IDENTIFICATION OF EXTENDED-SPECTRUM β-LACTAMASE TYPES, PLASMID-MEDIATED AMPC β-LACTAMASES AND STRAINS AMONG URINARY *ESCHERICHIA COLI* AND *KLEBSIELLA* IN NEW ZEALAND IN 2006

Helen Heffernan
Christopher Pope and
Philip Carter

Communicable Disease Group
ESR

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IDENTIFICATION OF EXTENDED-SPECTRUM β-LACTAMASE TYPES, PLASMID-MEDIATED AMPC β-LACTAMASES AND STRAINS AMONG URINARY *ESCHERICHIA COLI* AND *KLEBSIELLA* IN NEW ZEALAND IN 2006

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Helen Heffernan
Project Leader

Dr Tim Blackmore and Dr Naomi Runnegar
Capital and Coast District Health Board
Peer Reviewers

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CONTENTS

SUMMARY

Extended-spectrum β-lactamases (ESBLs) confer resistance to third- and fourth-generation cephalosporins, in addition to the earlier generation cephalosporins. ESBLs are most common in *Klebsiella pneumoniae* and *Escherichia coli,* but do occur in other Enterobacteriaceae and in *Pseudomonas aeruginosa*. ESBLs can be grouped into three main types: TEM, SHV or CTX-M.

Another class of β-lactamases, the AmpC β-lactamases, confer resistance to third-generation cephalosporins and cephamycins (eg, cefoxitin). The genetic determinants for AmpC β-lactamases are commonly found on the chromosomes of genera such as *Enterobacter* and *Citrobacter,* but have now transferred onto plasmids and spread to other organisms, including *E. coli* and *Klebsiella*. Six families of plasmid-mediated AmpC β-lactamases (PMACBLs) have been identified.

A survey among urinary *E. coli* and *Klebsiella* isolated throughout New Zealand during a one-month period in 2006, identified that 0.7% (57/8707) of *E. coli* and 4.2% (31/746) of *Klebsiella* produced an ESBL. In addition, 38 isolates were resistant to cefoxitin and therefore potential producers of PMACBLs.

In this follow-up study, the ESBL-producing isolates identified in the 2006 survey were used to investigate the relative prevalence of ESBL types and the degree of clonality among ESBL-producing *E. coli* and *Klebsiella* in New Zealand. A secondary aim was to investigate whether cefoxitin resistance in these two organisms was due to PMACBLs. ESBL types and PMACBLs were identified by PCR and sequencing. Pulsed-field gel electrophoresis (PFGE) was used to identify strains and clonality among isolates.

Eighty-four ESBLs were identified among the 55 ESBL-producing *E. coli* and 28 ESBL-producing *K. pneumoniae* tested. One *K. pneumoniae* isolate had two ESBLs. Eighty-one (96.4%) of the 84 ESBLs were a CTX-M type, 2 (2.4%) were an SHV type, and 1 (1.2%) was a TEM type. Among the 81 CTX-M ESBLs, 63 (77.8%) were CTX-M-15 and 11 (13.6%) were CTX-M-14. CTX-M-14 was only identified in *E. coli*. One novel ESBL, designated CTX-M-68, was identified. With one exception, CTX-M-15 ESBLs were identified in isolates from every district health board in which ESBL-producing isolates were identified. There were no significant associations between ESBL types and whether the isolates were from community-acquired or hospital-acquired infections.

PFGE showed that 58.0% of the 50 typable ESBL-producing *E. coli* were distinct strains and the remainder belonged to one of nine small, discrete clonal groups. In contrast, 78.6% of the 28 ESBL-producing *K. pneumoniae* belonged to one of three discrete clonal groups.

PMACBLs were identified in 6 of the 33 (18.2%) cefoxitin-resistant *E. coli*, but none of the 5 cefoxitin-resistant *Klebsiella*. All six PMACBLs identified belonged to the CIT-LAT family, with five being CMY-2 and the sixth being a novel PMACBL, designated CMY-29.
All isolates with PMACBLs were from infections categorised as community-acquired and they were all referred from the Auckland region. PFGE typing of the five isolates with CMY-2 showed they were distinct strains.

continued

SUMMARY *continued*

In conclusion, CTX-M ESBLs, especially CTX-M-15, accounted for the overwhelming majority of ESBL types among *E. coli* and *K. pneumoniae* from urinary sources. There was a wide diversity of strains among ESBL-producing *E. coli*, whereas ESBL-producing *K. pneumoniae* were more clonal. PMACBLs were relatively uncommon and accounted for only a minority of the cefoxitin resistance in *E. coli*.

RECOMMENDATIONS

- Investigate and publish the basic properties of the novel ESBL, CTX-M-68, identified in this study.
- Investigate and publish the basic properties of the novel plasmid-mediated AmpC β-lactamase, CMY-29, identified in this study.

1. INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) hydrolyze third- and fourth-generation cephalosporins and monobactams, as well as the earlier generation cephalosporins and penicillins. ESBLs have been identified in many different genera of Enterobacteriaceae and in *Pseudomonas aeruginosa*. However, they are most common in *Klebsiella pneumoniae* and *Escherichia coli*. ESBLs are designated as class A β-lactamases in the Ambler molecular classification scheme and group 2be β-lactamases in the Bush-Jacoby-Medeiros functional classification scheme. They are plasmid-mediated and are inhibited by β-lactamase inhibitors, such as clavulanic acid.

There are three main types of ESBLs: TEM, SHV and CTX-M. The TEM and SHV ESBLs have evolved from broad-spectrum β-lactamases of the same type, specifically TEM-1, TEM-2, SHV-1 and SHV-11. Often the ESBL derivative differs by only one amino acid from the parent enzyme, but the difference is sufficient to confer an extended spectrum of activity. Almost all CTX-M type β-lactamases described to date are ESBLs. The total number of ESBLs now characterised exceeds 200.[1]

The first ESBL described, SHV-2, was identified in an isolate of *Klebsiella ozaenae* in Germany in 1983. Analysis of the SHV-2 gene showed that it was a result of a point mutation in the SHV-1 gene which resulted in an amino acid change from glycine to serine at position 238.[2] TEM-3, the first TEM-type ESBL reported, arose from point mutations in the TEM-2 gene which resulted in two amino acid changes: glutamic acid to lysine at position 104 and glycine to serine at position 238.[3]

CTX-M ESBLs were first reported in the second half of the 1980s and appear to have spread rapidly in the last decade to become the most common ESBL type in many countries.[4] These ESBLs were designated 'CTX-M' as they characteristically display a higher level of resistance to cefotaxime than ceftazidime. CTX-M ESBLs are subdivided into five groups on the basis of amino acid sequence similarity: CTX-M-1 group, CTX-M-2 group, CTX-M-8 group, CTX-M-9 group, and CTX-M-25 group.

Reference work carried out at ESR has identified several ESBL types among New Zealand isolates. These include TEM-10, TEM-29, SHV-2, SHV2a, SHV-5, SHV-12, SHV-42, CTX-M-9, CTX-M-14 and CTX-M-15. However, little is known about the relative prevalence of different ESBL types, although CTX-M-15 has been identified as the ESBL in all ESBL-producing outbreak strains investigated to date, including two *E. coli* strains and four *K. pneumoniae* strains.

AmpC β-lactamases hydrolyze third-generation cephalosporins and cephamycins (eg, cefoxitin), as well as the earlier generation cephalosporins and penicillins. AmpC β-lactamases are designated as class C β-lactamases in the Ambler classification scheme and group 1 β-lactamases in the Bush-Jacoby-Medeiros classification scheme. Features that distinguish AmpC β-lactamases from ESBLs include their hydrolysis of cephamycins and resistance to β-lactamase inhibitors. Several genera of Enterobacteriaceae, including *Enterobacter*, *Serratia* and *Citrobacter,* possess inducible chromosomal AmpC β-lactamases. Isolates with inducible AmpC β-lactamases are characteristically cefoxitin resistant, as cefoxitin is a strong inducer of the β-lactamase. However, they remain susceptible to third-generation cephalosporins in the absence of an inducer. Following a mutation, the production of the β-lactamase can become derepressed and such derepressed mutants will display resistance to third-generation cephalosporins.

In the mid-1970s, plasmid-mediated AmpC β-lactamases (PMACBLs) began to be identified in organisms such as *E. coli*, *Klebsiella* and *Salmonella*. The genes for these PMACBLs are derived from the chromosomal AmpC genes of organisms like *Enterobacter cloacae*, *Citrobacter freundii and Morganella morganii*. Six families of PMACBLs have been recognised.[5] In contrast to the chromosomally mediated AmpC β-lactamases, PMACBLs are usually produced constitutively. Prior to this study, nothing was known about the prevalence of PMACBLs in New Zealand, although cefoxitin resistance has been observed among *E. coli* and *Klebsiella* and could be due to PMACBLs.

A survey of the prevalence of ESBLs among 8707 *E. coli* and 746 *Klebsiella* isolated from urinary sources in laboratories throughout New Zealand during a 1-month period in 2006, identified 57 (0.7%) ESBL-producing *E. coli* and 31 (4.2%) ESBL-producing *Klebsiella*.[6] In addition, 33 cefoxitin-resistant *E. coli* and five cefoxitin-resistant *Klebsiella* were identified. The aims of this follow-up study were to use these ESBL-producing and cefoxitin-resistant isolates to:

- identify the relative prevalence of ESBL types among ESBL-producing *E. coli* and *Klebsiella* in New Zealand
- to investigate whether the cefoxitin resistance identified in *E. coli* and *Klebsiella* is due to PMACBLs
- to investigate clonality among ESBL-producing *E. coli* and *Klebsiella*
- to investigate clonality among any isolates with PMACBLs.

2. METHODS

2.1. Bacterial isolates

Fifty-five ESBL-producing *E. coli* and 28 ESBL-producing *Klebsiella* identified in the 2006 survey of ESBLs among urinary isolates were available for this study.[6] All 28 ESBL-producing *Klebsiella* were identified as *K. pneumoniae*. The β-lactamases in these 83 isolates were identified and the isolates were typed by macrorestriction digestion using pulsed-field gel electrophoresis (PFGE).

Thirty-three cefoxitin-resistant *E. coli* and five cefoxitin-resistant *Klebsiella,* also identified during the 2006 survey of ESBLs, were tested for the presence of PMACBLs. Any PMACBLs were fully identified. All isolates with a PMACBL were typed by PFGE.

Epidemiological data collected with the isolates was used to analyse associations between ESBL types, PMACBLs and strains, and whether the isolate was hospital- or community-acquired and the geographic source of the isolate. Hospital-acquired isolates were defined as isolates from in-patients who had been admitted to hospital at least 48 hours. Community-acquired isolates were defined as isolates from specimens referred from general practitioners, rest homes, hospital outpatient clinics, accident and emergency units, or from hospital in-patients within 48 hours of admission. For the geographic analysis, district health board (DHB) boundaries were used. As the patient's place of residence was not usually known, the location of the referring diagnostic laboratory was used to assign cases to DHBs. The three Auckland DHBs (Waitemata, Auckland and Counties Manukau) and the two Canterbury DHBs (Canterbury and South Canterbury) were combined for these analyses.

2.2. PCR assays for TEM, SHV and CTX-M β-lactamase genes

ESBL-producing isolates were tested for the genes encoding TEM, SHV and CTX-M β-lactamases by PCR using the primers listed in Table 1 and the amplification conditions specified in Table 2. PCRs were performed using 47 μL of Platinum PCR Supermix (Invitrogen), 0.5 μL of the forward and reverse primers (20 pmol each) and 2 μL of DNA template. The DNA template was prepared by a simple boiling method.

Initial PCRs were carried out with the SHV, TEM and universal CTX-M primers. Further PCRs using the CTX-M group 1 and CTX-M group 9 primers were performed with isolates that were positive with the universal CTX-M primers.

Table 1. Primers used for the detection and sequencing of ESBL genes

Primer name	Target gene	Primer sequence 5'-3'	Product size	Reference
TEM-1[1] TEM-2[1]	*bla*$_{TEM}$	GTA TCC GCT CAT GAG ACA ATA TCT AAA GTA TAT ATG AGT AAA C	966 bp	7
SHV-1[1] SHV-2[1]	*bla*$_{SHV}$	GCC GGG TTA TTC TTA TTT GTC GC ATG CCG CCG CCA GTC A	1007 bp	8
CTX-1-universal[2] CTX-2-universal[2]	*bla*$_{CTX-M}$	SCS ATG TGC AGY ACC AGT AA CCG CRA TAT CRT TGG TGG TG	543 bp	9
CTX-M-1gpF[1] CTX-M-1gpR[1]	*bla*$_{CTX-M-group\ 1}$	CCC ATG GTT AAA AAA TCA CTG CCG TTT CCG CTA TTA CAA AC	891 bp	10
CTX-M-9gpF[1] CTX-M-9gpR[1]	*bla*$_{CTX-M-group\ 9}$	GTG ACA AAG AGA GTG CAA CGG ATG ATT CTC GCC GCT GAA GCC	857 bp	11

1 PCR and sequencing primers
2 PCR primers

Table 2. PCR conditions used for the detection of ESBL genes

Target gene	PCR conditions
bla$_{TEM}$	Denaturation for 5 min at 94ºC; 35 cycles of 94ºC for 60 s, 55ºC for 60 s, 72ºC for 60 s; and final extension of 72ºC for 10 min.
bla$_{SHV}$	Denaturation for 5 min at 94ºC; 35 cycles of 94ºC for 30 s, 68ºC for 30 s, 72ºC for 60 s; and final extension of 72ºC for 7 min.
bla$_{CTX-M}$	Denaturation for 5 min at 94ºC; 30 cycles of 94ºC for 30 s, 60ºC for 30 s, 72ºC for 60 s; and final extension of 72ºC for 7 min.
bla$_{CTX-M-group\ 1}$	Denaturation for 10 min at 94ºC; 35 cycles of 94ºC for 60 s, 55ºC for 60 s, 72ºC for 2 min; and final extension of 72ºC for 5 min.
bla$_{CTX-M-group\ 9}$	Denaturation for 10 min at 94ºC; 25 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 60 s; and final extension of 72ºC for 10 min.

2.3. PCR assays for plasmid-mediated AmpC β-lactamase genes

Cefoxitin-resistant isolates were tested for the genes encoding PMACBLs by multiplex PCR using the multiplex PCR primers listed in Table 3.[5] The DNA template was prepared as for the ESBL gene PCRs above. The PCR was performed using 25 µL of AmpliTaq Gold PCR Master Mix (Applied Biosystems); 0.6 µM of the primers MOX-M-F, MOX-M-R, CIT-M-F, CIT-M-R, DHA-M-F and DHA-M-R; 0.5 µM of the primers ACC-M-F, ACC-M-R, EBC-M-F and EBC-M-R; 0.4 µM of the primers FOX-M-F and FOX-M-R; and 2 µL of DNA template. The final reaction volume of 50 µL was made up with sterile distilled water. The amplification conditions were 10 min at 95^{o}C; 25 cycles of 94^{o}C for 30 s, 64^{o}C for 30 s, 72^{o}C for 60 s; and final extension of 72^{o}C for 7 min.

The complete CIT-like PMACBL gene in isolates which were positive with the CIT-M primers in the multiplex PCR was amplified again using the sequencing primers listed in Table 3.[12] The PCR was performed using 25 µL of AmpliTaq Gold PCR Master Mix; 0.2 µM of the primers

ampC-CIT-F and ampC-CIT-R; and 2 µL of DNA template. The final reaction volume of 50 µL was made up with sterile distilled water. The amplification conditions were 10 min at 94°C; 30 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 60 s; and final extension of 72°C for 10 min.

Table 3. Primers used for the detection and sequencing of plasmid-mediated AmpC β-lactamase genes

Primer name	Primer sequence 5'-3'	Product size
MOX-M-F[1]	GCT GCT CAA GGA GCA CAG GAT	
MOX-M-R[1]	CAC ATT GAC ATA GGT GTG GTG C	520 bp
CIT-M-F[1]	TGG CCA GAA CTG ACA GGC AAA	
CIT-M-R[1]	TTT CTC CTG AAC GTG GCT GGC	462 bp
DHA-M-F[1]	AAC TTT CAC AGG TGT GCT GGG T	
DHA-M-R[1]	CCG TAC GCA TAC TGG CTT TGC	405 bp
ACC-M-F[1]	AAC AGC CTC AGC AGC CGG TTA	
ACC-M-R[1]	TTC GCC GCA ATC ATC CCT AGC	346 bp
EBC-M-F[1]	TCG GTA AAG CCG ATG TTG CGG	
EBC-M-R[1]	CTT CCA CTG CGG CTG CCA GTT	302 bp
FOX-M-F[1]	AAC ATG GGG TAT CAG GGA GAT G	
FOX-M-R[1]	CAA AGC GCG TAA CCG GAT TGG	190 bp
ampC-CIT-F[2]	ATG ATG AAA AAATCG TTA TGC	
ampC-CIT-R[2]	TTG CAG CTT TTC AAG AAT GCG C	1144 bp

1 multiplex PCR primers
2 sequencing primers

2.4. Sequencing of TEM, SHV, CTX-M and PMACBL PCR products

Products from the TEM, SHV, CTX-M group 1, CTX-M group 9 and ampC-CIT PCRs were sequenced to identify the β-lactamases. The products were purified using the QIAquick PCR Purification Kit (Qiagen). Sequencing was performed on an ABI 3130XL Genetic Analyser (Applied Biosystems) using the sequencing primers listed in Tables 1 and 3.

Translation of the nucleotide sequences into protein sequences was carried out over the internet (http://www.biophp.org/minitools/dna_to_protein/). The nucleotide sequences and predicted amino acid sequences were analysed using BioNumerics version 4.61 (Applied Maths, Kortrijk, Belgium). Protein sequences were compared with known SHV, TEM, CTX-M and PMACBL amino acid sequences and were positively identified if 100% homology was found with a known sequence.

2.5. Pulsed-field gel electrophoresis

Genomic DNA was digested with *Xba*I and separated on a 1% agarose gel using a BioRad Chef Mapper II (parameters: 6 V/cm, 22 h, 120°, 5-35 s). *Salmonella* Braenderup H9812 (NZRM Acc 4085) was used as the size standard. Banding patterns were analysed with BioNumerics software version 4.61. Isolates were considered to belong to the same clonal group if their PFGE profiles shared ≥90% similarity.

3. RESULTS

3.1. ESBL types

The ESBL types identified among the 55 *E. coli* and 28 *K. pneumoniae* ESBL-producing isolates tested are shown in Table 4.

Table 4. Distribution of ESBL types among ESBL-producing *E. coli* and *K. pneumoniae* isolates from urinary sources

ESBL type	Number (%)		
	E. coli **n=55**	***K. pneumoniae*** **n=29[1]**	**All isolates n=84[1]**
CTX-M-15	37 (67.3)	26 (89.7)	63 (75.0)
CTX-M-14	11 (20.0)	0	11 (13.1)
CTX-M-3	2 (3.6)	0	2 (2.4)
CTX-M-27	2 (3.6)	0	2 (2.4)
CTX-M-9	1 (1.8)	1 (3.4)	2 (2.4)
CTX-M-68	0	1 (3.4)	1 (1.2)
CTX-M total	**53 (96.4)**	**28 (96.6)**	**81 (96.4)**
SHV-2a	0	1 (3.4)	1 (1.2)
SHV-12	1 (1.8)	0	1 (1.2)
SHV total	**1 (1.8)**	**1 (3.4)**	**2 (2.4)**
TEM-19	1 (1.8)	0	1 (1.2)
TEM total	**1 (1.8)**	**0**	**1 (1.2)**

1 one isolate had both CTX-M-15 and SHV2a ESBLs

A novel CTX-M-type ESBL was identified in a *K. pneumoniae* isolate (lab number ARS06/441) and assigned as CTX-M-68 following submission of its sequence data to the Lahey Clinic Medical Centre (Burlington, MA, USA), which curates and assigns β-lactamase types. CTX-M-68 belongs to the Group 1 CTX-M ESBLs. It is most closely related to CTX-M-10 from which it differs by three amino acids (see Appendix 1). CTX-M-68 conferred the classical CTX-M phenotype of cefotaxime, but not ceftazidime, resistance.

Most (62, 74.7%) of the 83 ESBL-producing isolates also had one or more broad-spectrum β-lactamases, namely TEM-1, SHV-1 and SHV-11. One isolate also had an inhibitor-resistant β-lactamase, TEM-40. The distribution of these broad-spectrum β-lactamases in the two species was:

- 61.8% (34/55) of the *E. coli* had TEM-1
- 85.7% (24/28) of the *K. pneumoniae* had TEM-1
- 82.1% (23/28) of the *K. pneumoniae* had SHV-11
- 14.3% (4/28) of the *K. pneumoniae* had SHV-1
- 82.1% (23/28) of the *K. pneumoniae* had both TEM-1 and either SHV-1 or SHV-11, with most (22) having TEM-1 and SHV-11

The combination of β-lactamases in each isolate is shown in Figures 1 and 2.

Table 5. Source of *E. coli* and *K. pneumoniae* isolates with CTX-M-15 and CTX-M-14 ESBLs

	Number (%)		
	Hospital-acquired	**Community-acquired**	**P value[1]**
CTX-M-15-producing isolates (n=61)[2]	18 (29.5)	43 (70.5)	0.6359
CTX-M-14-producing isolates (n=11)	1 (9.1)	10 (90.9)	0.2194
All ESBL-producing isolates (n=80)[2]	21 (26.3)	59 (73.8)	

1 Chi-square test for significance of difference between distribution of all isolates and those with CTX-M-15 or CTX-M-14

2 Source not reported for two of the 63 isolates with CTX-M-15 and for three of the total of 83 ESBL-producing isolates

A comparison of the distribution of ESBL types according to whether the isolate was categorised as hospital- or community-acquired, showed that the distribution of the two most common types, CTX-M-15 and CTX-M-14, did not differ significantly from the overall distribution of ESBL-producing isolates (Table 5). This result is not surprising for CTX-M-15, as this type accounted for the majority (75.0%) of ESBL types.

The geographic distribution of ESBL types is shown in Table 6. CTX-M-15 ESBLs were identified in isolates from every DHB in which ESBL-producing isolates were identified except Otago.

Table 6. Geographic distribution of CTX-M-15 and CTX-M-14 ESBL types

District Health Board	Total number of ESBL-producing isolates	Number (%) CTX-M-15 n=63	Number (%) CTX-M-14 n=11	Number (%) of other ESBL types n=10[1]
Northland	0	-	-	
Auckland	50	40 (80.0)	5 (10.0)	5 (10.0)
Waikato	4	2 (50.0)	1 (25.0)	1 (25.0)
Lakes	0	-	-	-
Bay of Plenty	0	-	-	-
Tairawhiti	0	-	-	-
Taranaki	0	-	-	-
Hawke's Bay	13	13 (100)	0	0
Whanganui	0	-	-	-
MidCentral	1	1 (100)	0	0
Hutt	2	1 (50.0)	1 (50.0)	0
Capital and Coast	5	3 (60.0)	1 (20.0)	1 (20.0)
Wairarapa	0	-	-	-
Nelson Marlborough	1	1 (100)	0	0
West Coast	0	-	-	-
Canterbury	6[2]	2 (28.6)	3 (42.9)	2 (28.6)
Otago	1	0	0	1 (100)
Southland	0	-	-	-

1 The other ESBL types identified were:
2 x CTX-M-3, 1 x CTX-M-9, 1 x CTX-M-27, and 1 x SHV-12 from the Auckland DHBs
1 x CTX-M-9 from Waikato DHB
1 x CTX-M-68 from Capital and Coast DHB
1 x CTX-M-27 and 1 x SHV-2a from the Canterbury DHBs
1x TEM-19 from the Otago DHB

2 There were six ESBL-producing isolates from Canterbury, one isolate had two ESBLs: CTX-M-15 and SHV2a

3.2. PFGE profiles among the ESBL-producing isolates

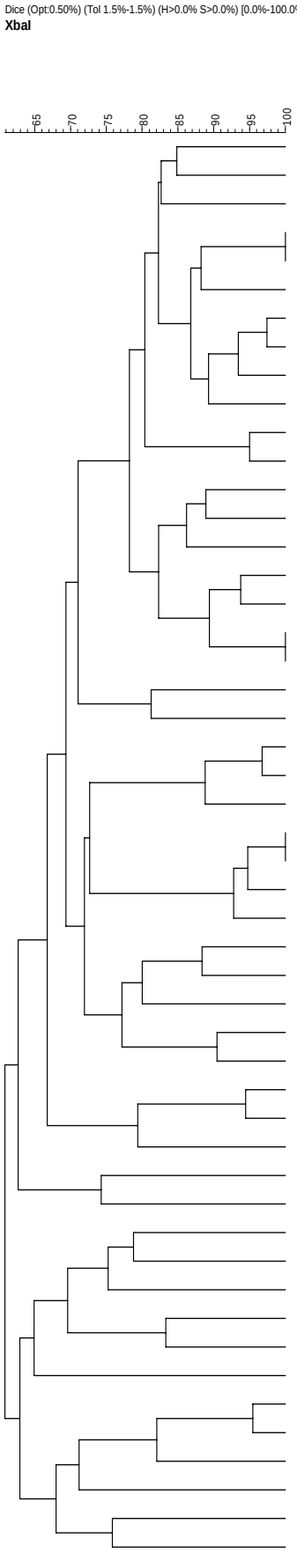
Five of the 55 ESBL-producing *E. coli* could not be typed. The PFGE profiles of the remaining 50 isolates are shown in Figure 1. The majority (29/50) of the isolates were considered distinct as they demonstrated <90% similarity with any other isolate. Among the remaining 21 ESBL-producing *E. coli*, there were nine small, discrete 'clonal groups' each consisting of 2-4 isolates which shared ≥90% similarity. With three exceptions, the extended- and broad-spectrum β-lactamases were the same in each of the isolates belonging to a clonal group (Figure 1). In all nine clonal groups, the ESBL type was CTX-M-15.

For five of the nine clonal groups of ESBL-producing *E. coli*, the isolates belonging to each group came from the same DHB area. And for seven of the nine groups, the isolates belonging to each group were either all categorised as community-acquired or all categorised as hospital-acquired.

All 28 of the ESBL-producing *K pneumoniae* were typable and their PFGE profiles are shown in Figure 2. In contrast to the ESBL-producing *E. coli,* the majority (22/28) of the *K. pneumoniae* belonged to one of three clonal groups. The extended- and broad-spectrum β-lactamases were the same in all isolates belonging to any of the three clonal groups: CTX-M-15, SHV-11 and TEM-1 (Figure 2). Clonal groups 1, 2 and 3 are indistinguishable from three previously recognised ESBL-producing *K. pneumoniae* strains designated KpB, KpA and KpA2, respectively. The KpB and KpA strains were initially identified among ESBL-producing *K. pneumoniae* from the Auckland area and the KpA2 strain, which is closely related to strain KpA, was identified among isolates from the Hawkes Bay area.

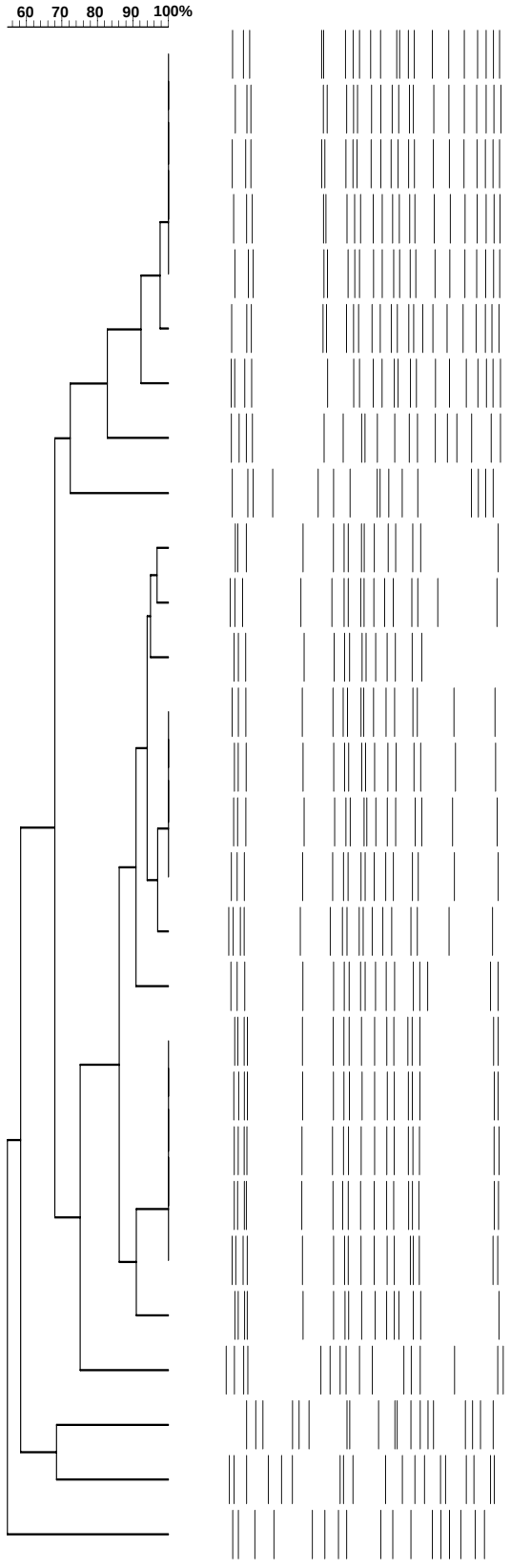
In this study, all ESBL-producing *K. pneumoniae* belonging to clonal groups 1 and 2 were isolated in the Auckland area and all isolates belonging to group 3 were from the Hawkes Bay area. The isolates belonging to all three groups included a mix of those categorised as community-acquired and hospital-acquired.

Figure 1. PFGE patterns among ESBL-positive *E. coli*



ID Number	CTX-M	SHV	TEM	Clonal Group
ARS06-038	15			
ARS06-519	15		1	
ARS06-906	15		1	
ARS06-919	15			1
ARS06-937	15			
ARS06-875	15		1	
ARS06-858	15		1	2
ARS06-936	15		1	
ARS06-926	15		1	
ARS06-866	15			
ARS06-274	15			3
ARS06-836	15			
ARS06-277	15		1	
ARS06-322			19	
ARS06-870	15		40	
ARS06-784	15		1	4
ARS06-862	15			
ARS06-901	15			5
ARS06-923	15			
ARS06-275	14		1	
ARS06-865	15		1	
ARS06-193	15		1	6
ARS06-882	15		1	
ARS06-544	15			
ARS06-089	15			7
ARS06-828	15		1	
ARS06-823	15		1	
ARS06-829	15		1	
ARS06-799	14		1	
ARS06-846	27			
ARS06-864	15			
ARS06-911	14		1	
ARS06-941	15			
ARS06-825	15		1	8
ARS06-893	15		1	
ARS06-518		12	1	
ARS06-092	15		1	
ARS06-795	9		1	
ARS06-343	14			
ARS06-761	27		1	
ARS06-879	14		1	
ARS06-765	15		1	
ARS06-920	14		1	
ARS06-885	14		1	
ARS06-838	15			9
ARS06-847	15			
ARS06-871	3		1	
ARS06-315	15		1	
ARS06-824	15		1	
ARS06-932	14		1	

Figure 2. PFGE patterns among ESBL-positive *K. pneumoniae*



ID number	CTX-M	SHV	TEM	Clonal group
ARS06-012	15	11	1	
ARS06-036	15	11	1	
ARS06-521	15	11	1	
ARS06-532	15	11	1	1
ARS06-909	15	11	1	
ARS06-895	15	11	1	
ARS06-855	15	11	1	
ARS06-276	15	11		
ARS06-903	15	1		
ARS06-082	15	11	1	
ARS06-528	15	11	1	
ARS06-910	15	11	1	
ARS06-042	15	11	1	
ARS06-081	15	11	1	2
ARS06-083	15	11	1	
ARS06-896	15	11	1	
ARS06-520	15	11	1	
ARS06-894	15	11	1	
ARS06-820	15	11	1	
ARS06-821	15	11	1	
ARS06-822	15	11	1	3
ARS06-826	15	11	1	
ARS06-830	15	11	1	
ARS06-090	15	11	1	
ARS06-441	68	1		
ARS06-316	15	2a	1	
ARS06-918	9	1		
ARS06-002	15	1	1	

3.3. Plasmid-mediated AmpC β-lactamases

PMACBLs were identified in six of the 33 (18.2%) cefoxitin-resistant *E. coli* isolates tested. No PMACBLs were found in the five cefoxitin-resistant *Klebsiella*.

Five of the six PMACBLs were CMY-2. The sixth PMACBL, identified in lab number ARS06/913, was novel and assigned as CMY-29 by the Lahey Clinic. It is most closely related to CMY-7 from which it differs by one amino acid (see Appendix 2).

All six of the isolates with PMACBLs were from infections categorised as community-acquired and they were all referred from the Auckland region. PFGE typing of the five *E. coli* isolates with CMY-2 showed they were distinct strains, with no two isolates sharing ≥90% similarity.

4. DISCUSSION

A 2006 survey of the prevalence of ESBLs among *E. coli* and *Klebsiella* isolated from urinary sources indicated that these β-lactamases have increased significantly in New Zealand in recent years.[6] This follow-up study aimed to identify the prevalent ESBL types and the strain diversity among the ESBL-producing organisms isolated during the 2006 survey. This was the first systematic study of ESBL types in New Zealand.

Clearly CTX-M ESBLs, in particular CTX-M-15, are almost wholly dominant among ESBL-producing *E. coli* and *K. pneumoniae* in New Zealand. All but three of the 84 ESBLs identified were CTX-Ms and most of these (63/81, 78%) were CTX-M-15. A similar predominance of CTX-M ESBLs has been reported in recent years in many countries throughout the world.[4,13,14] This is a dramatic turnaround from the situation in the 1990s when TEM and SHV ESBLs were dominant and CTX-Ms were rarely recognised.

Among the CTX-M types, CTX-M-15 seems to be the most widespread globally, while many of the other CTX-M ESBLs tend to be more limited in their distribution. Not only is CTX-M-15 widespread, it is now also being reported to be the dominant CTX-M in several countries, including the United Kingdom, France, Switzerland, Italy, Canada, Lebanon and India.[15-21] While CTX-M ESBLs are common in Asian countries, CTX-M-15 is not usually predominant. CTX-M-14, our second most common CTX-M-type and identified only in *E. coli*, is one of the two CTX-M types (along with CTX-M-3) reported to be dominant in Asia.[22-24]

The predominance of CTX-M ESBLs has not only been observed in the hospital environment but also in community settings. In fact, there is increasing evidence that the CTX-M family of ESBLs has a particular propensity for community spread.[15, 25-27] Dissemination of CTX-M-15-producing *E. coli* and *Shigella* in the community in this country has already been documented.[28]

Our results indicate that CTX-M-producing *E. coli* and *K. pneumoniae* are probably already established in the community in several areas of New Zealand. Among the 80 ESBL-producing isolates included our study and for which the source of infection was reported, the majority (74%) were from infections reported to be community-acquired, with 71% of isolates with CTX-M-15 and 91% of isolates with CTX-M-14 from community-acquired infections. These results indicate that ESBLs, and especially CTX-M types, are more frequently isolated from *E. coli* and *K. pneumoniae* from urinary sources in the community than in hospitals. However, our data do not allow us to estimate the relative prevalence among community- and hospital-acquired isolates due to our denominator data not being categorised according to source.[6]

There was a wide diversity of strains among the ESBL-producing *E. coli,* with little evidence of clonality. It has been suggested that CTX-M ESBLs are associated with mobile genetic elements that facilitate the spread of CTX-M genes between host bacteria.[14] The gene encoding CTX-M-15 is carried on a plasmid and often flanked by the insertion element IS*Ecp1* which has been shown to mobilise itself and adjacent DNA.[29] Other studies have also reported a wide diversity of strains among *E. coli* with CTX-M-15, although often with the co-existence of epidemic or outbreak strains.[15,17,19-21]

In contrast to the ESBL-producing *E. coli*, 79% of the ESBL-producing *K. pneumoniae* belonged to one of three clonal groups. All isolates belonging to each of these clonal groups were isolated in the same geographic area. However, and perhaps somewhat surprisingly, each of the clonal groups of *K. pneumoniae* comprised isolates that were reported to be from both community and hospital sources. However, it is quite possible that some of the patients, who were reported to

have community-acquired infections, had a history of prior hospitalisation which was not reported.

Our results support the premise that the accumulation of CTX-M ESBLs has involved a mixture of clonal expansion and plasmid spread, with the former being more common in *K. pneumoniae* and the latter more common in *E. coli*. In addition, some of the diversity among the CTX-M-producing *E. coli* could be due to the importation of strains from overseas with returning travellers and immigrants, as has already been reported.[28] Clonal expansion has been observed previously in New Zealand among both ESBL-producing *K. pneumoniae* and *E. coli,* with several outbreak strains being identified, all of which have produced CTX-M-15 (ESR, unpublished observations). One of the *E. coli* strains was associated with an outbreak in the community in the Auckland area,[30] while the other strains have been associated with predominantly hospital-based outbreaks.

A coincidental finding in the 2006 survey of ESBL prevalence was the identification of a relatively large number of cefoxitin-resistant isolates, especially among *E. coli*. Thirty-three cefoxitin-resistant *E. coli* were identified among the same pool of screened isolates from which 55 ESBL producers were identified. However, only a small proportion (18%) of this cefoxitin resistance in *E. coli*, and none in *Klebsiella,* was due to PMACBLs. There are at least three possible mechanisms of cefoxitin resistance in *E. coli.* These include (1) PMACBLs; (2) a change in the outer membrane protein affecting permeability to β-lactams; and (3) hyperproduction of AmpC β-lactamase following a mutation resulting in the upregulation of the organism's own chromosomal AmpC gene, which usually produces only very small amounts of AmpC β-lactamase. As *Klebsiella* do not have a chromosomal AmpC gene, only the first two of the above three mechanisms may account for cefoxitin resistance in this genus.

All six PMACBLs identified, including the novel CMY-29, belonged to the CIT-LAT family which are among the most commonly reported PMACBLs.[31-34] While five of the six PMACBLs were CMY-2, they were identified in distinct strains of *E. coli* despite the five isolates all being from community-acquired infections in the Auckland area. As with the ESBL-producing *E. coli* this suggests spread of the AmpC plasmid or importation of strains into New Zealand, rather than clonal spread.

In conclusion, CTX-M-15 and CTX-M-14 accounted for the overwhelming majority of ESBL types in *E. coli* and *K. pneumoniae* from urinary sources. There was a wide diversity of strains among ESBL-producing *E. coli*, whereas ESBL-producing *K. pneumoniae* were more clonal. PMACBLs were relatively uncommon and accounted for only a minority of the cefoxitin resistance in *E. coli*. Finally, the basic properties of the novel β-lactamases discovered in this study, CTX-M-68 and CMY-29, should be published for the benefit of the international scientific community.

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APPENDIX 1

Complete predicted amino acid sequence of the novel CTX-M-68 ESBL from isolate ARS06/441 (GenBank accession number EU177100), compared with the predicted amino acid sequence of CTX-M-10 (GenBank accession number AY598759)

The amino acid sequence of CTX-M-68 differs from CTX-M-10 by Tyr23His, Gln38Arg and Glu158Asp (Ambler numbering) amino acid substitutions.

```
ARS06/441   MVKKSLRQFTLMATATVTLLLGSVPLHAQTVDVQQKLAELERQSGGRLGVALINTADNSQ
CTX-M-10    MVKKSLRQFTLMATATVTLLLGSVPLYAQTVDVQQKLAELEQQSGGRLGVALINTADNSQ

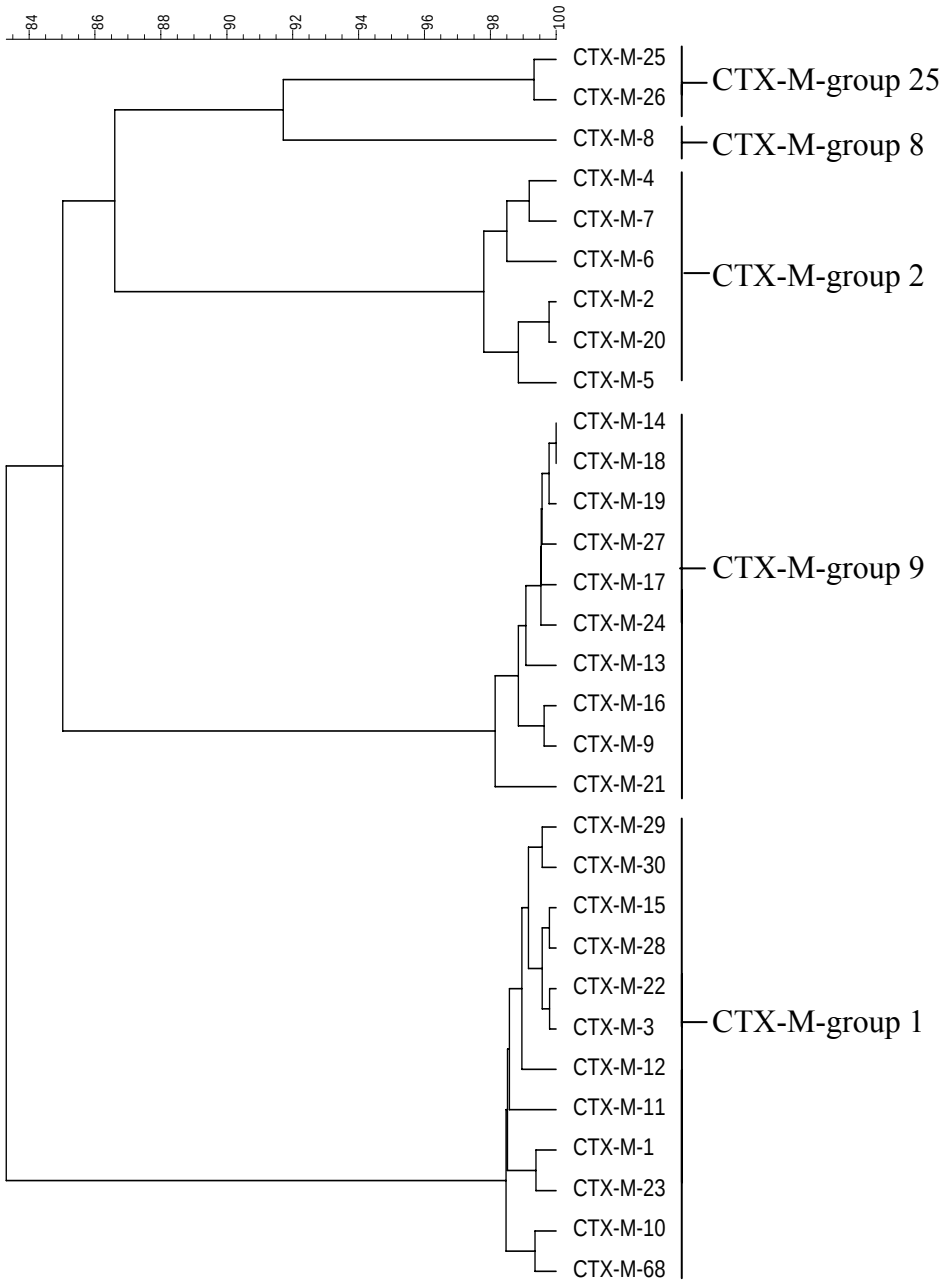
ARS06/441   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM
CTX-M-10    ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM

ARS06/441   SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDDTFRLDRTEPTLNTAIPGDP
CTX-M-10    SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP

ARS06/441   RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS
CTX-M-10    RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS

ARS06/441   GDYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL
CTX-M-10    GDYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL
```

Dendrogram comparing the amino acid sequence of CTX-M-68 with the most common CTX-M ESBL types.



APPENDIX 2

Complete predicted amino acid sequence of the novel CMY-29 plasmid-mediated β-lactamase from isolate ARS06/913 compared with the predicted amino acid sequence of CMY-7 (GenBank accession number AJ011291).

The amino acid sequence of CMY-29 differs from CMY-7 by an Ile141Phe amino acid substitution. Another isolate (ARL05/909) with CMY-29 was identified among reference specimens referred to ESR at about the same time as CMY-29 was identified in ARS06/913. Isolate ARL05/909 was designated the index strain for CMY-29 and submitted to GenBank and assigned accession number EF685371**.**

```
ARS06/913   MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY 60
CMY-7       MMKKSLCCALLLTASFSTFAAAKTEQQIADIVNRTITPLMQEQAIPGMAVAVIYQGKPYY 60

ARS06/913   FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ 120
CMY-7       FTWGKADIANNHPVTQQTLFELGSVSKTFNGVLGGDAIARGEIKLSDPVTKYWPELTGKQ 120

ARS06/913   WQGIRLLHLATYTAGGLPLQFPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGE 180
CMY-7       WQGIRLLHLATYTAGGLPLQIPDDVRDKAALLHFYQNWQPQWTPGAKRLYANSSIGLFGA 180


ARS06/913   LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA 240
CMY-7       LAVKPSGMSYEEAMTRRVLQPLKLAHTWITVPQNEQKDYAWGYREGKPVHVSPGQLDAEA 240

ARS06/913   YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA 300
CMY-7       YGVKSSVIDMARWVQANMDASHVQEKTLQQGIALAQSRYWRIGDMYQGLGWEMLNWPLKA 300

ARS06/913   DSIINGSDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLA 360
CMY-7       DSIINGSDSKVALAALPAVEVNPPAPAVKASWVHKTGSTGGFGSYVAFVPEKNLGIVMLA 360

ARS06/913   NKSYPNPVRVEAAWRILEKLQ 381
CMY-7       NKSYPNPVRVEAAWRILEKLQ 381
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Dendrogram comparing the amino acid sequence of CMY-29 and other closely related CMY β-lactamases.