Detection of the Carbapenem-hydrolyzing CTX-M-15 Variant, CTX-M-33, among Carbapenem-nonsusceptible *Klebsiella pneumoniae* Isolates from Poland

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Introduction

- Production of extended spectrum β-lactamases (ESBLs) is the most common β-lactam resistance mechanism among Enterobacterales.
  - Among ESBLs, CTX-M-15 is the most frequently detected ESBL worldwide.
- Carbapenems are often used to treat infections caused by ESBL producers; consequently, prevalence of carbapenem-resistant bacteria, including carbapenemase producers, is increasing.
- Resistance to carbapenems can be caused by diverse mechanisms, including the production of enzymes that hydrolyze carbapenems, changes in cell permeability, and overexpression of efflux genes.
- Recently, a CTX-M-15-like enzyme that hydrolyzes carbapenems, CTX-M-33, was described.
- We identified and characterized 4 carbapenem-nonsusceptible, CTX-M-33-producing *K. pneumoniae* isolates from Poland.

Materials and Methods

- A total of 75 *K. pneumoniae* isolates were received from a medical center in Poland during 2020 as part of the SENTRY Antimicrobial Surveillance Program.
- Isolates were tested for susceptibility by reference broth microdilution as described by the Clinical and Laboratory Standards Institute (CLSI) M07 (2018) and M100 (2021) documents.
  - Quality control (QC) was performed according to the CLSI M100 (2021) criteria.
- Carbapenem-nonsusceptible isolates were submitted to whole genome sequencing and analysis.
  - Total genomic DNA was used as input material for library construction.
  - DNA libraries were prepared using the Nextera XT™ library construction protocol and index kit (Illumina, San Diego, CA, USA).
  - DNA libraries were sequenced on a MiSeq Sequencer using MiSeq Reagent Kit v3 (600 cycle; Illumina).
  - FASTQ format sequencing files for each sample were assembled independently using *de novo* assembler SPAdes 3.13.0.
- An in-house designed software was used for *in silico* screening of resistance genes and multilocus sequence typing (MLST) from the assembled contigs.
  - Mutational resistance mechanisms were identified by comparing sequences with *K. pneumoniae* ATCC 13883.
- Expression of genes encoding efflux pumps and outer membrane proteins were measured by quantitative RT-PCR.
  - Total RNA was extracted, and residual DNA was eliminated by treatment with RNAse-free DNase (Promega, Madison, WI, USA).
  - mRNA quantification and sample quality were assessed using the RNA 6000 Pico kit (Agilent, Santa Clara, CA, USA) on the Agilent 2100 Bioanalyzer according to manufacturer instructions.
  - The transcription levels of *acrA* and outer membrane protein genes were determined in triplicate against a housekeeping reference gene (*gyrA*) using real-time quantitative PCR ($\Delta\Delta C_t$) assays in a StepOne Plus instrument (Life Technologies, Foster City, CA, USA).

Results

- Among 26 (34.7%) of 75 carbapenem-nonsusceptible *K. pneumoniae* isolates from Poland, 4 isolates carried $bla_{CTX\text{-}M\text{-}33}$.
  - $bla_{CTX\text{-}M\text{-}33}$ was detected along with a bla_{SHV} ubiquitous of *K. pneumoniae* and a $bla_{TEM\text{-}1}$ (Table 2).
  - $bla_{OXA\text{-}1}$ was detected in 2 isolates.
  - None of these isolates harbored other carbapenemase-encoding genes.
- Of the 4 isolates carrying $bla_{CTX\text{-}M\text{-}33}$, one each was collected from infections of the bloodstream, intra-abdominal, skin and soft tissue, and urinary tract.
  - These isolates belonged to ST147 (2 isolates), ST215 (1), and ST5082 (1).
- Imipenem MIC values for the CTX-M-33 producers ranged from 4-8 mg/L while meropenem MICs ranged from 16-32 mg/L (Table 1).
  - The MIC results for CTX-M-33-producing isolates were elevated for all other β-lactam agents.
  - Among the newer β-lactam/β-lactamase inhibitor combinations, MIC values were 1-4 mg/L for ceftazidime-avibactam, 0.25-2 mg/L for imipenem-relebactam, and 4-8 mg/L for meropenem-vaborbactam, all except one imipenem-relebactam MIC value were in the susceptible range per CLSI criteria.
  - Amikacin (1-8 mg/L), ciprofloxacin (1->4 mg/L), tetracycline (2->16 mg/L), and tigecycline (0.25-2 mg/L) MIC values varied among the CTX-M-33 producers.
  - All CTX-M-33-producing isolates were resistant to trimethoprim-sulfamethoxazole (MIC, >4 mg/L) and displayed intermediate MIC values to colistin (MIC, 0.12-0.25 mg/L).
- All isolates had a premature stop codon on *ompK35*, while 3 of 4 isolates exhibited reduced (≤0.1X compared to control) expression of this gene (Table 2).
- *ompK36* was disrupted with a premature stop codon in 2 isolates with expression levels similar to the baseline.
  - This gene could not be detected in the remaining 2 isolates.
- Two *K. pneumoniae* isolates displayed >5X expression of the multidrug efflux pump AcrAB-TolC, compared to the control (Table 2).

Table 1. Antimicrobial susceptibility of CTX-M-33-producing *K. pneumoniae* isolates

Isolate	MLST	Ceftazidime	Ceftazidime-avibactam	Imipenem	Imipenem-relebactam	Meropenem	Meropenem-vaborbactam	Amikacin	Tobramycin	Ciprofloxacin	Tetracycline	Tigecycline	Colistin	Trimethoprim-sulfamethoxazole
1162455	147	>32	1	4	0.25	16	4	8	>16	>4	>16	2	0.25	>4
1162485	215[a]	>32	4	8	2	32	8	2	0.5	1	2	0.25	0.12	>4
1163119	147	32	1	4	1	16	4	1	0.25	>4	>16	1	0.12	>4
1183346	5082[a]	>32	2	4	0.25	16	4	4	>16	>4	>16	2	0.25	>4

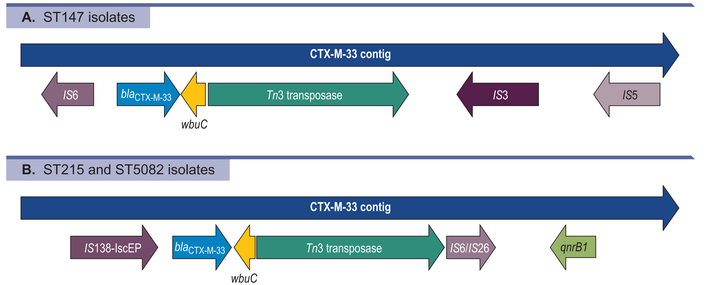
[a] ST215 and ST5082 have 4 of 7 alleles in common.

Table 2. Resistance mechanisms detected among the CTX-M-33-producing *K. pneumoniae* isolates

Characteristic	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Infection type	Intra-abdominal infection	Skin/soft tissue infection	Bloodstream infection	Urinary tract infection
MLST	147	215	147	5082
Resistance mechanisms				
Genes encoding β-lactamases	CTX-M-33, OXA-1, SHV-11, TEM-1	CTX-M-33, SHV-1, TEM-1	CTX-M-33, SHV-1, TEM-1	CTX-M-33, OXA-1, SHV-27, TEM-1
Other resistance mechanisms	*aac(6´)-Ib-cr, aph(6)-Ia, aph(6)-Id, dfrA1, oqxA, qnrS1, sul1, tet(A)*	*aph(6)-Ia, aph(6)-Id, dfrA14, fosA, oqxA, qnrB1, sul2*	*aph(6)-Ia, aph(6)-Id, catA1, dfrA1, oqxA, qnrS1, sul1, tet(A)*	*aac(3)-IIa, aac(6´)-Ib-cr, aph(6)-Ia, aph(6)-Id, dfrA14, oqxA, oqxB19, qnrB1, sul2, tet(A)*
Porin sequence analysis				
OmpK35	Disrupted[a]	Disrupted	Disrupted	Disrupted
OmpK36	Not detected	Disrupted	Not detected	Disrupted
OmpK37	Alterations (V19A, S88T, V295G, D350G, S353K, K356E)	Alterations (V19A, S88T, V295G, D350G, S353K, K356E)	Alterations (V19A, S88T, V295G, D350G, S353K, K356E)	Alterations (V19A, S88T, N230A, M233Q, T234H, Q235F, N237S, N237_A238insDNNF, R239T, N274S, D275T, D275_G276insSSTNGG, V277I, V295G, D350G, S353K, K356E)
Gene expression[b]				
acrA	**Elevated expression** (17.9X)	Similar to baseline (1.4X)	Similar to baseline (2.0X)	**Moderate expression** (6.3X)
ompK35	**Reduced expression** (0.1X)	Similar to baseline (0.7X)	**Reduced expression** (0.1X)	**Reduced expression** (0.1X)
ompK36	No amplification	Similar to baseline (0.3X)	No amplification	Similar to baseline (0.2X)
ompK37	Similar to baseline (6.8X)	Similar to baseline (1.1X)	Similar to baseline (1.0X)	Similar to baseline (3.8X)

[a] Gene disruption in all cases due to a premature stop codon
[b] Fold expression compared to a control strain is indicated in the parentheses

Figure 1. Genetic context of $bla_{CTX\text{-}M\text{-}33}$ in the *K. pneumoniae* isolates



- Isolates belonging to ST147 showed similar genetic context for $bla_{CTX\text{-}M\text{-}33}$.
  - The β-lactamase gene was flanked by *IS*6, *IS*3, and *IS*5 sequences (Figure 1A).
  - No other resistance gene was detected in the vicinities of $bla_{CTX\text{-}M\text{-}33}$, although both isolates carried a *qnrS1*.
- Isolates belonging to ST215 and ST5082 shared a similar $bla_{CTX\text{-}M\text{-}33}$ context that included a *IS*138-like *IS*Ecp1 family transposase and a *Tn*3 transposase (Figure 1B).
  - *qnrB1* was present downstream of *IS*6 transposase in these isolates.

Conclusions

- Alterations in β-lactamases that expand the activity of these enzymes to other agents have been documented.
  - The CTX-M-15-derivative CTX-M-33 (single amino acid Asp to Ser substitution at Ambler position 109) observed in 4 isolates from Poland led to carbapenem resistance.
  - Other non-carbapenemase mechanisms complementing resistance to carbapenems were present in the isolates producing this enzyme.
- This set of isolates showed genetic diversity as well as variability in the $bla_{CTX\text{-}M\text{-}33}$ genetic context.
  - Furthermore, 2 isolates harboring $bla_{CTX\text{-}M\text{-}33}$ belonged to ST147, globally recognized as an emerging high-risk clone that carries CTX-M-15 and has recently acquired carbapenemases.
- Surveillance for CTX-M-33 should be performed to understand the dissemination of this resistance gene and evaluate treatment options for the infections caused by CTX-M-33 producers.

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