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Dominance of *bla*$_{CTX-M}$ within an Australian Extended-Spectrum β-Lactamase Gene Pool[▽]

Zhiyong Zong,[1,3] Sally R. Partridge,[1] Lee Thomas,[2] and Jonathan R. Iredell[1]*

Centre for Infectious Diseases and Microbiology, University of Sydney,[1] and Institute for Clinical Pathology and Medical Research, Westmead Hospital,[2] Sydney, New South Wales, Australia, and Department of Infectious Diseases, West China Hospital, Sichuan University, Chengdu, China[3]

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***bla*$_{CTX-M}$ genes, particularly *bla*$_{CTX-M-15}$, are the dominant extended-spectrum β-lactamase (ESBL) genes among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* in Sydney, Australia, where we also found one example of *bla*$_{CTX-M-62}$, encoding a novel enzyme conferring ceftazidime resistance. ESBL genes were present in diverse community isolates and in a variety of associated conjugative plasmids.**

The dominant mechanism of resistance to expanded-spectrum cephalosporins and monobactams among members of the family *Enterobacteriaceae* is the production of Ambler class A extended-spectrum β-lactamases (ESBLs), with more than 200 variants described (23). SHV-type ESBLs have been sporadically reported in *Klebsiella pneumoniae* isolates from Australia (24, 27), and a single isolate from Queensland, Australia was reported to carry a *bla*$_{CTX-M-3}$-like gene in a study of ESBLs in invasive *K. pneumoniae* from 1996 to 1997 (24). SHV- and TEM-type ESBLs were dominant all over the world in members of the family *Enterobacteriaceae* during the 1990s (3, 5) but now appear less important than the widely distributed CTX-M enzymes (1, 5).

Reduced susceptibility (MIC ≥ 2 μg/ml) (Phoenix NMIC/ID-101 panel; Becton, Dickinson & Co., Franklin Lakes, NJ) to cefotaxime (CTX) and/or ceftazidime (CAZ) was observed in 206 of 9,946 *Escherichia coli* (2.1%) and 64 of 1,391 *K. pneumoniae* clinical isolates (4.6%) submitted to our laboratory from four regional hospitals and two associated community clinics in the western Sydney area of New South Wales, Australia, from March 2005 to May 2007. Of these, 81 randomly selected isolates (61 *E. coli* and 20 *K. pneumoniae* isolates) from different patients had been stored and were screened for *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$ genes by PCR (Table 1).

The majority (50 of 61 *E. coli* isolates and 10 of 20 *K. pneumoniae* isolates) yielded amplicons with *bla*$_{CTX-M}$ universal primers (18). Subsequent multiplex PCR (30) and analysis of sequences (ABI PRISM 3100 genetic analyzer; Applied Biosystems, Foster City, CA) revealed genes encoding CTX-M-3 (n = 4), CTX-M-15 (n = 33), and CTX-M-62 (n = 1) from the CTX-M-1 group and genes encoding CTX-M-9 (n = 3), CTX-M-14 (n = 17), CTX-M-24 (n = 2), and CTX-M-27 (n = 1) from the CTX-M-9 group (Table 2) (*bla*$_{CTX-M-9}$ and *bla*$_{CTX-M-14}$ coexisted in one isolate).

The CTX-M-3 enzymes identified in this study are encoded by the first reported and now widespread *bla*$_{CTX-M-3}$ gene (e.g., GenBank accession no. Y10278) (here designated *bla*$_{CTX-M-3a}$) (12), which is closely related to *bla*$_{CTX-M-15}$. A novel variant of CTX-M-3 (Pro167Ser) has the CAZ resistance characteristic of this substitution (26) and was designated CTX-M-62. Ceftazidime resistance, but not cefotaxime resistance, was transferred to *E. coli* with *bla*$_{CTX-M-62}$ on a conjugative plasmid, but the incompatibility group of the plasmid could not be determined. *bla*$_{CTX-M-62}$ is a G509T variant of a *bla*$_{CTX-M-3}$ gene, here designated *bla*$_{CTX-M-3b}$ (e.g., GenBank accession no. AB059404), previously reported from Asia, which differs from *bla*$_{CTX-M-3a}$ at 8 nucleotide positions (Table 3). Additional novel (silent) *bla*$_{CTX-M}$ variants were seen (Table 3): *bla*$_{CTX-M-9b}$ is a C109T variant of all previously deposited *bla*$_{CTX-M-9}$ sequences (e.g., GenBank accession no. AF174129) and *bla*$_{CTX-M-24}$ variants, including *bla*$_{CTX-M-24a}$ (e.g., GenBank accession no. AY143430) and the novel *bla*$_{CTX-M-24e}$ with AGG at codon 275. Using primers located in ISEcp*1* (ISEcp1IR-F) and IS*CR1* (CR1-F) combined with primers located in *bla*$_{CTX-M-1}$ group genes (CTXM1-R) and *bla*$_{CTX-M-9}$ group genes (CTXM9-R), *bla*$_{CTXM-9}$ was found adjacent to IS*CR1*, while all other *bla*$_{CTX-M}$ genes were associated with ISEcp*1*, as expected.

Four *E. coli* isolates yielded amplicons with *bla*$_{SHV}$ primers, all found to be the ESBL gene *bla*$_{SHV-12}$. Sequencing of amplicons obtained from all 20 *K. pneumoniae* isolates suggested single *bla*$_{SHV}$ genes in 17 (3 *bla*$_{SHV-1}$, 11 *bla*$_{SHV-11}$, and 1 each of *bla*$_{SHV-27}$, *bla*$_{SHV-28}$, and *bla*$_{SHV-109}$). SHV-109 is a novel variant most similar to SHV-61 (Thr268Met) and SHV-11 (Thr268Met with Leu10Arg in the signal peptide). The remaining three isolates appeared to have *bla*$_{SHV-12}$ plus another *bla*$_{SHV}$ gene, with uncut and cut amplicons evident on electrophoresis after digestion with NheI (New England Biolabs, Ipswich, MA), which cuts at the position of a relevant sequence variation in *bla*$_{SHV-12}$. Separate sequencing of purified uncut and cut bands revealed that one isolate had both *bla*$_{SHV-12}$ and *bla*$_{SHV-1}$ and that two isolates had both *bla*$_{SHV-12}$ and *bla*$_{SHV-11}$. Fifty-nine isolates also had *bla*$_{TEM}$ genes, all encoding (non-ESBL) TEM-1.

The 66 isolates (54 *E. coli* and 12 *K. pneumoniae* isolates) with *bla*$_{CTX-M}$ (n = 60) and/or *bla*$_{SHV-12}$ (n = 7) (one isolate, JIE162, had both *bla*$_{SHV-12}$ and *bla*$_{CTX-M-15}$), were subjected to

* Corresponding author. Mailing address: CIDM, Level 3, ICPMR Building, Westmead Hospital, Westmead, NSW 2145, Australia. Phone: 61-2-9845-6255. Fax: 61-2-9891-5317. E-mail: jon.iredell@swahs.health.nsw.gov.au.

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TABLE 1. PCR primers used in this study

Primer	Sequence (5′–3′)[a]	Target	GenBank accession no.	Position	Reference
CTX-M-U1	ATGTGCAGYACCAGTAARGTKATGGC	*bla*$_{CTX-M}$ genes	AY458016	20993–20218	18
CTX-M-U2	TGGGTRAARTARGTSACCAGAAYCAGCGG			20426–20454	
CTXM1-F	AAAAATCACTGCGCCAGTTC	*bla*$_{CTX-M-1}$ group	AY458016	21202–21221	30
CTXM1-R	AGCTTATTCATCGCCACGTT			20807–20826	
CTXM2-F	CGACGCTACCCCTGCTATT	*bla*$_{CTX-M-2}$ group	X92507	49–67	30
CTXM2-R	CCAGCGTCAGATTTTTCAGG			581–600	
CTXM8-F	TCGCGTTAAGCGGATGATGC	*bla*$_{CTX-M-8}$ group	AF189721	285–304	30
CTXM8/25-R	AACCCACGATGTGGGTAGC			954–973	
CTXM9-F	CAAAGAGAGTGCAACGGATG	*bla*$_{CTX-M-9}$ group	AF174129	6343–6364	30
CTXM9-R	ATTGGAAAGCGTTCATCACC			6528–6547	
CTXM25-F[b]	GCACGATGACATTCGGG	*bla*$_{CTX-M-25}$ group	AF518567	2673–2689	30
42bp-F	GGATTGACCGTATTGGGAGTT	*bla*$_{CTX-M-9}$ group	AF252622	1716–1736	This work
5′orf903-R	CGGTTGATGAGGGCTTTATT	IS*903*	AF252622	2738–2757	This work
5′orf3-R	GGCGGAAACAATGAGAAAAC	ORF3	AF252622	7478–7497	10
orf477-F	GGTGGCATAATTTTTGAAGT	ORF477	AY458016	20151–20170	This work
ISEcp1IR-F	CAATGTGTGAGAAGCAGTCTAAA	Near IRR ISEcp*1*	AY458016	21332–21354	This work
CR1-F	ACAAATCGGAAGGTCTCG	IS*CR1*	AF174192	5702–5719	11
SHV-F	CGCCGGGTTATTCTTATTTGTCGC	*bla*$_{SHV}$ and adjacent regions	X98101	3–27	21
SHV-R	TCTTTCCGATGCCGCCGCCAGTCA			995–1018	
FIN	ATTCTTGAAGACGAAAGGGC	*bla*$_{TEM}$ and adjacent regions	AY458016	23841–23860	4
DEB	ATGAGTAAACTTGGTCTGAC			24913–24912	
blaTEM-F	GAGTATTCAACATTTTCGT	*bla*$_{TEM}$	AY458016	24052–24070	16
blaTEM-R	ACCAATGCTTAATCAGTGA			24890–24908	
VEB-F	CGACTTCCATTTCCCGATGC	*bla*$_{VEB}$	AF010416	343–362	19
VEB-R	GGACTCTGCAACAAATACGC			985–966	
GES-1A	ATGCGCTTCATTCACGCA	*bla*$_{GES}$	AF156486	1332–1349	25
GES-1B	CTATTTGTCCGTGCTCAG			2195–2178	
BES-1F	AGCGGCGAGAGTTACAGCTA	*bla*$_{BES}$	AF234999	343–362	This work
BES-1R	AGAGGATGGCGATATCGTTG			931–912	
SFO-F	GTTCGGTAGCGCACCATTAT	*bla*$_{SFO}$	AB003148	1477–1496	This work
SFO-R	TTGCCCAAAGTTAGGGTTTG			2028–2009	
PER-UF	CCTGACGATCTGGAACCTTT	*bla*$_{PER}$	Z21957	645–666	This work
PER-UR	TCATCGASGTCCAGTTTTGA			1055–1036	
CA1	ATGTCGCASAYHGAAAATGC	IncFII *copA* or *oriR*	AY458016	88558–88577	22
OR1	CCTTGCAGTTWWHTGTGRRTAA			90150–90171	

[a] H = A, C, or T; K = G or T; M = A or C; R = A or G; S = C or G; W = A or T; Y = C or T.
[b] Paired with CTXM8/25-R.

pulsed-field gel electrophoresis after XbaI (New England Biolabs) restriction of DNA purified from whole-cell extracts (14), and *E. coli* phylogenetic groups were assigned (7) (Table 2). Forty-eight unique strains were identified in this way among the 54 *E. coli* isolates, and 8 unique strains were identified from the 12 *K. pneumoniae* isolates.

Most (47/60) *bla*$_{CTX-M}$ genes transferred on conjugative plasmids to rifampin-resistant *E. coli* DH5α(Δ*lacZ*) selected with rifampin (80 or 200 μg/ml) (Sigma, St. Louis, MO) plus ampicillin (80 μg/ml), CTX (2 μg/ml), or CAZ (2 μg/ml) by filter (29) and/or broth mating methods (9). Plasmid replicon typing of transconjugants as previously described (6), with an additional PCR for IncFII (22), revealed significant plasmid diversity (Table 2). Consistent with previous reports, multiple

TABLE 2. Isolates with ESBL genes

Species[a]	Isolate (JIE)[b]	Drug resistance profile[c]	bla_{TEM} variant[d]	bla_{SHV} variant	Inc group[e]
Isolates with $bla_{CTX\text{-}M\text{-}15}$ (n = 33)					
E. coli B2	101	CTX CAZ GEN TOB SXT CIP	1b		FIA+FIB+FII+N
E. coli B2	118,157,224	CTX CAZ GEN TOB SXT CIP	−, 1b, 1b		FIA+FII
E. coli B2	186/295	CTX CAZ GEN TOB SXT CIP	1b/1b		FII
E. coli B2	250	CTX CAZ GEN TOB FOX SXT CIP	1b		FIA+FIB+FII
E. coli D	085,166,204	CTX CAZ GEN TOB FOX SXT CIP	1b, −, −		FIA+FIB+FII
E. coli B2	106, 110	CTX CAZ GEN TOB SXT	1a, 1b		−
E. coli D	236/242	CTX CAZ GEN TOB SXT	1b/1b		I1
E. coli D	174	CTX CAZ GEN TOB CIP	1b		I1
E. coli A	189, 291	CTX CAZ GEN TOB CIP	−, 1i		−
E. coli B2	188	CTX CAZ GEN TOB CIP	1b		−
E. coli B2	100	CTX CAZ CIP	1b		FII
E. coli B2	143	CTX CAZ CIP	1b		ND
E. coli A	222	CTX CAZ CIP	1b		−
E. coli B2	097/154/286	CTX TOB SXT CIP	1b/−/−		FIA+FII
E. coli D	134	CTX CAZ GEN TOB FOX CIP			FIA+FIB+FII
E. coli D	098	CTX CAZ GEN TOB FOX CIP	1i		FIA+FIB+FII
E. coli B1	113	CTX CAZ SXT CIP	1b		I1
E. coli D	139	CTX CAZ SXT CIP	1b		I1
E. coli B2	289	CTX GEN TOB CIP			−
K. pneumoniae	120/127,146	CTX CAZ GEN TOB SXT CIP	1b (all)	11 (all)	ND
K. pneumoniae	162[f]	CTX CAZ GEN TOB FOX SXT CIP	1b	11, 12	−
Isolates with $bla_{CTX\text{-}M\text{-}3}$ (n = 4)					
E. coli D	161	CTX GEN TOB FOX SXT CIP	1b		FII+B
E. coli D	095	CTX GEN TOB FOX SXT	1b		−
E. coli D	077	CTX GEN FOX SXT CIP	1b		N
E. coli B2	251	CTX GEN CIP	1b		FII+Y
Isolate with $bla_{CTX\text{-}M\text{-}62}$ (n = 1)					
K. pneumoniae	137	CAZ SXT	1b	1	ND
Isolates with $bla_{CTX\text{-}M\text{-}14}$ (n = 17)					
E. coli A	081	CTX GEN TOB SXT	1b		FII
E. coli D	084[g], 110b, 196	CTX GEN TOB SXT	1b, 1b, 1i		FII
E. coli D	182	CTX GEN TOB SXT	1b		B
E. coli D	153, 180	CTX SXT CIP	−, 1b		FII
E. coli D	201	CTX GEN TOB FOX SXT CIP	1b		K
E. coli D	168	CTX GEN TOB SXT CIP	1b		ND
E. coli D	121	CTX GEN TOB FOX CIP	1b		−
E. coli A	088	CTX GEN CIP	1b		I1
E. coli B2	052	CTX CAZ GEN TOB SXT	1b		B
K. pneumoniae	014/021/025/056	CTX GEN TOB SXT	1b (all)	11 (all)	ND
K. pneumoniae	223	CTX		11	FII
Isolates with $bla_{CTX\text{-}M\text{-}9}$ (n = 3)					
E. coli D	059/084[g]/277	CTX GEN TOB SXT	−/1b/−		FIB
Isolates with $bla_{CTX\text{-}M\text{-}24}$ (n = 2)					
E. coli D	216	CTX GEN TOB FOX SXT CIP	1b		FII
E. coli B2	298	CTX			FII
Isolate with $bla_{CTX\text{-}M\text{-}27}$ (n = 1)					
E. coli A	058	CTX CAZ GEN TOB FOX SXT CIP	1b		FII
Isolates with $bla_{SHV\text{-}12}$ as the only ESBL gene (n = 6)					
E. coli D	163	CAZ SXT	1b	12	FIB
E. coli B2	038	CAZ SXT	1b	12	−

Continued on following page

TABLE 2—*Continued*

Species[a]	Isolate (JIE)[b]	Drug resistance profile[c]	bla_{TEM} variant[d]	bla_{SHV} variant	Inc group[e]
E. coli D	119	CTX CAZ GEN TOB FOX SXT CIP	1b	12	−
E. coli B2	124	CAZ GEN TOB SXT		12	A/C
K. pneumoniae	024	CTX CAZ GEN TOB FOX SXT CIP	1b	1, 12	−
K. pneumoniae	205	CTX CAZ GEN TOB FOX SXT CIP	1b	11, 12	−

[a] *E. coli* phylogenetic groups are shown.
[b] JIE isolates (shown in the table without JIE prefix) with identical DNA fingerprints (pulsed-field gel electrophoresis) are separated by slashes (e.g., 097/154/286), and JIE isolates with dissimilar DNA fingerprints are separated by commas. JIE isolates from which the ESBL gene was transferred to *E. coli* by conjugation are underlined. −, absence of bla_{TEM} gene.
[c] Isolates were not susceptible by NCCLS/CLSI guidelines (20) to drugs unless left blank. None were resistant to imipenem or amikacin. Resistance phenotypes transferred to *E. coli* by conjugation are underlined. CTX, cefotaxime; CAZ, ceftazidime; GEN, gentamicin; TOB, tobramycin; FOX, cefoxitin; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin.
[d] bla_{TEM-1i} is identical to bla_{TEM} in GenBank accession no. EF035590 (*E. coli*, India), a C228T variant of bla_{TEM-1a} (e.g., EMBL accession no. X54604); slashes and commas reflect identity relationships as in footnote *a* above.
[e] Inc group, incompatibility groups of conjugative plasmids; ND, Inc group not determined; −, no plasmid transferred.
[f] Neither $bla_{CTX-M-15}$ nor bla_{SHV-12} was transferred from isolate JIE162 by conjugation.
[g] The JIE084 isolate carried both $bla_{CTX-M-9}$ and $bla_{CTX-M-14}$; only $bla_{CTX-M-14}$ was found in the IncFII-positive transconjugant.

plasmid replicons were present in some transconjugants but IncF plasmids were numerically most important (13). All non-IncF amplicons and several IncF amplicons were sequenced, confirming the specificity of PCR typing. Several different HpaI (New England Biolabs) restriction patterns were observed among $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$ plasmids (IncF and IncI1) extracted from transconjugants by alkaline lysis (28), but none matched the recently described epidemic IncFII plasmids in Europe (8) (not shown).

Three-quarters of the 66 isolates were not susceptible to gentamicin or tobramycin, and most were resistant to both (Table 2). Three-quarters were also resistant to trimethoprim-sulfamethoxazole. Aminoglycoside resistance was cotransferred with $bla_{CTX-M-15}$ particularly. Although more than 60% of the original isolates were ciprofloxacin resistant, this phenotype was not transferred to transconjugants (Table 2). Variable associations of $bla_{CTX-M-15}$ with genes conferring β-lactam and aminoglycoside resistance have been previously documented (2, 8), and further investigation is ongoing.

Nearly three-quarters of the 66 isolates with ESBL genes were recovered from urine. Two-thirds (35/54) of the *E. coli* isolates were from community-acquired infections, almost all of unique clonal type. *K. pneumoniae* isolates were more commonly (8/12) collected in the hospital setting and were less diverse (Table 2).

We detected no ESBL-type bla_{SHV}, ESBL-type bla_{TEM}, or bla_{CTX-M} in 15 isolates. Despite having reduced susceptibility to CTX or CAZ (MIC ≥ 2 μg/ml), there was no zone enhancement to suggest an ESBL in any of these isolates by disk approximation test (15, 17), and none of the several less common ESBL genes were detected by PCR (Table 1). The majority (13/15) were cefoxitin resistant, and most carried either a plasmid-borne *ampC* gene (bla_{DHA} or $bla_{CMY-2-like}$; $n = 7$) or a metallo-β-lactamase gene (bla_{IMP-4}; $n = 3$).

In summary, bla_{CTX-M} genes are well-established in the general community here, and $bla_{CTX-M-15}$ (and, to a lesser extent, $bla_{CTX-M-14}$) is particularly dominant despite the presence of novel local variants. Our data indicate that these genes, including $bla_{CTX-M-15}$, are associated with a variety of plasmid replicons and are present in a wide range of bacterial strains.

Nucleotide sequence accession numbers. The nucleotide sequences of bla_{CTX-M} and bla_{SHV} genes from representative isolates have been submitted to GenBank under accession nos. EU418908 to EU418920. The $bla_{CTX-M-62}$ sequence is available under GenBank accession no. EF219134.

TABLE 3. CTX-M gene variants

bla_{CTX-M} gene variant[a]	Original GenBank accession no.[b]	Reported location(s)	Variation(s)[c]
3a	**Y10278**	Various	
3b	AB059404	Japan, Taiwan	8 nt
15a	**AY044436**	Various	
62	**EF219134**	Australia	
9a	**AF174129**	Various	
9b	EU418915	Australia	C109T
14	**AF252622**	Various	
24a	**AY143430**	Mainland China, Taiwan	823–825 CGC
24b	AJ972953	France	823–825 CGT
24c	DQ343293	Mainland China	823–825 AGG; G153A
24d	EF374096	Latin America	823–825 AGA
24e	EU418918	Australia	823–825 AGG
27	**AY156923**	France, Australia	

[a] Letters designated in order of identification; other variants of some genes exist but are not relevant here.
[b] Accession nos. listed in http://www.lahey.org/Studies/ are in bold typeface.
[c] nt, nucleotides. Nucleotides 823 to 825 encode Arg at Ambler position 275 in CTX-M-24.

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