New OXY-variants from *Klebsiella oxytoca* Bloodculture Clinical Isolates Collected in USA Hospitals

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AMENDED ABSTRACT

Background: Hyperproduction of *bla*$_{OXY}$ in *K. oxytoca* (KOX) can cause variable resistance (R) levels to β-lactams. We previously evaluated the presence of β-lactamases among 214 Enterobacteriaceae bloodculture isolates and here 10 KOX were analyzed for the presence of *bla*$_{OXY}$ and new variants were characterized.

Methods: KOX collected from USA hospitals during 2010 were selected according to the CLSI ESBL screening criteria. Isolates were tested for the presence of genes encoding ESBLs, pAmpC and carbapenemases (when imipenem [IMI] and/or meropenem [MER] MIC at >1 µg/mL). *bla*$_{OXY}$-like enzymes were sequenced on both strands and entire genes were cloned into PCRScript/XL1 Blue *E. coli*. Susceptibility testing was performed using CLSI reference methods for aztreonam (AZT), 9 other β-lactams and piperacillin/tazobactam (P/T).

Results: Among 135 KOX, 10 were positive for CLSI-ESBL criteria and *bla*$_{OXY}$. Additionally, one strain carried *bla*$_{SHV-12}$ and *bla*$_{TEM-1}$ and one carbapenem-R KOX harbored *bla*$_{KPC-2}$ and *bla*$_{OXA-2}$. Sequencing of *bla*$_{OXY}$ showed 5 new variants among 6 isolates. Alteration at position 155 was noted in all 5 variants with or without other substitutions (**Table**). In the same genetic background, MIC results for AZT, cefepime and ceftriaxone exhibited differences. One variant displayed a deletion of A15, but the susceptibility profile was not significantly altered and this gene conferred R to ampicillin, AZT, P/T and cefepime. Ceftazidime, IMI and MER MIC values were not significantly increased.

Conclusions: Five new variants of *bla*$_{OXY}$ in USA KOX with distinct R profiles against several β-lactams tested were identified. Promoter regions on the clinical isolates remain under investigation.

		MIC (µg/mL)[a]:						
PCRScript Construct	Amino acid changes	AMP	FOX	CRO	CAZ	FEP	AZT	P/T
1115A	A15 deletion, H155R	>256	4	48	1	8	>256	>64
49A	H155R, D199N	>256	8	8	0.25	≤0.5	32	32
1012A	H155R, D199N	>256	4	64	0.5	4	>256	>64
10052A	H155R, D255N	>256	16	6	0.5	2	48	>64
34322A	H155R	>256	8	16	0.5	1	128	>64
17424A	OXY-2 (C861G mutation)	>256	2	48	1	2	>256	>64
36003A	Y143H	>256	16	48	0.5	4	>256	>64
49685A	OXY-2	>256	2	48	0.25	2	>256	>64

a. Ampicillin (AMP), cefoxitin (FOX), ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEP), aztreonam (AZT) and piperacillin/tazobactam (P/T).

INTRODUCTION

Klebsiella oxytoca is a ubiquitous organism that has the ability to colonize the human gut and it is an important opportunistic pathogen causing serious infections in hospitalized patients, including neonates. This bacterial species produces an intrinsic Ambler class A β-lactamase, initially named K1, KOXY and currently known as OXY. When up regulation occurs due to mutations in the promoter region of these genes, OXY β-lactamases encode resistance to aztreonam and extended spectrum cephalosporins such as ceftriaxone.

Six main variants of OXY chromosomal β-lactamases have been reported and the majority of *K. oxytoca* strains produce OXY-1 or OXY-2, that share 87.3% homology. Additionally, OXY-3 to OXY-6 variants were detected in a limited number of samples, and with the exception of OXY-3, all these uncommon enzymes cluster within the OXY-1 family. A correlation between the presence of OXY-variants and phylogenetic groups of *K. oxytoca* has been established and apparently, OXY-enzymes evolved within each of these groups.

In this study, we analyzed 10 *K. oxytoca* strains that displayed elevated MIC values to ceftazidime, ceftriaxone and/or aztreonam (CLSI ESBL epidemiological criteria) collected from USA hospitals during 2010 and characterized new OXY enzymes.

MATERIALS AND METHODS

Bacterial isolates. A total of 135 *K. oxytoca* bloodstream isolates were collected from USA hospitals during the SENTRY Antimicrobial Surveillance Program (2010). Only one isolate per patient from documented bloodstream infections were included in the study. Species identification was confirmed by standard biochemical tests and the Vitek System (bioMerieux, Hazelwood, Missouri), when necessary.

Antimicrobial susceptibility testing. All isolates were susceptibility tested using the broth microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI, M07-A9). Categorical interpretations for all antimicrobials were those found in M100-S22 (2012) and quality control (QC) was performed using *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. All QC results were within specified ranges as published in CLSI documents (M100-S22).

Genotypic detection of β-lactamases. Strains were selected based on the CLSI criteria for ESBL epidemiological screening (M100-S22). PCR screening was performed for *bla*$_{OXY}$, *bla*$_{TEM}$, *bla*$_{SHV}$ (singleplex reactions), *bla*$_{CTX-M}$, *bla*$_{GES}$, *bla*$_{VEB}$, *bla*$_{PER}$, *bla*$_{PSE}$, *bla*$_{BEL}$, and oxacillinases with ESBL spectrum (*bla*$_{OXA-2}$-, *bla*$_{OXA-10}$- and *bla*$_{OXA-30}$-group, *bla*$_{OXA-18}$ and *bla*$_{OXA-45}$) in a combination of multiplex reactions. Additionally, *bla*$_{CMY-1-41}$, *bla*$_{CMY-43-44}$, *bla*$_{CMY-49}$, *bla*$_{FOX-1-7}$, *bla*$_{ACC-1-4}$, *bla*$_{ACT-1-7}$, *bla*$_{DHA-1-3}$, *bla*$_{LAT-1}$, *bla*$_{MIR-1-5}$, *bla*$_{MOM-1-7}$ were also amplified in a multiplex reaction. One isolate with reduced susceptibility to imipenem and meropenem (MIC, ≥2 µg/mL) was screened for the following carbapenemases: *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SPM-1}$, *bla*$_{KPC}$, *bla*$_{SME}$, *bla*$_{IMI}$, *bla*$_{NMC-A}$, *bla*$_{GES}$ and *bla*$_{OXA-48}$ by PCR. Amplicons were sequenced on both strands and the nucleotide sequences and deduced amino acid sequences were analyzed using the Lasergene software package (DNASTAR, Madison, WI). Sequences were compared to others available via internet sources (http://www.ncbi.nlm.nih.gov/blast/).

Cloning of *bla*$_{OXY}$-variants. Amplicons containing the open reading frame and promoter region of *bla*$_{OXY}$ were cloned into pPCRScriptCam SK+ (Stratagene, California, USA). The colonies obtained after transformation in XL10-Gold® Kan ultracompetent *E. coli* were selected on plates containing 30 µg/mL chloramphenicol. The presence and orientation of inserts was confirmed by PCR and sequencing. MIC testing was performed as described above.

RESULTS

- During 2010, 135 *K. oxytoca* strains were collected in USA hospitals and 10 (7.4%) strains displayed elevated MIC values for aztreonam and/or cephalosporins. Isolates were screened for β-lactamases and, as expected, were positive for *bla*$_{OXY}$ (**Table 1**).
- One strain from Akron, Ohio carried *bla*$_{SHV-12}$ and *bla*$_{TEM-1}$ and another from Charlottesville, Virginia that displayed elevated carbapenem MIC values (imipenem and meropenem MIC, 4 µg/mL) harbored *bla*$_{KPC-2}$ and *bla*$_{OXA-2}$.
- Sequencing of the OXY encoding gene revealed that eight genes were similar or identical to *bla*$_{OXY-2}$ and one displaying one silent mutation compared to *bla*$_{OXY-1}$, but among six isolates aminoacid substitutions were detected and isolates were further analyzed (**Figure 1**). Five new OXY-variants were detected and alteration at position 155 was noted in all variants. Other substitutions were also observed (**Figure 2**).
- The results of the *bla*$_{OXY}$ open reading frame and its promoter cloned and expressed in an *E. coli* background showed differences in the MIC results when compared to OXY-2-producing strains (**Table 1**).
- Aztreonam MIC values were elevated among all constructs, but OXY variants displaying H155R, D199N and H155R, D255N substitutions had 8- to 16-fold lower aztreonam MIC results when compared to OXY-2-producing constructs (**Table 1**).
- Cefoxitin MIC values were greater in all new variants compared to OXY-2 in the same genetic background.
- Cefepime also showed significant differences in two new OXY variants. One isolate displaying H155R and D199N had cefepime MIC values ≤0.5 µg/mL, whereas OXY-2-producing constructs displayed a cefepime MIC of 2 µg/mL. One isolate showing an A15 deletion and H155R substitution had a cefepime MIC of 8 µg/mL.
- The variant displaying a deletion of A15 did not show significantly altered susceptibility profiles and the gene seems functional.
- Ceftazidime, imipenem, meropenem and piperacillin/ tazobactam MIC results were not significantly altered among the new variants.

Table 1. Demographic information, susceptibility testing and β-lactamases present among ESBL-phenotype positive *K. oxytoca* strains collected from blood cultures in USA hospitals during 2010. Susceptibility results for *E. coli* host carrying OXY-variants cloned and expressed are also displayed.

				MIC (µg/mL):								
Isolate	City/State	Culture Date	OXY-variant/amino acid substitution	Ampicillin	Aztreonam	Cefoxitin	Ceftriaxone	Ceftazidime	Cefepime	Piperacillin/ Tazobactam	Imipenem	Meropenem	Additional β-lactamases
K. oxytoca clinical strains													
49A	New Brunswick, NJ	02-Jan-10	H155R, D199N	>8	2	16	0.25	0.5	0.25	8	≤0.12	≤0.12	
1012A	New York, NY	18-Jan-10	H155R, D199N	>8	>16	>16	>8	1	2	>64	0.25	≤0.12	
1115A	Charlottesville, VA	04-Jan-10	A15 deletion, H155R	>8	>16	>16	>8	8	8	>64	4	4	KPC-2, OXA-2
10052A	Akron, OH	NA[a]	H155R, D255N	>8	8	1.9	4	4	0.25	1	≤0.12	≤0.12	SHV-12, TEM-1
17424A	Ewa Beach, HI	01-May-10	OXY-1 (C861G mutation)	>8	>16	1.9	4	0.12	≤0.12	64	≤0.12	≤0.12	
34322A	Detroit, MI	04-Sep-10	H155R	>8	1	16	0.25	0.5	≤0.12	8	≤0.12	≤0.12	
36003A	Burlington, MA	26-Jun-10	Y143H	>8	>16	≤2	>8	0.25	4	>64	≤0.12	≤0.12	
49685A	Milwaukee, WI	06-Nov-10	OXY-2	>8	1	16	0.5	0.5	≤0.12	16	≤0.12	≤0.12	
18295A	Indianapolis, IN	07-Jul-10	OXY-2[b]	>8	8	1.9	1	0.12	0.25	>64	0.25	≤0.12	
20203A	Charlottesville, VA	12-Jun-10	OXY-2[b]	>8	8	>16	>8	2	2	8	1	≤0.12	
E. coli carrying OXY-plasmid constructs													
11180J			H155R, D199N	>8	8	8	1	0.25	≤0.5	32	0.25	≤0.06	
11181J			H155R, D199N	>8	>16	4	>8	0.5	4	>64	0.25	≤0.06	
11179J			A15 deletion, H155R	>8	>16	4	>8	1	8	>64	0.5	≤0.06	
11182J			H155R, D255N	>8	16	16	8	0.5	2	>64	0.25	≤0.06	
11198J			OXY-1 (C861G mutation)	>8	>16	2	>8	1	2	>64	0.25	≤0.06	
11183J			H155R	>8	>16	8	8	0.5	1	>64	0.25	≤0.06	
11199J			Y143H	>8	>16	16	>8	0.5	4	>64	0.25	≤0.06	
11200J			OXY-2	>8	>16	2	8	0.25	2	>64	0.25	≤0.06	

a. NA= Not available.
b. Isolates 18295A and 20203A were not cloned.

Figure 1. Philogenetic tree of *bla*$_{OXY}$ variants, including sequences identified in this study (red box).

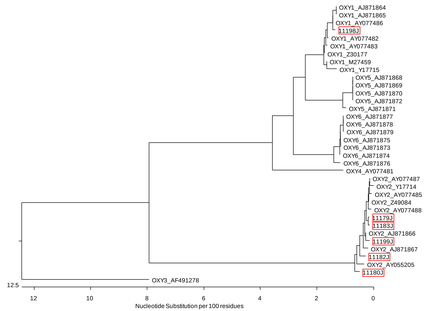
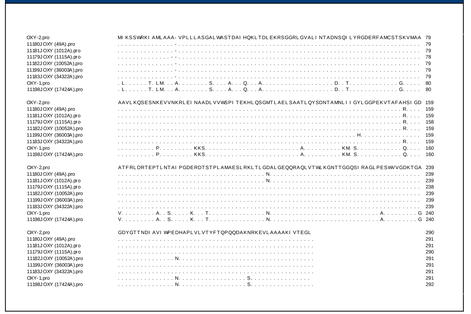


Figure 2. Alignment of the new OXY-variants comparing to OXY-1 and OXY-2. Amino acids identical to OXY-2 are not displayed.



CONCLUSIONS

- Five new OXY variants have been identified among *K. oxytoca* strains collected in USA in 2010. Limited studies of OXY β-lactamases have been reported in USA sampling and strains producing new variants might be widespread in this country.
- An investigation to evaluate the promoter region and genetic relations among these isolates is still in progress.

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