae* carbapenemases (KPCs) were present in 35% of the CTX-M-negative *Klebsiella* sp. isolates, but KPCs were not present in any of those that were CTX-M positive. A total of 56% and 17% of the CTX-M-negative and -positive *Klebsiella* spp., respectively, were found to be amikacin resistant but gentamicin susceptible. This phenotype was less common among the *E. coli* isolates, with 3% and 9% CTX-M-negative and -positive *E. coli* bacteria, respectively, having that phenotype.

Twenty-three of the 43 CTX-M-positive isolates contained TEM, SHV, or OXA-1 β-lactamases. This included 16 of 30 CTX-M-positive *E. coli* isolates that were PCR positive for OXA-1 (11 isolates), TEM (4 isolates), and TEM plus OXA-1 (1 isolate). The six CTX-M-positive *Klebsiella* sp. isolates were PCR positive for SHV (three isolates); TEM plus SHV (one isolate); TEM, OXA-1, and SHV (one isolate); and OXA-1 and SHV (one isolate). No CTX-M-positive *P. mirabilis* isolate contained detectable SHV, TEM, or OXA-1.

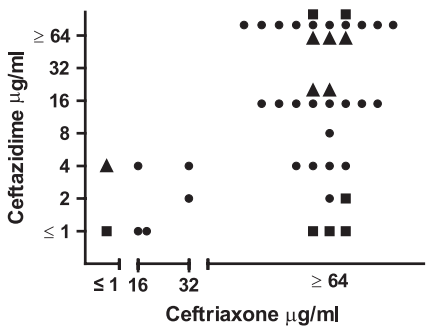

FIG. 1. Ceftazidime MICs versus ceftriaxone MICs (determined with the Vitek 2 system) for 42 CTX-M-positive isolates. Symbols: circles, *E. coli*; squares, *P. mirabilis*; triangles, *Klebsiella* spp.

PFGE analysis of 28 CTX-M-positive *E. coli* isolates showed that there were seven small clusters of highly related strains; however, the overall group was genetically heterogeneous (Fig. 2A). The CTX-M-positive groups did not fall within a particular cluster and were distributed among the entire dendrogram. Furthermore, neither CTX-M-15 nor CTX-M-18 was found in a particular clonal group. Within some of these clusters there was variability in the CTX-M group detected, for example, in isolates 16 and 49, one of which was in CTX-M group IV and the other of which was in CTX-M group II; this is not unexpected, as PFGE may not detect nucleotide differences within one gene (4). Of interest, the PFGE profile of one CTX-M-positive *E. coli* isolate submitted from the same patient 2 months after the first isolate was submitted (the second isolate was not included in the data analysis) did not match the PFGE profile of the first isolate and had acquired TEM in the interim. Of six CTX-M-positive *K. pneumoniae* isolates, two isolates were highly related (Fig. 2B). In contrast, all six CTX-M-positive *P. mirabilis* isolates that were analyzed were identical to or differed by one band from several random clinical *P. mirabilis* isolates, which showed genetic heterogeneity (Fig. 2C).

DISCUSSION

CTX-M ESBLs are common among our extended-spectrum cephalosporin-resistant *E. coli* and *P. mirabilis* isolates and are less common among the multiresistant *K. pneumoniae* isolates. The CLSI guidelines for screening and confirmation of ESBL production are sufficient for the detection of these ESBLs, as long as cefotaxime is included in the confirmatory test, as suggested in the guidelines. However, distinguishing these isolates from other ESBL-producing bacteria on the basis of their antibiograms or the preferential inactivation of cefotaxime in the confirmatory test for ESBL production can be difficult because many isolates are ceftazidime resistant.

CTX-M-15 was found in all three CTX-M group I isolates sequenced and may be the most common group I type in our geographic region. CTX-M-15 was the most common CTX-M type detected in the study conducted in Texas (7) and was also frequently common in a recent study of U.S. ESBL isolates (2). This CTX-M type is relatively common among European isolates and has been responsible for community outbreaks of multiresistant *E. coli* infections in the United Kingdom and

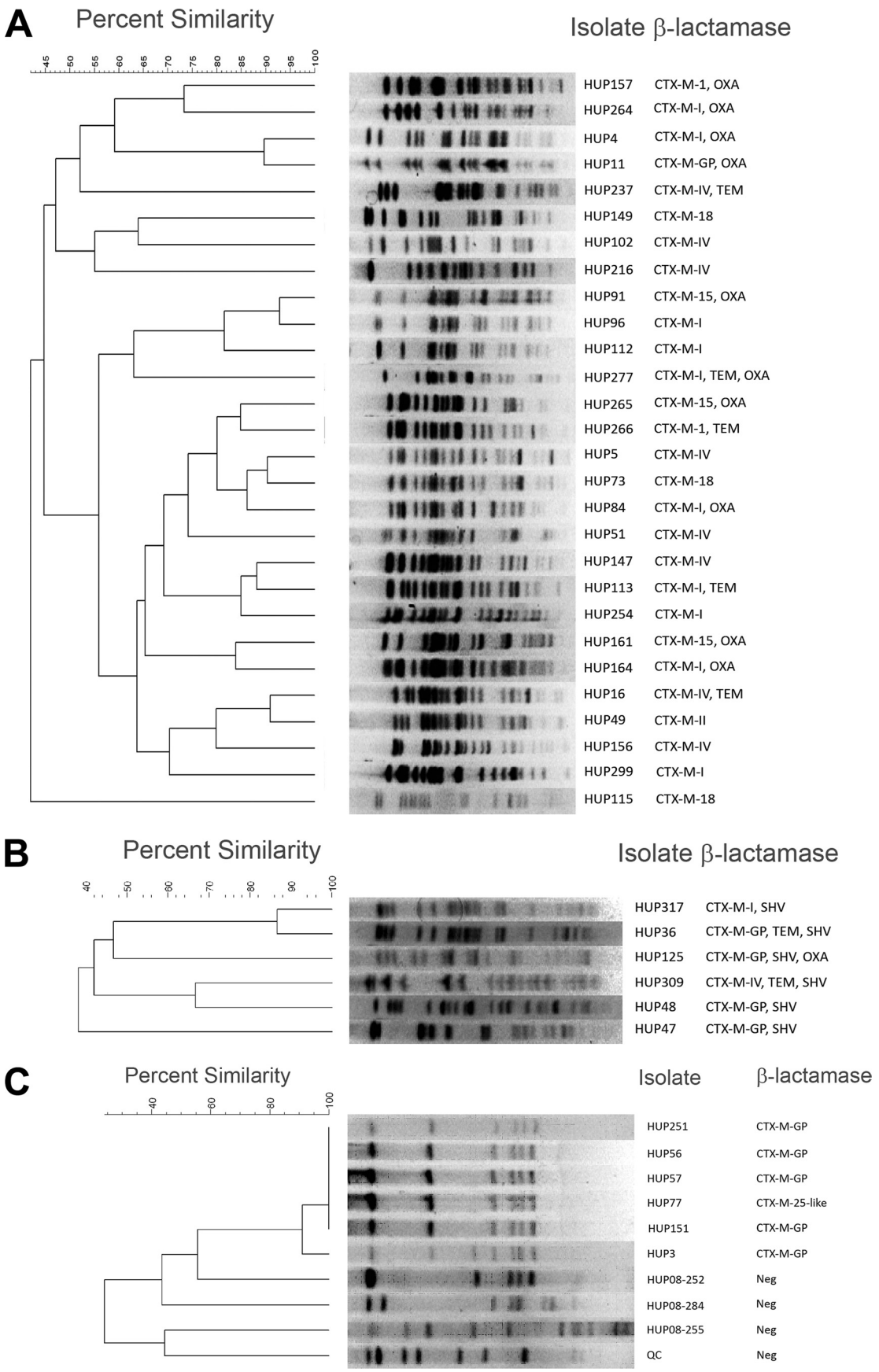

FIG. 2. PFGE analysis of *E. coli* isolates (A), *K. pneumoniae* isolates (B), and *P. mirabilis* isolates (C) positive for a CTX-M-type β-lactamase. Isolates HUP 08-252, HUP 08-284, and HUP 08-255 are random clinical isolates. Isolate QC is an isolate used for in vitro susceptibility testing quality control. Neg, negative. CTX-M-GP, positive with pan-CTX-M primers only but unable to be grouped with the CTX-M group I to IV primer set.

elsewhere (8, 11). The discovery of an apparently novel CTX-M type, CTX-M-25-like, in clonal and predominantly invasive *P. mirabilis* isolates is notable.

Lewis and colleagues recently described the emergence of CTX-M-positive bacteria in a single medical center in Texas (7). Our results differ significantly from those of Lewis and colleagues in several aspects. First, our isolates were isolated predominately (60%) from invasive sites, whereas most (60%) of the CTX-M-positive isolates in the study in Texas were recovered from urinary tract sources. Second, a minority (40%) of our isolates were in CTX-M group I, which was the predominant (75%) group in which the Texas isolates were located; in addition, about one-third of our isolates were in groups other than group I or IV, in contrast to the 12% rate for the Texas isolates. Finally, we observed the presence of an invasive and clonal *P. mirabilis* strain that was not detected among the Texas isolates. The types and distributions of CTX-M among our ESBL-producing isolates also differed from what has been reported from some European outbreaks, which have tended to be clonal and to be cause by a high fraction of isolates from urinary tract sources (1, 8). On the basis of these differences, it seems that the spread of CTX-M within the United States is heterogeneous and may differ in important aspects from other described outbreaks. The heterogeneity of CTX-M types that we observed may represent an early stage of their emergence in our region that will develop into a clonal predominance with time. Alternatively, this could represent the importation or the de novo emergence of multiple equally fit clones.

A weakness of our study is that we do not know whether genetically related strains of *E. coli*, *K. pneumoniae*, or *P. mirabilis* are linked epidemiologically, nor do we know how rapidly these strains have emerged.

We found that the use of a modified primer set for the detection of CTX-M-positive bacteria was more sensitive and no less specific than the use of the originally described pan-CTX-M primers. Use of the modified PCR primer set appeared to increase the rate of detection of CTX-M enzymes not in groups I to IV and most likely in CTX-M group V. Additional studies should be conducted with this primer set to determine if it can replace the current pan-CTX-M primer set.

The high ceftazidime MICs and the high rate of positive results by confirmatory testing for ESBL production by using ceftazidime is likely because most of our CTX-M-positive *E. coli* and *K. pneumoniae* isolates possess other β-lactamases and possibly also because their CTX-M enzymes may have ceftazidimase activity. Some CTX-M ESBLs, such as CTX-M-15, CTX-M-18, CTX-M-19, and CTX-M-32, efficiently hydrolyze ceftazidime (5, 8, 11); and two of these ESBLs were documented to be present in our isolates. In contrast, the CTX-M-positive *P. mirabilis* isolates had more classical CTX-M antimicrobial resistance phenotypes, probably because of their lack of other β-lactamases. The ceftazidime resistance of most of our CTX-M-positive isolates makes inference of the CTX-M genotype on the basis of the antibiogram difficult, requiring molecular testing for their identification.

The emergence and predominance of CTX-M in ESBL-positive members of the *Enterobacteriaceae* family, especially *E. coli*, in our geographic region suggests that the same strains are also dominant but unrecognized in the northeastern region of the United States. While the current CLSI guidelines for the detection of ESBL-positive bacteria and confirmation of the presence of ESBLs appear to be sufficient to detect these isolates, it is unclear whether this will be the case in the future as these bacteria acquire multiple β-lactamases and CTX-M moves into genera for which there are no CLSI guidelines for confirmatory testing for ESBL production (7). Clinical laboratories not performing CTX-M-specific molecular testing are unlikely to recognize that many ESBL-positive bacteria produce CTX-M because of the presence of nonclassical phenotypes.

In conclusion, CTX-M-producing isolates of the *Enterobacteriaceae* family are common in our region. Because many such isolates produce other β-lactamases, the specific detection of these bacteria by the use of phenotypic properties alone can be difficult. The high frequency of invasive strains, including a clonal *P. mirabilis* strain, is worrisome. Although ESBLs are difficult to detect specifically, use of the current CLSI guidelines for the detection of ESBLs correctly classifies these multidrug-resistant organisms as ESBL producers.

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ADDENDUM IN PROOF

The CTX-M-25-like gene has been designated CTX-M-89 by George A. Jacoby.

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