

ESBL genotypes in fluoroquinolone-resistant and fluoroquinolone-susceptible ESBL-producing *Escherichia coli* urinary isolates in Manitoba

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OBJECTIVE: Extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* are increasingly common in nosocomial and community settings. Furthermore, fluoroquinolone (FQ) and even multidrug resistance (MDR) appear to be associated with certain ESBL genotypes. The purpose of the present study was to determine which ESBL genotypes are associated with FQ and MDR in *E coli* urinary isolates in Manitoba.

METHODS: The authors determined the antimicrobial susceptibility, genetic similarity and ESBL genotype of 27 FQ-resistant and seven FQ-susceptible, ESBL-producing urinary isolates submitted to the clinical microbiology laboratories of two teaching hospitals between October 2000 and April 2005. Susceptibilities to beta-lactams, FQs, trimethoprim-sulfamethoxazole (SXT), doxycycline (DOX), gentamicin (GM) and tigecycline were determined by microbroth dilution; pulsed-field gel electrophoresis (PFGE) was used to determine genetic relatedness, and ESBL genotype was determined by polymerase chain reaction and sequencing.

RESULTS: Of 34 ESBL-producing organisms, 27 (79.4%) were found to be ciprofloxacin (CIP) resistant, 27 (79.4%) were SXT resistant, eight (23.5%) were GM resistant and 29 (85.3%) were DOX resistant. Twenty-three (67.6%) had MDR, with concomitant resistance to CIP and SXT; 16 had concomitant resistance to CIP, SXT and DOX; and seven (20.6%) had MDR, with concomitant resistance to CIP, SXT, DOX and GM. All isolates were susceptible to tigecycline. Of 27 FQ-resistant ESBL-producing organisms, seven (25.9%) were genotype CTX-M-14, 19 (70.4%) were genotype CTX-M-15 and one (3.7%) was genotype CTX-M-24. Among the seven FQ-susceptible strains, three (42.8%) expressed SHV-type enzymes, three (42.8%) expressed TEM-type enzymes and one (14.3%) expressed CTX-M-9. CTX-M-15 was the most common MDR-associated genotype. Of a total of 19 strains, 18 (94.7%) were resistant to FQs and SXT; 15 (78.9%) were resistant to FQs, SXT and DOX; and five (26.3%) were resistant to FQs, SXT, DOX and GM. PFGE analysis revealed genetic similarity within CTX-M-15-producing isolates only.

CONCLUSION: CTX-M-15 in *E coli* is strongly associated with an MDR phenotype compared with other genotypes. CTX-M-14 is associated with FQ resistance only. PFGE suggests clonality of CTX-M-15-producing isolates within and among hospitals.

Key Words: CTX-M-15; ESBL; *Escherichia coli*; Fluoroquinolone-resistant; Molecular epidemiology; Multidrug-resistant

Génotypes de bêta-lactamases à spectre étendu dans des isolats urinaires d'*Escherichia coli*, producteurs de BLSE, résistants ou sensibles aux fluoroquinolones, au Manitoba

BUT : Les souches d'*Escherichia coli* productrices de bêta-lactamases à spectre étendu (BLSE) sont de plus en plus souvent mises en causes dans les infections nosocomiales et les infections extrahospitalières. De plus, la résistance aux fluoroquinolones (FQ) et même la multirésistance aux antibiotiques (MRA) semblent associées à certains génotypes de BLSE. La présente étude avait pour but de déterminer quels génotypes de BLSE étaient associés aux FQ et à la MRA dans des isolats urinaires d'*E. coli*, au Manitoba.

MÉTHODE : Les auteurs ont procédé à la détermination de la sensibilité antimicrobienne, de la ressemblance génétique et du génotype de BLSE de 27 isolats urinaires, producteurs de BLSE, résistants aux FQ et de 7 isolats sensibles aux FQ, soumis aux laboratoires de microbiologie clinique de deux hôpitaux universitaires, entre octobre 2000 et avril 2005. La sensibilité aux bêta-lactamines, aux FQ, au triméthoprim, au sulfaméthoxazole (Sul), à la doxycycline (Dox), à la gentamicine (Gen) et à la tigécycline a été déterminée par la technique de microdilution en milieu liquide; les ressemblances génétiques ont été déterminées par l'électrophorèse en champ pulsé (ECP) et le génotype des BLSE a été déterminé par l'amplification en chaîne par polymérase et par le séquençage.

RÉSULTATS : Sur 34 micro-organismes producteurs de BLSE, 27 (79,4 %) étaient résistants à la ciprofloxacine (Cip); 27 (79,4 %), au Sul; 8 (23,5 %), à la Gen; et 29 (85,3 %), à la Dox. Vingt-trois (67,6 %) présentaient une résistance concomitante à la Cip et au Sul; 16, une résistance concomitante à la Cip, au Sul et à la Dox; et 7 (20,6 %) une résistance concomitante à la Cip, au Sul, à la Dox et à la Gen. Tous les isolats se sont montrés sensibles à la tigécycline. Sur les 27 micro-organismes producteurs de BLSE, résistants aux FQ, 7 (25,9 %) présentaient le génotype CTX-M-14; 19 (70,4 %), le génotype CTX-M-15; et 1 (3,7 %), le génotype CTX-M-24. Parmi les 7 souches sensibles aux FQ, 3 (42,8 %) exprimaient des enzymes de type SHV; 3 (42,8 %), des enzymes de type TEM; et 1 (14,3 %), le génotype CTX-M-9. Le génotype le plus souvent associé à la MRA était le CTX-M-15. Sur un total de 19 souches, 18 (94,7 %) étaient résistantes aux FQ et au Sul; 15 (78,9 %), aux FQ, au Sul et à la Dox; et 5 (26,3 %), aux FQ, au Sul, à la Dox et à la Gen. L'analyse par ECP a révélé des ressemblances génétiques parmi les isolats ayant le génotype CTX-M-15 seulement.

CONCLUSIONS : Le génotype CTX-M-15 présent dans les souches d'*E. coli* est fortement associé au phénotype de la MRA, comparativement aux autres génotypes. Le génotype CTX-M-14 est associé à la résistance aux FQ seulement. Les résultats de l'ECP semblent confirmer la clonalité des isolats producteurs du génotype CTX-M-15, que ce soit dans les hôpitaux ou entre les hôpitaux.

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Extended-spectrum beta-lactamase (ESBL)-producing organisms have spread worldwide since their first description in 1983 (1). Over 300 Ambler class A ESBL genotypes have now been described, and they have been reported in numerous species of the *Enterobacteriaceae* family, including *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella* species, as well as in non-*Enterobacteriaceae* (1). Furthermore, the spread of ESBL producers is increasingly becoming a local issue. Recent studies (2,3) from Calgary, Alberta, have clearly demonstrated the increasing prevalence of ESBL-producing *E coli* isolates in the community and hospital setting; local surveillance data from one hospital microbiology laboratory in Winnipeg, Manitoba, demonstrated that the number of ESBL-producing *E coli* isolates had increased dramatically from one (0.05% of *E coli* isolates) in 2000, when surveillance of ESBL isolates began, to 24 (0.8% of *E coli* isolates) in 2005.

ESBLs, named because of their activity against third- and fourth-generation cephalosporins, have a broad spectrum of activity and hydrolyze, to some degree, all cephalosporins and penicillins (1). In addition, there are increasing reports of ESBL-producing clinical isolates expressing multidrug resistance (MDR), defined as concomitant resistance to at least three different antibiotic classes (1,4-6). Organisms with varying resistance to fluoroquinolones (FQs), sulfonamides, aminoglycosides and tetracyclines, in addition to their ESBL phenotype, are not uncommon and may severely limit therapeutic options (1,5,7). In fact, carbapenems are the only antimicrobials with consistent activity against almost all ESBL-producing organisms and remain the drug of choice in severe infections (1,7).

Most ESBLs are members of the Ambler class A beta-lactamases and consist of three main genotypes based on sequence and structure: SHV, TEM and CTX-M. These and other rarer ESBLs exist and have recently been summarized in a review by Paterson and Bonomo (1). SHV and TEM ESBLs are derivatives of the narrow-spectrum SHV-1 and TEM-1/2 enzymes that are commonly found in many members of the *Enterobacteriaceae* family. CTX-M enzymes, named because of their strong hydrolytic activity for cefotaxime, are newer additions to the ESBL family (1,8). Studies have shown that most ESBLs are coded by plasmids, some of which also carry genes conferring resistance to other antimicrobials such as FQs, trimethoprim-sulfamethoxazole (SXT), tetracyclines and aminoglycosides (6,9,10). The purpose of the present study was to analyze and compare the ESBL genotypes and genetic relatedness of ESBL-producing *E coli* urinary isolates, with and without FQ resistance, in outpatient areas of Manitoba hospitals to determine whether any genotype is more strongly associated with FQ or MDR. In the present study, ESBLs with MDR were defined as those with concomitant resistance to two antimicrobial classes in addition to the resistance phenotype afforded by the ESBL.

METHODS

Isolates were obtained from urine specimens submitted to the clinical microbiology departments of the St Boniface General Hospital and the Health Sciences Centre in Winnipeg, Manitoba, between October 2000 and April 2005. Both laboratories are centralized and process samples from four community hospitals in addition to their own hospital specimens. Specimens were from outpatient hospital settings, including emergency departments and ambulatory care clinics. FQ-resistant organisms were collected from both hospitals as part of a larger study of all FQ-resistant

outpatient urinary isolates submitted to these laboratories (North American Urinary Tract Infection Collaborative Alliance) (11). FQ-susceptible isolates were collected from the same period of time, but only from the St Boniface General Hospital microbiology laboratory because it stocks all ESBL-producing isolates as a matter of surveillance policy. The organisms were identified as *E coli* with a potential ESBL phenotype using the Vitek automated system (bioMérieux, France). The ESBL phenotype was confirmed using the confirmation method described by the Clinical and Laboratory Standards Institute (CLSI; USA) (12) at the laboratory of origin and at the National Microbiology Laboratory in Winnipeg, Manitoba, using cefpodoxime, cefotaxime and ceftazidime disks (Mast Diagnostics, United Kingdom) with and without clavulanate. Isolates with ceftoxitin resistance were excluded from the study to exclude possible concomitant overproduction of a Class C (AmpC) beta-lactamase. Antimicrobial susceptibility to ampicillin, cephalixin, cefuroxime, cefotaxime, cefdinir, cefepime, ciprofloxacin (CIP), levofloxacin, meropenem, doxycycline (DOX), gentamicin (GM), SXT and tigecycline was determined using the microbroth dilution method in cation-adjusted Mueller-Hinton broth as per CLSI guidelines (13). Pulsed-field gel electrophoresis (PFGE) was performed as described by Barrett et al (14), with some modifications. *Xba*I restriction fragments were resolved on a Bio-Rad CHEF DRIII (Bio-Rad Laboratories, USA), with switch times of 2.2 s to 54.2 s at 14°C for 19 h. PFGE patterns were interpreted according to the criteria of Tenover et al (15) and digitized for analysis using BioNumerics software version 3.0 (Applied Maths, USA). Polymerase chain reaction (PCR) of the *bla*_{CTX-M}, *bla*_{SHV} and *bla*_{TEM} genes was performed using methods described by Mulvey et al (16). Briefly, whole DNA was extracted from cells, and was subjected to PCR amplification using universal in-house primers for *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{SHV}. Subsequently, amplicons were purified using amicon filters (Millipore Corporation, Canada) and sequenced using an ABI 3100 Genetic Analyser (Applied Biosystems, USA) at the DNA core facility at the National Microbiology Laboratory to identify the ESBL genotype responsible for cephalosporin resistance. The universal *bla*_{CTX-M} primers amplified only part of the CTX-M gene, insufficient for identification beyond the genetically distinct CTX-M groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. Isolates with CTX-M genes were subjected to a second PCR using a separate set of in-house primers specific to the CTX-M group, identified to sequence the entire CTX-M gene in question for final identification. In isolates coexpressing CTX-M and SHV genes, only the CTX-M gene was assumed to be the gene responsible for the ESBL phenotype. The authors justified this approach because a study by Mulvey et al (16) had shown that enzyme isoelectrical focusing of local ESBL producers with both CTX-M and SHV genes expressed only SHV-1. Statistical associations between resistance phenotypes and ESBL genotypes were determined using JMP software (SAS Institute, USA) with a Tukey-Kramer all-pairs comparison test, and possible associations between resistance phenotypes were determined by multiple regression analysis using a significance level of P=0.05.

RESULTS

The exact incidence and distribution of these urinary ESBL isolates cannot be determined by the present study because the sampling of FQ-resistant and FQ-susceptible isolates came from different pools of ESBL-producing organisms, and, therefore, the denominators were not known. Because the FQ-resistant isolates all came from both hospital microbiology laboratories,

TABLE 1
Distribution of genotypes by resistance phenotype

Genotype	CIP resistance, n (%)	SXT resistance, n (%)	DOX resistance, n (%)	GM resistance, n (%)
CTX-M-15 (n=19)	19 (100)	18 (94.7)	16 (84.2)	5 (26.3)
CTX-M-14 (n=7)	7 (100)	4 (57.1)	6 (85.7)	1 (14.3)
CTX-M-9 (n=1)	0 (0)	1 (100)	1 (100)	0 (0)
CTX-M-24 (n=1)	1 (100)	1 (100)	1 (100)	1 (100)
SHV (n=3)	0 (0)	2 (66.7)	3 (100)	0 (0)
TEM (n=3)	0 (0)	1 (33.3)	2 (66.7)	1 (33.3)

Resistance breakpoints were ciprofloxacin (CIP) (minimal inhibitory concentration [MIC] of at least 4 mg/L), trimethoprim-sulfamethoxazole (SXT) (MIC of at least 8:152 mg/L), doxycycline (DOX) (MIC of at least 16 mg/L) and gentamicin (GM) (MIC of at least 16 mg/L)

an estimate can be made of the incidence of FQ-resistant, ESBL-producing *E coli* submitted to these laboratories. Twenty-seven isolates were identified over a period of 54 months, or one isolate every two months. However, there has been a great increase in the rate of isolation, from one isolate in 2000 to 21 isolates in 2004.

PCR and sequencing of ESBL genes identified 34 ESBL enzymes in 34 isolates: 19 CTX-M-15 (members of the CTX-M-1 group), seven CTX-M-14, one CTX-M-24, one CTX-M-9 (all members of the CTX-M-9 group), three TEM (TEM-11, -52 and -52) and three SHV (SHV-2a, -5 and -12) genes. Three of the CTX-M-15-producing organisms also expressed an SHV enzyme, which was not sequenced because previous studies had shown that all local CTX-M-producing strains coexpressed only SHV-1 (see Methods). As expected, all ESBL-producing isolates were resistant to ampicillin (minimal inhibitory concentration [MIC] of at least 32 mg/L) and had reduced susceptibility to at least one third-generation cephalosporin. Furthermore, many isolates had additional resistance phenotypes. Relationships between genotype and resistance phenotypes are shown in Table 1. One isolate was resistant only to beta-lactams, three had one additional resistance phenotype, 10 had two additional resistance phenotypes, 13 had three additional resistance phenotypes and seven had four additional resistance phenotypes. For all 34 ESBL-producing isolates, coresistance to SXT, DOX and GM were significantly correlated ($P=0.0376$). Within the CTX-M-15 ESBL genotype, resistance to SXT and CIP were correlated ($P<0.001$), as was resistance to DOX and CIP ($P=0.0419$). No correlations for antibiotic resistance were noted within other genotype subgroups. The CTX-M-15 ESBL genotype was significantly associated with CIP resistance ($P<0.01$), SXT resistance ($P<0.01$) resistance, and the combination of CIP and SXT resistance ($P<0.01$) compared with other genotypes. The CTX-M-14 genotype was significantly associated with CIP resistance only ($P<0.01$) compared with other non-CTX-M-15 ESBL producers. Figure 1 demonstrates the relative frequency of the resistance phenotypes by ESBL genotype.

MDR occurred more commonly with the CTX-M-15 genotype than with other observed genotypes. The majority of the FQ-resistant isolates (70.3%) were CTX-M-15 ESBL producers. Furthermore, 94.7% of CTX-M-15 ESBL producers were resistant to FQs and SXT, and 84.2% were resistant to FQs, SXT and DOX, although only the former resistance phenotype was significantly and independently associated with CTX-M-15. Although five antibiotic class resistance was more common in

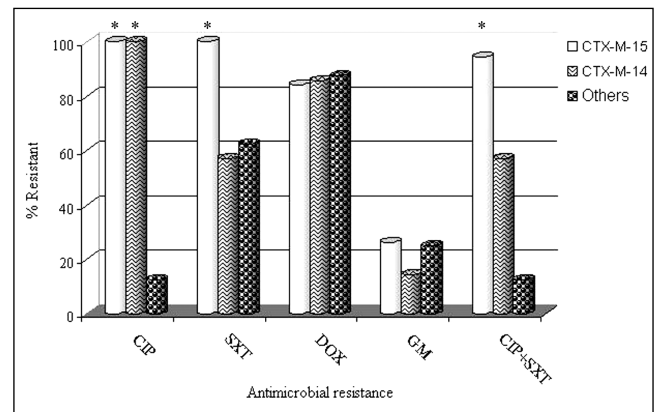


Figure 1 Comparative frequency of ciprofloxacin (CIP), trimethoprim-sulfamethoxazole (SXT), doxycycline (DOX), gentamicin (GM) and multidrug resistance by extended-spectrum beta-lactamase genotype. Multidrug resistance phenotypes not included were not independently associated with a particular genotype. * $P<0.01$

CTX-M-15 isolates (26.3%) than either CTX-M-14 (14.2%) or other (12.5%) isolates, this did not achieve significance. CTX-M-14 was also associated with FQ resistance compared with non-CTX-M-producing isolates, but it was not significantly associated with either SXT, GM, DOX or an MDR phenotype compared with other ESBL-producing organisms in the present study (Figure 1). All ESBL-producing isolates in the present study were susceptible to tigecycline, with an MIC of 1 mg/L or less.

Figure 2 demonstrates the genetic relationship among the isolates harbouring an ESBL. PFGE revealed that the isolates of the CTX-M-15 genotype were more genetically homogeneous than other ESBL genotypes. Dendrogram analysis identified a predominant cluster with a coefficient of similarity of at least 75% containing 14 of 19 (73.7%) of the CTX-M-15 isolates. A small degree of clustering, although to a much lesser extent, was also observed among the CTX-M-14 isolates.

DISCUSSION

One possible explanation for the significant coresistance among CTX-M-15 ESBL-producing isolates is the presence of multiple plasmid-borne resistance loci (9). Many resistance genes, including most ESBLs, are encoded by plasmid-integrated transposable elements (1,6). When analyzing our resistance data from all the ESBL-producing isolates for any associations among resistance phenotypes, the only significant correlation was coresistance to DOX, SXT and GM. This correlation may be due to a single plasmid carrying resistance to these three agents, as well as the ESBL gene. Indeed, this phenomenon has been observed in a number of plasmid analysis studies. In one such study of an ESBL producer outbreak in Toronto, Ontario (6), the CTX-M-15 plasmid also carried transposable resistance determinants to multiple classes of antimicrobials, including aminoglycosides via drug modifying enzymes and tetracyclines via efflux pumps. Earlier studies of plasmids harbouring ESBLs also demonstrated multiple resistance genes, including aminoglycoside, sulfonamide and tetracycline resistance genes (9). The presence of a similar plasmid could explain MDR in our CTX-M-15 isolates. FQ resistance was not correlated with any other antibiotic resistance overall, but was associated with SXT and DOX resistance in the CTX-M-15 and CTX-M-14 genotypes. Because most FQ resistance is chromosomally mediated through

units (1). Furthermore, the finding of similar CTX-M-15 PFGE profiles from geographically distinct centres in our study suggests that there is a significant asymptomatic carrier population causing widespread distribution of ESBL-producing organisms by patient or caregiver-to-patient transfer. Indeed, recent studies (25,26) have documented significant asymptomatic carriage of ESBL organisms, even in nonoutbreak situations. Carriage rates were reported to be high as 11.8% in the inpatient population and 3.7% in the general population. Recent reports from Calgary, Alberta, have also reported increasing rates of CTX-M-15-producing *E coli* in the community setting (2,3). The possibility of clonal spread and significant carriage of such organisms in Canadian hospital outpatient areas and in the community is worrisome and warrants further study. Of particular concern is the common practice of transferring patients between hospital facilities to optimize bed utilization. Such practices may unknowingly lead to the clonal spread of ESBL-producing organisms and other antimicrobial-resistant organisms from unrecognized carriers to noncarriers, both within and between hospital departments. Interestingly, it was the FQ-resistant, CTX-M-15-producing isolates that appeared to be spreading in a clonal fashion in this study. Although the exact

cause of this clonal spread remains unclear, horizontal spread of CTX-M-15-bearing organisms with acquired chromosomal FQ resistance may explain the high levels of FQ coresistance in CTX-M-14- and CTX-M-15-producing isolates in Manitoba.

CONCLUSION

MDR is common in *E coli*-expressing ESBLs. In particular, the CTX-M-15 genotype is associated with three- and four-class MDR. Although the exact mechanism of coresistance remains to be determined, plasmid-borne transposable resistance genes, besides those encoding for ESBL genes, are likely playing a role. This is particularly true of resistance genes commonly found on plasmids, such as those conferring aminoglycoside, DOX and SXT resistance. The transferable nature of these resistance genes is particularly worrisome, and treatment options for infections caused by these organisms are very limited. In addition, the clonal spread of these organisms with MDR may be occurring, and may be contributing to the burden of organisms with MDR in both the community and nosocomial settings. This calls for enhanced infection control and a better understanding of the resistance mechanisms, molecular epidemiology and the means by which spread occurs.

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