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# Exploration of Genetic Mutations Associated with Reduced Susceptibility to Ertapenem in *Enterobacterales* Clinical Isolates: CANWARD 2007-2018

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## Background

Antimicrobial resistance is increasing globally in *Enterobacterales* [1]. Of particular concern is resistance to carbapenems, the broadest spectrum class of antimicrobials, and one of the only remaining classes available to treat resistant pathogens [2, 3]. Carbapenem resistance can often be attributed to the presence of carbapenemase enzymes; in the USA, carbapenemase-producing *Enterobacterales* (CPE) account for ~35-59% of all carbapenem-resistant *Enterobacterales* [4]. In Canada, the number of CPE collected increased from 81 in 2015 to 261 in 2019 [3]. However, β-lactam/carbapenem resistance in *Enterobacterales* can be associated with a number of acquired resistance elements and gene alterations, for example altered/truncated outer membrane porin genes [4].

This study utilized whole genome sequencing data of a cohort of ertapenem-nonsusceptible *Enterobacterales* clinical isolates collected from patients in Canadian hospitals. The purpose of this study was to identify the resistance mechanisms associated with reduced susceptibility to ertapenem in Canada.

## Materials and Methods

**Bacterial Isolates:**
CANWARD is an ongoing national Public Health Agency of Canada/Canadian Antimicrobial Resistance Alliance (PHAC/CARA) partnered surveillance study evaluating *in vitro* activities of antimicrobial agents against bacterial pathogens isolated by clinical laboratories from patients attending tertiary care hospitals across Canada. Hospitals in 8 of the 10 Canadian provinces submitted clinically relevant isolates from inpatients and outpatients attending hospital clinics, medical and surgical wards, emergency rooms, and intensive care units to the CANWARD coordinating laboratory (Health Sciences Centre, Winnipeg, Canada). The CANWARD study sets annual quotas for respiratory, wound, urine and bloodstream isolates and requires isolates to be collected consecutively, one per patient, per site of infection. Isolates are deemed clinically significant by the submitting sites local testing criteria and the identities of the isolates are confirmed, by colonial appearance, spot testing and/or MALDI-TOF MS (Bruker Daltonics, Billerica, MA, USA), upon receipt at the CANWARD coordinating laboratory.

A total of 18,027 *Enterobacterales* isolates were collected from January 2007 to December 2018 as part of the CANWARD surveillance study. Antimicrobial susceptibility testing for ertapenem and comparator agents was performed using reference CLSI broth microdilution [5] and MICs were interpreted using CLSI M100 breakpoints [6]. From this isolate collection, 179 ertapenem-nonsusceptible *Enterobacterales* (79 ertapenem-intermediate, MIC = 1 µg/mL; 100 ertapenem-resistant, MIC ≥2 µg/mL) were identified, including 96 *Enterobacter cloacae*, 47 *Klebsiella* spp., 26 *E. coli* and 10 isolates of other species.

**Whole Genome Sequencing and Analysis:**
Ertapenem-nonsusceptible isolates, plus 51 ertapenem-susceptible *Enterobacterales* controls, were sequenced using the Illumina NextSeq platform. Carbapenemases and other acquired β-lactamases were identified using ResFinder 4.0 [7]. Alterations in genes *ompC/F* (and homologues) and *ftsI* (encoding PBP3) were identified by comparing extracted sequences to the appropriate NCBI reference gene. Porin alterations were analyzed with Provean v1.1.3 to predict those that may have a negative impact on protein function. Specific alterations of interest in PBP3 included a YRIN or YRIK insertion after P333.

## Results

**Table 1. Various β-lactam resistance mechanisms identified in 179 ertapenem-nonsusceptible and 51 ertapenem-susceptible *Enterobacterales*.**

| ERT MIC (n) [µg/mL] | Percentage of Isolates with Genome Feature | | | | | |
|---|---|---|---|---|---|---|
| | CP [a] | ESBL [b] | Class C BL [c, d] | Other BL [e] | Altered Porins [f] | PBP3 Insertion [g] |
| **All ERT-S (51)** | 0 | 5.9 | 9.8 | 19.6 | 86.3 | 0 |
| **≤0.25 (30)** | 0 | 0 | 3.3 | 26.7 | 83.3 | 0 |
| **0.5 (21)** | 0 | 14.3 | 19.0 | 9.5 | 90.5 | 0 |
| **ERT-I (79)** | | | | | | |
| **1 (79)** | 1.3 | 16.5 | 13.9 | 24.1 | 88.6 | 0 |
| **All ERT-R (100)** | 15.0 | 26.0 | 17.0 | 42.0 | 93.0 | 2.0 |
| **2 (49)** | 2.0 | 18.4 | 26.5 | 32.7 | 89.8 | 2.0 |
| **4 (11)** | 0 | 27.3 | 18.2 | 45.5 | 90.9 | 0 |
| **8 (9)** | 11.1 | 44.4 | 11.1 | 55.6 | 88.9 | 0 |
| **16 (10)** | 20.0 | 20.0 | 10.0 | 40.0 | 80.0 | 0 |
| **32 (7)** | 42.9 | 42.9 | 0 | 57.1 | 100 | 0 |
| **>32 (14)** | 57.1 | 35.7 | 0 | 57.1 | 100 | 7.1 |
| **All ERT-NS (179)** | 8.9 | 21.8 | 15.6 | 34.1 | 89.9 | 1.1 |

[a] CP, carbapenemases: genes identified include KPC-2, KPC-3, NDM-1, NDM-5, OXA-48 (and OXA-48-like) and SME-3. [b] ESBL genes identified include CTX-M-3, 9, 14, 15, 67 and 71, and SHV-12 and 38; [c] BL, β-lactamases. [d] Class C genes identified include acquired genes only: CMY-, DHA-, FOX-, MIR-, and PAO-family genes. [e] Includes all other BL that are not CP, ESBL or class C enzymes. [f] Includes ompC/ompK36 and ompF/ompK35, plus ompK37 for *K. pneumoniae* only; includes isolates where gene was either truncated due to a premature stop codon or altered in such a way (point mutations, insertions/deletions) that Provean predicted a negative impact on biological protein function. [g] Four amino acid insertion (YRIN, YRIK) after P333 of PBP3. S, susceptible. I, intermediate. R, resistant.

**Table 2. Ertapenem MIC distribution (µg/mL) versus 16 *Enterobacterales* with carbapenemase genes.**

| Carbapenemase (organism) | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | > 32 | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| KPC-2 (All *K. pneumoniae*) | | | | 1 | | | 1 | | 2 | 4 |
| KPC-3 [a] | | | | | | 1 | 1 | 1 | 3 | 6 |
| NDM-1 + OXA-232 (All *K. pneumoniae*) | | | | | | | | 2 | | 2 |
| NDM-5 + OXA-181 (*E. coli*) | | | | | | | | | 1 | 1 |
| OXA-48 (*K. pneumoniae*) | | | | | | | | | 1 | 1 |
| OXA-181 (*K. pneumoniae*) | | | 1 | | | | | | | 1 |
| SME-3 (*S. marcescens*) | | | | | | | | | 1 | 1 |

[a] Includes two *E. coli*, two *K. pneumoniae*, one *Klebsiella oxytoca* and one *S. marcescens*.
Light orange shading: ertapenem-susceptible; medium orange shading: ertapenem-intermediate; dark orange shading: ertapenem-resistant.

**Table 3. Expanded gene content of 21 ertapenem-nonsusceptible *Enterobacterales* with MICs ≥32 µg/mL.**

| ERT MIC (µg/mL) | Organism (ST) | Clinical Source | CPs | ESBLs | Other BL [a] | Porin Alterations [b] | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | OmpC (OmpK36) | OmpF (OmpK35) | OmpK37 (KP only) |
| 32 | ECL (ST113) | Resp | - | - | - | g.C511T → premature stop codon (truncated 170aa protein) | N48Y | NA |
| | ECL (ST135) | Blood | - | - | - | g.C244T → premature stop codon (truncated 81aa protein) | N48Y | NA |
| | KA (NF) | Wound | - | - | - | A12_V15del | - | NA |
| | KO (NF) | Wound | KPC-3 | - | OXY-1-7, TEM-1B | D189G, T222N | - | NA |
| | KP (ST16) | Blood | NDM-1, OXA-232 | CTX-M-15 | OXA-9, SHV-1, TEM-1C | N304delinsER | - | - |
| | KP (ST16) | Blood | NDM-1, OXA-232 | CTX-M-15 | OXA-9, SHV-1, TEM-1C | A190W, N304delinsER | - | - |
| | KP (ST967) | Blood | - | CTX-M-3, SHV-27 | TEM-1B | g.T75A → premature stop codon (truncated 24aa protein) | - | - |
| >32 | ECL (ST141) | Resp | - | - | - | G104A, G358Q, L359F | N48Y | NA |
| | EC (ST405) | Resp | - | CTX-M-71 | - | N165D, F182_R195delinsMTTNGRDDVFE, D208N, D225W | g.756_757insGAAC → frameshift, premature stop codon (truncated 256aa protein) | NA |
| | EC (ST361) | Blood | NDM-5, OXA-181 | - | TEM-1B | D192G | - [c] | NA |
| | EC (ST131) | Wound | - | CTX-M-15 | - | D192G, T229_A231delinsFGLNGYGER | g.525_529delCGCTG → frameshift, premature stop codon (truncated 179aa protein) | NA |
| | KA (ST135) | Wound | - | - | - | g.G281A → premature stop codon (truncated 93aa protein) | - | NA |
| | KA (NF) | Blood | - | - | - | g.C582T → premature stop codon (truncated 194aa protein) | - | NA |
| | KP (ST258) | Blood | KPC-3 | - | OXA-9, SHV-11, TEM-1A | A183_T184insLSP, T222L | g.121_122insG → frameshift, premature stop codon (truncated 88aa protein) | N230G, M233_R239delinsQHYTHTERYAK |
| | KP (ST101) | Wound | OXA-48 | CTX-M-15 | OXA-1, SCO-1, SHV-1, TEM-1A | G134_D135insDG, A190W, N304delinsER | g.185delG → frameshift, premature stop codon (truncated 62aa protein) | - |
| | KP (ST45) | Blood | - | CTX-M-15 | OXA-1, SHV-1, TEM-1B | - | - | N230G, M233_R239delinsQHYTHTERYAK |
| | KP (ST834) | Resp | KPC-2 | - | SHV-11, TEM-1B | T222N | - | N230G, M233_R239delinsQHYTHTERYAK |
| | KP (NF) | Wound | KPC-3 | - | SHV-11 | G134_D135insDG, A183_T184insLSP, T222L | g.121_122insG → frameshift, premature stop codon (truncated 88aa protein) | N230G, M233_R239delinsQHYTHTERYAK |
| | KP (ST258) | Blood | KPC-2 | SHV-12 | OXA-9 | A183_T184insLSP, T222L | g.121_122insG → frameshift, premature stop codon (truncated 88aa protein) | N230G, M233_R239delinsQHYTHTERYAK |
| | SM (NA) | Blood | KPC-3 | - | OXA-9, TEM-1C | D157G, V252T | D229K, D351K, G359K | NA |
| | SM (NA) | Resp | SME-3 | - | - | - | I360T | NA |

ST, sequence type; CP, carbapenemase; BL, β-lactamase. EC, *E. coli*; ECL, *Enterobacter cloacae*; KA, *Klebsiella aerogenes*; KP, *Klebsiella pneumoniae*; KO, *Klebsiella oxytoca*; SM, *Serratia marcescens*. NF, sequence type not found; NA, no MLST database available.
[a] Includes all other BL that are not CP, ESBL or class C enzymes; [b] Including truncations due to a premature stop codon, or alterations predicted by Provean to have a negative impact on biological protein function; [c] EC isolate possesses Y333_R334insYRIN in PBP3.

## Discussion and Conclusions

- The presence of carbapenemase genes generally increased in prevalence with increasing ertapenem MIC, with 52.4% of isolates with ertapenem MIC ≥32 µg/mL possessing a carbapenemase gene.
- ESBLs were present in 26.0% of ertapenem-resistant isolates.
- In isolates with ertapenem-resistant MICs, acquired class C β-lactamase genes were less common overall than ESBL and other BL genes (excluding carbapenemase genes) and became less common with increases in ertapenem MICs.
- Alterations in the coding region of major porin genes were very common regardless of ertapenem MIC.
- PBP3 insertions were uncommon in either ertapenem-susceptible or ertapenem-nonsusceptible isolates.
- A limitation of this study is that expression of chromosomal AmpC genes or porin genes was not studied.
- Isolates with high level resistance to ertapenem (MIC ≥32 µg/mL) tended to possess a carbapenemase, ESBL gene(s), other β-lactamases and/or had at least one truncated or frameshifted porin gene.
- Continued genomic surveillance of antimicrobial-resistant *Enterobacterales* is crucial to understand the evolving mechanisms of carbapenem resistance.

The specimen sources of the 179 ertapenem-nonsusceptible *Enterobacterales* were as follows: 43.0% (n=77), 38.5% (n=69), 9.5% (n=17) and 8.9% (n=16) from respiratory, blood, urine and wound specimens, respectively.

**Table 4. Ertapenem MIC distribution (µg/mL) versus 42 *Enterobacterales* with ESBL genes.**

| ESBL (organism) | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | > 32 | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| CTX-M-3 + SHV-27 (*K. pneumoniae*) | | | | | | | | 1 | | 1 |
| CTX-M-9 (*E. cloacae*) | | 1 | | | | | | | | 1 |
| CTX-M-14 (*E. coli*) | | | 1 | | | | | | | 1 |
| CTX-M-15 [a] | | | 10 | 4 | 2 | 4 | 1 | 2 | 3 | 26 |
| CTX-M-67 (*E. coli*) | | | | | 1 | | | | | 1 |
| CTX-M-71 (*E. coli*) | | | | | | | | | 1 | 1 |
| SHV-12 [b] | | 1 | 2 | 5 | | | 1 | | 1 | 10 |
| SHV-38 (*K. pneumoniae*) | | 1 | | | | | | | | 1 |

[a] Includes 15 *K. pneumoniae*, 10 *E. coli* and one *E. cloacae*.
[b] Includes eight *E. cloacae* and two *K. pneumoniae*.
Light orange shading: ertapenem-susceptible; medium orange shading: ertapenem-intermediate; dark orange shading: ertapenem-resistant.

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