Molecular Characterization of Genes Encoding CTX-M-134, TEM-207 and TEM-212 Detected among Clinical Isolates from USA Hospitals

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ECCMID 2015
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AMENDED ABSTRACT

Objectives: To characterize three new beta-lactamase-encoding genes detected in clinical isolates collected in USA hospitals during the SENTRY Antimicrobial Surveillance Program.

Methods: Genes encoding new variants and its closest known beta-lactamase genes were amplified and cloned into a plasmid vector and transformed into a common *E. coli* background. Clinical isolates were submitted to conjugation and/or transformation. Genetic location of new genes was assessed by S1 nuclease and ICeuI digestion followed by Southern blot and probe hybridization. All clinical isolates, transformants and transconjugants were susceptibility tested by reference broth microdilution methods. Incompatibility factors of plasmids carrying new genes was determined by a multiplex PCR method. Primer walking was used to reveal the genetic environment of the new genes. MLST was also performed.

Results: $bla_{CTX\text{-}M\text{-}134}$ was detected in an *E. coli* clinical strain collected on 5/4/2011 from a bile specimen of a 70 y/o female patient hospitalized in the ICU after surgery at a hospital in Lexington, KY. $bla_{TEM\text{-}207}$ was observed in an *E. coli* isolate collected on 4/25/2012 in Gainsville, FL from a sputum specimen of a 60 y/o male patient. Two *Providencia stuartii* isolates carrying $bla_{TEM\text{-}212}$ were detected in a hospital located at Sun City, AZ. The isolates were collected from different ICU patients, both males of 87 and 19 y/o in a three month interval. All isolates had modestly elevated MIC values (1-8 mg/L) for broad-spectrum cephalosporins and TEM-producing isolates were also resistant to piperacillin/tazobactam (MIC, >64 mg/L). All isolates carried no other beta-lactamase encoding genes. When expressed in an *E. coli* background, CTX-M-134 encoded resistance to ceftazidime, cefepime and ceftriaxone (MIC, ≥16 mg/L) and MIC values were comparable to those for CTX-M-14 (closest variant; 99.7% similarity) in the same background. TEM-207 was an ESBL gene and TEM-212 was an inhibitor resistant narrow-spectrum enzyme. $bla_{CTX\text{-}M\text{-}134}$ was located in a 66-Kb self-conjugative FIA/FIB/FIC incompatibility plasmid and this gene was flanked upstream by IS*Ecp1* and downstream by IS903D. The gene encoding TEM-207 was located in a transposon element (*tnpR*-$bla_{TEM\text{-}207}$-Ins*A*-*yjcA* hypothetical protein encoded) carried by a 120-Kb plasmid that was transformed (but not conjugated) to an *E. coli* host. In both clinical isolates $bla_{TEM\text{-}212}$ was embedded in a 148-Kb A/C incompatibility type self-conjugative plasmid and this gene was flanked upstream by *tnpR* and downstream by IS*26*. Isolates carrying $bla_{CTX\text{-}M\text{-}134}$ and $bla_{TEM\text{-}207}$ belonged to ST131.

Conclusions: We report three new beta-lactamase genes two detected in *E. coli* and one in two *P. stuartii* clinical isolates collected in USA hospitals. Two of these clinical isolates belonged to ST131, a strain with known virulence factors that cause severe infections. The other gene was observed in isolates collected from the same hospital within a short time interval, all being potential causes of concerns for microbiologists and infectious diseases practitioners.

INTRODUCTION

Resistance mechanisms to β-lactam agents include (i) alteration of the penicillin-binding proteins, targets of these agents in the bacterial cell, (ii) lack or diminished membrane permeability of the antimicrobial agents due to the reduced expression of the outer membrane proteins (OMP), (iii) extrusion of the β-lactam molecule by increased expression of efflux pumps, and (iv) production of β-lactamases.

Production of β-lactamases is the most common resistance mechanism among Enterobacteriaceae species. These enzymes are worrisome due to their ability to disseminate among other organisms since they are carried by transferable genetic elements such as plasmids. Additionally, amino acid alterations in specific motifs can broaden their spectrum of activity to other β-lactam agents or cause resistance to clinically available β-lactamase inhibitors.

Among the different β-lactamases detected in clinical isolates, extended-spectrum β-lactamases (ESBL) have been a matter of concern for more than two decades. These enzymes hydrolyze broad-spectrum cephalosporins that are widely used therapeutic agents. In this study, we describe three new β-lactamase encoding gene detected from clinical isolates collected in USA hospitals. Two of these enzymes, TEM-207 and CTX-M-134 are ESBLs and were detected in *Escherichia coli* isolates, whereas TEM-212 was determined to be an inhibitor-resistant narrow spectrum enzyme.

MATERIALS AND METHODS

Bacterial isolates. Two *E. coli* and two *P. stuartii* clinical isolates displaying the CLSI criteria for ESBL phenotype (MIC >1 mg/L for aztreonam and/or ceftazidime and/or ceftriaxone; M100-S25) were initially screened for β-lactamase-encoding genes using the microarray based assay Check-MDR CT101 kit (Check-points, Wageningen, Netherlands). The assay was performed according to the manufacturer's instructions. This kit has the capabilities to detect CTX-M Groups 1, 2, 8+25 and 9, TEM wild-type (WT) and ESBL, SHV WT and ESBL, CMYI/MOX, ACC, ACT/MIR, CMYII, DHA, FOX, KPC and NDM-1. Positive results were amplified using specific oligonucleotides and sequenced on both strands. Nucleotide and deduced amino acid sequences were analyzed using the Lasergene software package (DNASTAR, Madison, Wisconsin, USA). Amino acid sequences were compared with those available through the internet using NCBI/BLAST.

Susceptibility testing. Clinical isolates and recombinant strains were susceptibility tested using broth microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines using validated panels. Categorical interpretations for all antimicrobials were those found in the CLSI document M100-S25 and the EUCAST website, and quality control (QC) was performed using *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. All QC results were within specified ranges as published in CLSI documents.

Characterization of β-lactamase-encoding genes. The genes encoding CTX-M-134, TEM-207 and TEM-212 were cloned using the cloning vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning kit; Life Technologies) as recommended by the manufacturer and colony selection was performed on plates containing 50 mg/L of kanamycin. Additionally, genes encoding CTX-M-14 and TEM-1 were cloned as described above for comparison purposes. The presence and orientation of inserts was confirmed by PCR and sequencing. MIC testing was performed as described above.

Molecular typing. Pulsed-field gel electrophoresis (PFGE) was performed for two *P. stuartii* isolates. Genomic DNA was prepared in agarose blocks and digested with SfiI (New England, Beverly, Massachusetts, USA) and resolved in the CHEF-DR III (BioRad, Richmond, California, USA) using running conditions described elsewhere. Results were analyzed by GelCompar II software (Applied Math, Kortrijk, Belgium). Percent similarities were identified on a dendrogram derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. Band position tolerance and optimization were set at 1.2% and 0.5%, respectively.

Multilocus sequence typing (MLST) method for the two *E. coli* isolates, including PCR amplification, bi-directional sequencing, and ST assignment was performed in accordance with the *E. coli* PubMLST website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

Expression analysis of the chromosomally encoded *AmpC*, efflux pumps and porins. The relative expression of genes encoding the chromosomal cephalosporinase (AmpC), *ompC* and *ompF* and efflux system AcrAB-TolC was determined by quantitative real-time PCR (qRT-PCR) using DNA-free RNA preparations for two *E. coli* isolates. RNA extraction and treatment and relative quantification of target genes were prepared as previously described. Endogenous reference gene *(rspL)* and custom designed primers showing efficiencies >95.0% were used. Transcription levels were considered significantly different if at least a 10-fold difference was noted compared with the control isolates. *E. coli* ATCC 25922 was considered baseline in the experiments.

Transference of β-lactamase-encoding genes to *E. coli* background. Mating experiments were performed by mixing equal volume of donor (clinical isolate) and recipient (azide-resistant *E. coli* J53) bacterial cell suspensions in the exponential phase of growth. Transformation was perfomed by electroporation as described elsewhere using *E. coli* DH5α as recipient. Transconjugants were selected in plates containing 200 mg/L of sodium azide and 16 mg/L of ampicillin for TEM-212 or 1 mg/L ceftazidime for CTX-M-134. Transformants were selected in plates containing 16 mg/L of ampicillin. The presence of β-lactam resistance marker and species identification of the transconjugants/transformants strains was confirmed by PCR, PFGE and susceptibility testing.

Genetic location of β-lactamase-encoding genes. The genetic location of $bla_{CTX\text{-}M\text{-}134}$, $bla_{TEM\text{-}207}$ and $bla_{TEM\text{-}212}$ was determined using partial S1 nuclease digestion for clinical isolates, transconjugants and transformants strains. Total cellular DNA embedded in 2% agarose plugs was subjected to digestion. Preparations were resolved by electrophoresis performed on the CHEF-DR II (BioRad, Richmond, California, USA), with the following conditions: 0.5 x TBE, 1% agarose, 13°C, 200V, for 6 hours with switch time ramping from 5 to 25 seconds and 8 hours with the switch time from 30 to 45 seconds. DNA gels were transferred to nylon membranes by southern blotting and hybridized with a digoxigenin labeled probe (Roche Diagnostics GmbH, Mannheim, Germany) specific to each β-lactamase-encoding gene.

Plasmid incompatibility was determined by multiplex PCR as described previously. The surrounding sequences of $bla_{CTX\text{-}M\text{-}134}$, $bla_{TEM\text{-}207}$ and $bla_{TEM\text{-}212}$ was determined using primer walking strategy and plasmid template recovered from transconjugants/transformants.

RESULTS

- Three new β-lactamase encoding genes were detected among clinical isolates collected in USA hospitals. CTX-M-134 was closely related to CTX-M-14 (D242S; 99.7% homology) and CTX-M-27 (D242G; 99.7% homology), whereas TEM-207 and TEM-212 had one amino acid substitution (99.0% homology) compared to TEM-1: E238G and Y103N, respectively.
- The CTXM-134-producing *E. coli* was collected from a bile specimen in a hospital in Lexington, Kentucky and TEM-207-producing *E. coli* isolate was recovered from a sputum specimen in a hospital located in Gainsville, Florida (**Table 1**). Both *E. coli* isolates belonged to ST131.
- Two *Providencia stuartii* isolates carrying $bla_{TEM\text{-}212}$ were detected in a hospital located at Sun City, AZ. The isolates were collected from different ICU patients in a three month interval. These isolates were genetically identical by PFGE (**Table 1**).
- MIC values for the cephalosporins were modestly elevated (1-8 mg/L) for all three clinical isolates and TEM-producing isolates were resistant to piperacillin/tazobactam (MIC, >64 mg/L). Isolates were susceptible to carbapenems, aminoglycosides (*E. coli* only), but were all resistant to tetracycline and quinolones (**Table 1**).
- Cloning of the new genes in a common *E. coli* background demonstrated that CTX-M-134 encoded resistance to ceftazidime (MIC, 8 mg/L), cefepime (MIC, 16 mg/L) and ceftriaxone (MIC, >8 mg/L) and MIC values were comparable to those for CTX-M-14 expressed in the same background (**Table 1**).
- TEM-207-producing recombinant *E. coli* displayed elevated MIC results for ceftazidime (16 mg/L), cefepime (8 mg/L) and ceftriaxone (2 mg/L). TEM-212 MIC values were low for the extended spectrum cephalosporins, but elevated for piperacillin/tazobactam (**Table 1**).
- The $bla_{CTX\text{-}M\text{-}134}$-carrying plasmid was transferred by conjugation to *E. coli* J53. Further analysis demonstrated that this gene was located in a 66-Kb plasmid that was positive for the FIA/FIB/FIC incompatibility group. This gene was flanked upstream by IS*Ecp1* and downstream by IS*903D* (**Table 1**).
- The gene encoding TEM-207 was located in a transposon element (*tnpR*-$bla_{TEM\text{-}207}$-Ins*A*-*yjcA* hypothetical protein encoded) carried by a 120-Kb plasmid. Attempts to conjugate this gene failed, but the gene was transformed into an *E. coli* host. MIC results for the transconjugant were low for the broad spectrum cephalosporins when compared to the recombinant gene cloned into *E. coli* TOP10 (**Table 2**).
- The TEM-212-encoding gene was embedded in a 148-Kb A/C incompatibility type plasmid that was transferred by conjugation to *E. coli* J53. This gene was flanked upstream by *tnpR* and downstream by IS*26*.
- All isolates carried no other β-lactamase encoding genes. Expression of intrinsic resistance mechanisms to β-lactam agents demonstrated that the CTX-M-134-producing isolates had modestly low expression of *ompF* (**Table 1**).

Table 1. Characteristics and results for clinical isolates carrying genes encoding CTX-M-134, TEM-207 or TEM-212.

Test/Characteristic	CTX-M-134-producing *E. coli*	TEM-207-producing *E coli*	TEM-212-producing *P. stuartii*	TEM-212-producing *P. stuartii*
Demographic information				
City, State	Lexington, KY	Gainesville, FL	Sun City, AZ	Sun City, AZ
Age/Sex	70/F	60/M	87/M	19/M
Culture Date	04-May-11	25-Apr-12	31-May-12	24-Aug-12
Service	Surgery	NA	Intensive Care Unit	Intensive Care Unit
Antimicrobial susceptibility testing				
Ceftriaxone	>8	1	4	4
Ceftazidime	4	4	>32	32
Cefepime	4	4	16	8
Aztreonam	8	4	0.25	≤0.12
Ampicillin/sulbactam	>32	>32	>32	32
Piperacillin/tazobactam	2	64	64	32
Imipenem	≤0.12	≤0.12	2	2
Meropenem	≤0.06	≤0.06	0.12	0.12
Ciprofloxacin	>4	>4	>4	>4
Amikacin	2	4	1	4
Gentamicin	≤1	≤1	8	>8
Tobramycin	1	1	4	16
TMP/SMX	>4	≤0.5	>4	>4
Tetracycline	>8	>8	>8	>8
Tigecycline	0.06	0.12	1	0.5
Colistin	≤0.25	0.25	>8	2
Genetic location of β-lactamase genes				
Plasmid size	66-Kb + 170-Kb	120-Kb	148-Kb	-
Plasmid Incompatibility types	FIA/FIB/FIC + Y	FIA/FIB/FIC	A/C	-
Surrounding genes	IS*Ecp1*-$bla_{CTX\text{-}M\text{-}134}$-IS*903D*	*tnpR*-$bla_{TEM\text{-}207}$-InsA-yjcA hypothetical protein	*tnpR*-$bla_{TEM\text{-}212}$-IS*26*	-
Relative expression				
acrA	2.6	3.7	-	-
ampC	1.8	2.2	-	-
ompC	22562.4	18476.1	-	-
ompF	4.4	0.5	-	-
Molecular typing	ST131	ST131	PFGE pattern A	PFGE pattern A
Genbank accession no.	JX896165	KC818234	KF481968	-

CONCLUSIONS

- During screening for β-lactamase encoding genes for clinical isolates collected in USA hospitals, three new enzymes were detected: two ESBLs (TEM-207 and CTX-M-134) among *E. coli* strains and one inhibitor-resistant TEM (TEM-212) in *P. stuartii*.
- The two ESBL-producing *E. coli* strains belonged to the ST131 lineage that has been associated with severe infections due to its virulence factors.
- These results highlight the increase in diversity of β-lactamase-encoding genes in USA hospitals and the continuous need for surveillance for these genes.

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Table 2. Susceptibility testing results and genetic characteristics of *E. coli* isolates carrying β-lactamase genes prepared through conjugation, transformation or cloning as part of this study.

	Tranconjugants			Transformants		Recombinants				
Test/Characteristic	J53 pCTX-M-134	J53 pTEM-212	J53	DH5α pTEM-207	DH5α	TOP10 pZEROBlun tTOPO TEM-207	TOP10 pZEROBlun tTOPO TEM-212	TOP10 pZEROBlun tTOP TEM-1	TOP10 pZEROBlun tTOPO CTX-M-134	TOP10 pZEROBlun tTOPO CTX-M-14
Antimicrobial susceptibility testing										
Ceftriaxone	>8	≤0.06	0.12	≤0.06	≤0.06	2	0.12	0.12	>8	>8
Ceftazidime	16	0.12	0.25	0.25	0.12	16	1	0.5	8	8
Cefepime	>16	≤0.5	≤0.5	≤0.5	≤0.5	8	≤0.5	≤0.5	16	>16
Aztreonam	>16	≤0.12	≤0.12	≤0.12	≤0.12	8	1	0.25	>16	>16
Cefoxitin	8	4	8	8	4	16	8	16	8	8
Ampicillin	>8	>8	8	>8	4	>8	>8	>8	>8	>8
Ampicillin/sulbactam	>32	32	8	16	2	>32	>32	>32	>32	>32
Amoxicillin/clavulanate	>8	>8	8	8	8	>8	>8	>8	>8	>8
Piperacillin/tazobactam	8	32	4	1	1	>64	>64	>64	8	8
Imipenem	0.25	≤0.12	0.25	≤0.12	0.25	0.25	0.25	0.25	≤0.12	0.25
Meropenem	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06
Ciprofloxacin	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	-	-	-	-	-
Amikacin	1	1	1	0.5	≤0.25	-	-	-	-	-
Gentamicin	≤1	8	≤1	≤1	≤1	-	-	-	-	-
Tobramycin	0.25	0.5	0.25	≤0.12	0.25	-	-	-	-	-
Trimethoprim/sulfamethoxazole	>4	>4	≤0.5	≤0.5	≤0.5	-	-	-	-	-
Tetracycline	>32	2	2	32	2					
Genetic location of β-lactamase genes										
Plasmid size	66-Kb	148-Kb	-	120-Kb	-	-	-	-	-	-