Phenotypic and Genotypic Characterization of Enterobacteriaceae Producing Oxacillinase-48–Like Carbapenemases, United States


Oxacillinase (OXA)–48–like carbapenemases remain relatively uncommon in the United States. We performed phenotypic and genotypic characterization of 30 Enterobacteriaceae producing OXA-48–like carbapenemases that were recovered from patients during 2010–2014. Isolates were collected from 12 states and not associated with outbreaks, although we could not exclude limited local transmission. The alleles β-lactamase OXA-181 (bla_{OXA-181}) (43%), bla_{OXA-232} (33%), and bla_{OXA-48} (23%) were found. All isolates were resistant to ertapenem and showed positive results for the ertapenem and meropenem modified Hodge test and the modified carbapenem inactivation method; 73% showed a positive result for the Carba Nordmann–Poirel test. Whole-genome sequencing identified extended-spectrum β-lactamase genes in 93% of isolates. In all bla_{OXA-232} isolates, the gene was on a ColKP3 plasmid. A total of 12 of 13 isolates harboring bla_{OXA-181} contained the insertion sequence ΔISEcp1. In all isolates with bla_{OXA-48}, the gene was located on a TN1999 transposon; these isolates also carried IncL/M plasmids.

The prevalence of carbapenem-resistant Enterobacteriaceae (CRE) has been increasing in the United States since 2000 (1,2). This finding is problematic because treatment options for CRE infection are limited, and these infections are associated with a higher mortality rate than are infections with carbapenem-susceptible Enterobacteriaceae (3). Enterobacteriaceae might be resistant to carbapenems by a variety of mechanisms, the most concerning of which is production of carbapenemases (4). Although the Klebsiella pneumoniae carbapenemase is the most common carbapenemase reported in the United States, there have been reports of several other carbapenemases including the metallo-β-lactamases and, more recently, oxacillinase (OXA)–48–like carbapenemases (1,5–10).

OXA-48 is a member of the ambler class D β-lactamase family, first described in a K. pneumoniae isolate from Turkey in 2004 (11). The OXA-48 enzyme hydrolyzes penicillins efficiently, carbapenems slowly, and extended-spectrum cephalosporins poorly; it is not inhibited by tazobactam, sulbactam, or clavulanic acid (12). Since the initial report, OXA-48 has established reservoirs in Turkey, the Middle East, countries in North Africa, and throughout Europe (12). These reservoirs have been reported in multiple Enterobacteriaceae species in addition to K. pneumoniae, including Citrobacter freundii, Enterobacter cloacae, Escherichia coli, K. oxytoca, Serratia marcescens, and Providencia rettgeri (12). In addition to OXA-48, several variants with similar enzymatic profiles have been described, including OXA-13, -162, -181, -204, -232, -244, -245, -370, -436, -438, and -484; each variant differs from OXA-48 by only a few amino acids (12–16). Other variants that do not hydrolyze carbapenems have also been described, including OXA-163, -247, and -405 (13,17,18).

The first description of isolates with β-lactamase OXA-48–like (bla_{OXA-48–like}) genes in the United States was from a surveillance study in 2013, which incidentally reported 2 K. pneumoniae isolates (6). This description was followed shortly afterward by a report of 2 clinical K. pneumoniae isolates with bla_{OXA-48–like} genes in patients from 1 institution in Virginia who had traveled internationally (7). More recently, CRE with bla_{OXA-232} genes have been isolated in the United States (8). The Centers for Disease Control and Prevention (CDC) has collected multiple isolates harboring bla_{OXA-48–like} genes from patients in the United States (19). We report the genotypic and phenotypic characterization of those isolates.

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Materials and Methods

Collection of Isolates
Isolates are submitted to CDC for antimicrobial susceptibility testing (AST) for many reasons, including outbreak response, AST confirmation, and surveillance studies. Surveillance studies include the Multi-Site Gram-Negative Surveillance Initiative, which is part of the Emerging Infections Program, and the Sentinel Study (5,20). All Enterobacteriaceae isolates received for AST at CDC during June 1, 2010–October 31, 2012, with reduced susceptibility to carbapenems (MIC ≥1 μg/mL for any carbapenem), a positive modified Hodge test result, and a PCR-negative result for bla K. pneumoniae were retrospectively screened for blaOXA-48–like genes (n = 115). During November 1, 2012–September 30, 2014, all Enterobacteriaceae received at CDC were routinely tested for blaOXA-48–like genes by real-time PCR (n = 1,399). Submitting institutions were characterized by state and US Department of Health and Human Services (HHS) region (https://www.hhs.gov/ash/about-ash/regional-offices/index.html).

Phenotypic Characterization of Isolates
We performed reference broth microdilution AST on all isolates by using in-house prepared frozen panels that included carbapenems, cephalosporins, aztreonam, penicillins, quinolones, trimethoprim/sulfamethoxazole, aminoglycosides, chloramphenicol, tetracyclines, tigecycline, polymyxin B, and colistin (21,22). The modified Hodge test, Carba Nordmann–Poirel test, and the modified carbapenem inactivation method (mCIM) were performed on all blaOXA-48–like isolates according to Clinical and Laboratory Standards Institute guidelines (22). We confirmed species identification by using the Biotype 3.1 MALDI System (Bruker Daltronics, Billerica, MA, USA).

Genotypic Characterization of Isolates
The PCR for blaOXA-48–like genes was developed at CDC and detects blaOXA-48, blaOXA-162, blaOXA-163, blaOXA-181, blaOXA-204, blaOXA-232, blaOXA-244, blaOXA-245, and blaOXA-247 by using 2 sets of blaOXA-48–like primers/probes and a bacterial 16S rRNA gene as an endogenous control for lysis validation and PCR amplification (Table 1). We extracted DNA by using the thermal/sodium hydroxide method for preparation of bacterial cell lysates (23). Cycling conditions were a 3-min enzyme activation step at 95°C, followed by 40 cycles for 3 s at 95°C, and a final step for 30 s at 60°C (24). We characterized all isolates positive for blaOXA-48–like genes by using whole-genome sequencing (WGS). We extracted DNA by using the Maxwell 16 Cell Low Elution Volume DNA Purification Kit (Promega, Madison, WI, USA) and fragmented input genomic DNA (gDNA) with an absorbance ratio of 1.8–2.0 to ≈800 bp by using an ultrasonic fragmentation system (Covaris, Woburn, MA, USA).

We prepared libraries by using the Ovation Ultralow DR Multiplex System 1-96 Kit (Nugen Technologies, Inc., San Carlos, CA, USA), then multiplexed, and sequenced with MiSeq V2.0 (Illumina, San Diego, CA, USA). We filtered raw Illumina sequencing reads for quality (average ≥Q20) and discarded trimmed reads <50 bp from the dataset by using SolexaQA version 3.1 (25). We then assembled clean reads into contigs by using SPAdes version 3.1.0 and 4 k-mer sizes (k = 41, 79, 85, and 97) (26). Afterward, we mapped trimmed reads back to each assembled genome by using the Burrows-Wheeler Alignment tool for minor contig error correction (27).

We randomly selected K. pneumoniae isolates 1, 11, and 23, encoding blaOXA-181, blaOXA-232, and blaOXA-48 respectively, as internal reference strains and sequenced them by using Single Molecule Real-Time Technology (Pacific Biosciences, Menlo Park, CA, USA) in addition to Illumina sequencing (Table 2). We extracted and purified gDNA by using the MasterPure Complete DNA and RNA Kit (Epicenter, Madison, WI, USA), according to the manufacturer’s recommended protocol. We generated 10-kb libraries by using the SMRTbell Template Prep Kit 1.0 (Pacific Biosciences) and sequenced libraries by using C4v2 Chemistry on the RSII Instrument (Pacific Biosciences). We assembled data by using Hierarchical Genome-Assembly Process

Table 1. Sequences of primers and probes used for identification of Enterobacteriaceae isolates with β-lactamase OXA-48–like carbapenemases, United States

<table>
<thead>
<tr>
<th>Primers and probes</th>
<th>Sequence, 5′→3′</th>
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<tr>
<td>16S rRNA, forward primer</td>
<td>TGG AGC ATG TGG TTT ATT ATG A</td>
</tr>
<tr>
<td>16S rRNA, reverse primer</td>
<td>TGG GGC ACT TAA CCC AAC A</td>
</tr>
<tr>
<td>16S rRNA, probe (CY5)</td>
<td>CY5-CA CGA GCT GAC GAC ARC CAT GCA-BHQ</td>
</tr>
<tr>
<td>OXA-48, forward 180</td>
<td>ACG GGC GAA CCA AGC AT</td>
</tr>
<tr>
<td>OXA-48, reverse 239</td>
<td>GCG ATC AAG CTA TTG GGA ATT T</td>
</tr>
<tr>
<td>OXA-48, probe 199</td>
<td>FAM-TT ACC CGG ATC TAC TAC C-BHQ</td>
</tr>
<tr>
<td>OXA-48, forward 722</td>
<td>TGC CCA CAT CAG ATG GTT</td>
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<tr>
<td>OXA-48, reverse 781</td>
<td>CCT GTT TGA GCC CTT CTG TTG TGA</td>
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<td>OXA-48, probe 741</td>
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</tr>
<tr>
<td>OXA-48 R1</td>
<td>CTA KGG AAT WAT YTT YTC CTG</td>
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</table>

*OXA, oxacillinase.
version 3.0 (Pacific Biosciences) and generated clean consensus sequences by using Quiver (28).

We deposited all raw sequencing reads, Pacific Biosciences assemblies, and MIC results in GenBank under BioProject PRJNA296771. We determined multilocus sequence types for each specimen by mapping clean Illumina reads to allele sequences (http://www.pubmlst.org) by using SRST2 software (29). We described antimicrobial resistance genotype profiles from assembled Illumina and Pacific Biosciences contigs by using SSTAR V1.0 (30) in combination with the ARG-ANNOT (31) and ResFinder (32) repositories.

We used the PlasmidFinder database (http://www.genomicepidemiology.org/) to detect plasmid replicon sequences among Illumina and Pacific Biosciences contigs to estimate the plasmid composition of each isolate (33). In addition, we predicted insertion sequences that might be associated with spread of antimicrobial resistance genes by using ISFinder (34). For isolates with bla<sub>CTX-M-15</sub> we estimated the copy number of IS<sub>R</sub> insertion sequences for determining Tn1999 variants by using blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and SPAdes K-mer coverage output (26,34–36). The clonality of our plasmids was also assessed, as was the location of bla<sub>CTX-M-15</sub>-like genes (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/4/17-1377-Techapp1.pdf). Because of a cluster of isolates from 1 state in this study, a phylogenetic tree and single-nucleotide polymorphism (SNP) tree matrix were produced by using RAxML version 8 (37) (online Technical Appendix).

**Transformation Experiments**

We randomly transformed 10 selected isolates (3 with bla<sub>CTX-M-15</sub>, 4 with bla<sub>SHV-12</sub> and 3 with bla<sub>TEM-1</sub>) for

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**Table 2. Phenotypic and genotypic characterization of Enterobacteriaceae harboring β-lactamase OXA-48–like carbapenemase, United States***

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<th>Isolate no.</th>
<th>Species</th>
<th>Year</th>
<th>Source</th>
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<th>MEM</th>
<th>mCIM</th>
<th>Carba NP</th>
<th>ST</th>
<th>OXA allele</th>
<th>NDM allele</th>
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*ESBLs, extended-spectrum β-lactamases; HHS, Health and Human Services; Ind, indeterminate; mCIM, modified carbapenem inactivation method; MEM, meropenem; MHT, modified Hodge test; NDM, New Delhi metallo-β-lactamase gene; NP, Nordmann–Poirel; OXA, oxacillinase; ST, sequence type (by multilocus sequence typing); –, negative; +, positive.

transformation experiments to better characterize plasmids harboring \textit{bla}_{OXA-48}-like genes. We subcultured parent isolates on trypticase soy agar containing 5% sheep blood, placed them in 50 mL of tryptic soy broth containing ertapenem (1 \mu g/mL), and incubated them overnight at 35°C. We extracted plasmid DNA by using Plasmid Midi Kits (QIAGEN, Valencia, CA, USA), according to the manufacturer’s protocol. We digested intact plasmid DNA and gDNA with \textit{HindIII} (New England Biolabs, Ipswich, MA, USA) and separated this DNA by electrophoresis on a 0.9% agarose gel.

We transformed 500 ng of plasmid DNA from each isolate into \textit{E. coli} DH10B cells (Invitrogen, Carlsbad, CA, USA) by electroporation and incubated at 35°C for 2 h. Potential transformants were plated on Luria–Bertani agar containing ertapenem (1 \mu g/mL) and incubated overnight at 35°C. Four colonies from each transformant plate were screened for \textit{bla}_{OXA-48}-like genes by using PCR. Transformant plasmid DNA was digested and separated by gel electrophoresis along with digested parent plasmid DNA to ensure that transformant plasmids were also present in parental cells.

We characterized confirmed transformants by using AST, the modified Hodge test, and WGS with MiSeq V2.0 (Illumina), as described previously. Trimmed reads from transformants were mapped to the genome sequence of \textit{E. coli} K12, substrain DH10B (GenBank accession no. NC_010473.1), by using Bowtie 2 software (38,39). Unmapped reads were extracted by using \textit{bam2fastq} (https://gsl.hudsonalpha.org/information/software/bam2fastq) and were considered to represent plasmid DNA harboring \textit{bla}_{OXA-48}-like genes (https://gsl.hudsonalpha.org/information/software/bam2fastq). We subsequently assembled these unmapped reads by using SPAdes software and screened generated contigs for antimicrobial drug resistance genes by using SSTAR V1.0 and for plasmid replicon sequences by using the PlasmidFinder database (26,30,33).

**Results**

**Epidemiology of Isolates**

We included all 30 US isolates in our collection that were positive for \textit{bla}_{OXA-48}-like carbapenemase gene in this study. Isolates were submitted from patients in 12 states representing 8 HHS regions: one from region 1, two from region 2, four from region 3, three from region 4, eight from region 5, three from region 6, eight from region 9, and one from region 10. \textit{K. pneumoniae} predominated (n = 27, 90%), although single isolates of \textit{K. ozacena}, \	extit{Enterobacter aerogenes}, and \textit{E. coli} (n = 1 each, 3%) were also found. Isolates were collected from a variety of sources: urine (n = 15, 50%), respiratory samples (n = 10, 33%), peritoneal fluids (n = 2, 7%), wounds (n = 1, 3%), rectal swab specimens (n = 1, 3%), and unknown sources (n = 1, 3%) (Table 2).

**Phenotypic Characterization of Isolates**

All submitted isolates with a \textit{bla}_{OXA-48}-like carbapenemase gene showed resistance to ertapenem and all penicillins tested (including those with β-lactamase inhibitors). Most showed intermediate resistance or resistance to imipenem (n = 30, 100%), meropenem (n = 28, 93%), doripenem (n = 28, 93%), ceftriaxone (n = 29, 97%), ceftazidime (n = 27, 90%), and cefepime (n = 28, 93%). In addition, all isolates had a colistin MIC < $2 \mu g/mL$ (Table 3). Results for the ertapenem modified Hodge test, meropenem modified Hodge test, and mCIM were positive for all isolates harboring \textit{bla}_{OXA-48}-like genes. The Carba Nordmann–Poirel test result was positive for 73% of isolates, indeterminate in 13%, and negative in 13% (Table 2).

**Transformation Experiments**

We purified plasmid DNA from 10 isolates (3 with \textit{bla}_{OXA-48}, 4 with \textit{bla}_{OXA-181}, and 3 with \textit{bla}_{OXA-232}) for transformation into \textit{E. coli} DH10B. Transformants were obtained for each preparation from strains harboring \textit{bla}_{OXA-48} and \textit{bla}_{OXA-252} as confirmed by PCR and phenotypic and genotypic characterization of each transformant (Table 4). Transformation was unsuccessful for all DNA preparations from strains with \textit{bla}_{OXA-181} (isolates 1, 2, 26, and 27).

When we compared transformants with parent strains, most of which harbored multiple plasmids and numerous resistance genes, transformants were confirmed to carry only 1 plasmid and typically showed greater susceptibility to extended-spectrum cephalosporins but retained resistance to $\geq 1$ carbapenem. As confirmed by WGS, we found that ESBL genes were not typically present on the same plasmid as \textit{bla}_{OXA-48}-like genes; only 1 transformant (23T) carried a plasmid harboring \textit{bla}_{CTX-M-14b} on the IncL/M plasmid carrying $\textit{bla}_{OXA-48}$. Similar to the parent strain, strain 23T showed increased MICs to cephalosporins and carbapenems, although the carbapenem MICs were lower than both the parent strain and other transformants carrying only an OXA-48–like carbapenemase (Table 4). None of the plasmids harboring \textit{bla}_{OXA-48}-like genes encoded additional carbapenemases.

**Genotypic Characterization of Isolates**

We confirmed by using WGS the presence of \textit{bla}_{OXA-48}-like genes in every isolate, including the alleles \textit{bla}_{OXA-48} (n = 7, 23%), \textit{bla}_{OXA-181} (43%), and \textit{bla}_{OXA-232} (33%). The gene \textit{ bla}_{NDM} was identified in 5 isolates with \textit{ bla}_{OXA-232}. Nearly all isolates (93%) contained >1 ESBL gene, including \textit{bla}_{SHV-12}, \textit{bla}_{CTX-M-14b}, and \textit{bla}_{CTX-M-15} (Table 2). We also found aminoglycoside, fluoroquinolone, sulfonamide, trimethoprim, tetracycline, chloramphenicol, macrolide, and fosfomycin resistance genes. Multilocus sequence typing of 27 \textit{K. pneumoniae} isolates showed ST34 (n = 7), ST14 (n = 7), ST16 (n = 3), ST43 (n = 3), and ST101 (n = 3) to be most common in this collection (Table 2).
Table 3. MIC results for *Enterobacteriaceae* harboring β-lactamase oxacillinase-48–like carbapenemases, United States*

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Species</th>
<th>Drug, MIC, µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Klebsiella pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td><em>K. oxytoca</em></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td><em>Enterobacter aerogenes</em></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td><em>K. pneumoniae</em></td>
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<td>16</td>
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<td><em>K. pneumoniae</em></td>
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<tr>
<td>21</td>
<td><em>K. pneumoniae</em></td>
<td></td>
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<tr>
<td>22</td>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td><em>Escherichia coli</em></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td><em>K. pneumoniae</em></td>
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<tr>
<td>29</td>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td><em>K. pneumoniae</em></td>
<td></td>
</tr>
</tbody>
</table>

*Not all drugs tested are listed. βla, β-lactamase; AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; COL, colistin; CRO, ceftriaxone; DOR, doripenem; ETP, ertapenem; FEP, cefepime; IMP, imipenem; MEM, meropenem; TIG, tigecycline; TZP, piperacillin/tazobactam.*

Isolates 11, 11, and 23 (carrying bla*oxa-48* and bla*oxa-48*, respectively) were randomly chosen for Pacific Biosciences WGS in addition to Illumina WGS. Isolate 1 had 2 plasmids and encoded 20 antimicrobial drug resistance genes, including 3 chromosomal copies of the ESBL CTX-M-15; *bla*oxa-181 was also chromosomally located, with an upstream Δ*ISEcp1* insertion sequence. The Δ*ISEcp1* insertion sequence has been described elsewhere (40–42). Isolate 11 had 4 plasmids and encoded 34 antimicrobial drug resistance genes, including plasmid-mediated *bla*CTX-M-15 and *bla*NDM-1 genes. The *bla*oxa-232 allele in isolate 11 was found on a ColK3 plasmid (plasmid size 6,139 bp, G + C content 52.17%); upstream of *bla*oxa-232, there was a Δ*ISEcp1* insertion sequence. The sequence

Table 4. Plasmid transformation of *Enterobacteriaceae* producing OXA-48–like carbapenemases, United States*

<table>
<thead>
<tr>
<th>Isolate no.†</th>
<th>ETP</th>
<th>MEM</th>
<th>CRO</th>
<th>TIZP</th>
<th>AMK</th>
<th>MHT MEM</th>
<th>OXA allele</th>
<th>Plasmid replicon type</th>
<th>ESBL CTX-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>&lt;0.12</td>
<td>&lt;0.12</td>
<td>&lt;1</td>
<td>&lt;4</td>
<td>&lt;1</td>
<td>–</td>
<td>232 ColK3, IncR, IncFIB(pQIL)</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>&gt;32</td>
<td>&gt;128</td>
<td>&gt;32</td>
<td>+</td>
<td>232 ColK3</td>
<td>IncFIB(K)</td>
<td>15</td>
</tr>
<tr>
<td>23</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>&gt;32</td>
<td>&gt;128</td>
<td>64</td>
<td>+</td>
<td>48 IncL/M</td>
<td>14b, 15</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>&lt;1</td>
<td>&gt;128</td>
<td>&gt;16</td>
<td>+</td>
<td>48 IncL/M</td>
<td>14b</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>&lt;1</td>
<td>&gt;128</td>
<td>64</td>
<td>+</td>
<td>48 IncL/M</td>
<td>14b</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>&lt;1</td>
<td>&gt;128</td>
<td>8</td>
<td>+</td>
<td>48 IncL/M</td>
<td>14b</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>&lt;1</td>
<td>&gt;128</td>
<td>8</td>
<td>+</td>
<td>48 IncL/M</td>
<td>14b</td>
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<td>30</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>&lt;1</td>
<td>&gt;128</td>
<td>8</td>
<td>+</td>
<td>48 IncL/M</td>
<td>14b</td>
<td></td>
</tr>
</tbody>
</table>

*AMK, amikacin; CRO, ceftriaxone; ESBL, extended-spectrum β-lactamase; ETP, ertapenem; MEM, meropenem; MHT, modified broth test; OXA, oxacillinase; R, recipient strain before transformation (*Escherichia coli* DH10B); T, transformant; TIZP, piperacillin/tazobactam; –, negative; +, positive.†Isolates 1, 2, 26, and 27 did not have any transformants.*
of this plasmid (pCoKP3_DHQP1300920) has been deposited in GenBank under accession no. CP016920.1. pCoKP3_DHQP1300920 was most similar to a CoKP3 plasmid previously deposited under GenBank accession no. JX423831 (100% query coverage, 99% sequence similarity) (Figure 1) (43).

Isolate 23 had 3 plasmids and encoded 16 antimicrobial drug resistance genes, including 2 ESBLs (plasmid-mediated CTX-M-14b and CTX-M-15). bla\textsubscript{OXA-48} was present on an IncL/M plasmid (plasmid size 72,093 bp, G + C content 50.55%). This plasmid contained 89 open reading frames, including those for several antimicrobial drug resistance genes (bla\textsubscript{CTX-M-14b} [streptomycin] strA, strB, and [aminoglycoside] ahp(3’)-Vib), in addition to bla\textsubscript{OXA-48}\textsuperscript{r} which appears to have been inserted into the plasmid by transposon Tn1999.2 (GenBank accession no. JN714122). The sequence of this plasmid (pIncL-M_DHQP1400954) has been deposited in GenBank under accession no. CP016927.1. This plasmid, pIncL-M_DHQP1400954, was most similar to pOXA48-Pm (GenBank accession no. KP025948) (95% query coverage, 99% sequence similarity) (Figure 2) (44).

We identified no SNPs when we compared Illumina and Pacific Biosciences genome sequences for the same isolate for isolates 1, 11, and 23. This finding indicates that Pacific Biosciences sequences can be used as a mapping reference. We compared Illumina sequence data for the remaining clinical isolates, which were not subjected to Pacific Biosciences sequencing, against the Pacific Biosciences reference. We compared Illumina sequence data for the same isolate for isolates 1, 11, and 23. This finding indicates that Pacific Biosciences sequences can be used as a mapping reference. We compared Illumina sequence data for the remaining clinical isolates, which were not subjected to Pacific Biosciences sequencing, against the Pacific Biosciences reference.

Figure 1. Sequence structure of 2 \beta-lactamase OXA-232 (bla\textsubscript{OXA-232}) plasmids tested during phenotypic and genotypic characterization of \textit{Enterobacteriaceae} producing OXA-48–like carbapenemases, United States. Top plasmid is from isolate 11 in this study (pCoKP3_DHQP1300920) (6,139 bp), and bottom plasmid is from Potron et al. (43) (GenBank accession no. JX423831). Arrows indicate direction of transcription. Unlabeled arrows indicate other genes. OXA, oxacillinase; repA, COLe type replicase.

in isolate 23 by Pacific Biosciences sequencing. However, in 4 isolates (14, 21, 25, and 30), coverage of the IS\textsubscript{R} insertion sequence was much higher than the overall assembly coverage, indicating multiple occurrences of this locus, suggestive of a different Tn1999 variant. Of the 13 isolates containing bla\textsubscript{OXA-181}, 12 had an upstream insertion sequence \textDelta IS\textsuperscript{Ecp1}. In isolate 1, which was sequenced by using Pacific Biosciences technology, bla\textsubscript{OXA-181} was confirmed as being chromosomally located. Finally, given the geographic association of several isolates carrying \textit{bla}\textsubscript{OXA-181\textsuperscript{r}}, we created a phylogenetic tree and SNP matrix table for the 7 \textit{K. pneumoniae} isolates from 1 state in HHS region 9 (Table 5; Figure 3).

Discussion

The increasing prevalence of CRE in the United States poses a challenge to patients, clinicians, and public health. The diversity of carbapenemases, including the OXA-48–like enzymes reported in this study, is an ongoing diagnostic challenge to clinical microbiology laboratories because of the variety of phenotypes displayed by isolates producing different, and sometimes multiple, carbapenemases. OXA-48 has been described as the phantom menace because of its subtle phenotype in the absence of co-resistance mechanisms (12).

In this study, all isolates with \textit{bla}\textsubscript{OXA-48}\textsuperscript{r} genes showed resistance to ertapenem, and most showed intermediate resistance or resistance to meropenem, ceftriaxone, ceftazidime, and cefepime. Three tests for carbapenemase production were performed on the isolates in this study. The modified Hodge test, performed for ertapenem or meropenem, and the mCIM showed positive results for all isolates with \textit{bla}\textsubscript{OXA-48}\textsuperscript{r} genes. The Carba Nordmann–Poirel test showed positive results for 73% of all isolates, which is consistent with other studies that have shown that this test had a sensitivity of 72%–76% for OXA-48–like carbapenemase producers (45,46). All isolates in this study would be identified as CRE by the current CDC and Council of State and Territorial...
**Figure 2.** Sequence structure of 2 β-lactamase OXA-48 (bla\textsubscript{OXA-48}) plasmids tested during phenotypic and genotypic characterization of *Enterobacteriaceae* producing OXA-48–like carbapenemases, United States. Top plasmid is from isolate 23 in this study (plnC-M\_DHP1400954) (72,093 bp), and bottom plasmid is from Chen et al. (44) (GenBank accession no. KP025948). Arrows indicate direction of transcription. Unlabeled arrows indicate other genes. Gray area indicates regions of homology, white lines indicate nonhomologous regions, and dark gray lines indicate inversions. *aph*, aminoglycoside; OXA, oxacillinase; *repA*, IncL/M type replicase; *str*, streptomycin.


The 10 isolates that harbored *bla*\textsubscript{OXA-232} were all found on a small ColKPl3 plasmid, and this association has been reported by Potron et al. (43). Likewise, the 7 isolates producing OXA-48 carried *bla*\textsubscript{OXA-48} on a similar genetic environment to those reported (44,48,49). Isolate 23, which was sequenced by using Illumina and Pacific Biosciences technology, harbored *bla*\textsubscript{OXA-48} on an IncL/M plasmid. The other 6 isolates, which were sequenced only by using Illumina technology, all had the IncL/M replicon gene. In addition, *bla*\textsubscript{OXA-48} was always associated with a variant of transposon TN1999, as discerned on the basis of the copy number of IS1IR insertion sequences (36). Because these IS1IR sequences are identical and duplicated, Illumina technology often fails to assemble these as separate loci but instead produces a single locus with high coverage. Comparing coverage of the IS1IR insertion sequence to the overall coverage of the assembly sequence enabled us to estimate the presence of the TN1999 variant by using isolate 23 as the reference. In 12 of 13 isolates with *bla*\textsubscript{OXA-181}, we found an upstream ΔISEcp1 element inserted upstream of the *bla*\textsubscript{CTX-M-14b} cassette. *bla*\textsubscript{OXA-181} is often associated with ISEcp1, which might facilitate its spread (50).

The transformation experiment helped to clarify our understanding of the plasmids harboring *bla*\textsubscript{OXA-48}–like genes. Transformation experiments were successful for each of the parent strains carrying *bla*\textsubscript{OXA-48} or *bla*\textsubscript{OXA-232}.

Carbapenem and penicillin MICs were not different between the parent and transformant, but transformant MICs were comparatively lower for cephalosporins and aminoglycosides. This finding supports the genotypic data, which indicated that ESBL genes and other β-lactamase genes did not cotransfer with the plasmid encoding *bla*\textsubscript{OXA-48}–like genes. One transformant (23T) did not have decreased cephalosporin MICs when compared with its parental strain, which is consistent with Pacific Biosciences sequencing of this isolate, which showed *bla*\textsubscript{CTX-M-14b} to be on the same IncL/M plasmid as *bla*\textsubscript{OXA-48}*. The unsuccessful transformation attempts of *bla*\textsubscript{OXA-181}–containing strains 1, 2, 26, and 27 were explained by WGS evidence that *bla*\textsubscript{OXA-181}, was chromosomally located in isolate 1.

We also detected a possible reservoir of isolates with *bla*\textsubscript{OXA-48}–like genes in the United States. Among the 13 isolates with *bla*\textsubscript{OXA-181}, 8 were from 1 state in HHS region 9 and contained *bla*\textsubscript{CTX-M-15}, *bla*\textsubscript{SHV-26}, and *ampH*. Seven of these isolates were *K. pneumoniae* belonging to ST34, and 5 were collected during June 2010–May 2011 (Tables 2, 5; Figure 3).

**Table 5.** SNP matrix for 7 *Klebsiella pneumoniae* isolates with β-lactamase oxacillinase-181–like carbapenemases from HHS region 9, United States*

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>26</th>
<th>27</th>
<th>4</th>
<th>5</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>0</td>
<td>31</td>
<td>33</td>
<td>17</td>
<td>32</td>
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<td>27</td>
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<td>13</td>
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<td>8</td>
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<td>8</td>
<td>1</td>
<td>9</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

*Genetic diversity ranged from 1 to 33 high-quality SNPs that were called in an ~5-Mb core genome, which equals ~90% of the reference genome size (isolate 1 sequenced by using Pacific Biosciences [Menlo Park, CA, USA] technology); *bla*, β-lactamase; HHS, Health and Human Services; SNP, single-nucleotide polymorphism.
This study had several limitations. The collection of isolates in this study might not be representative of all isolates with bla_{OXA-48}–like genes in the United States. There is also a reporting bias because only isolates sent to CDC were included. CDC receives isolates as part of outbreak investigations, surveillance studies, and to confirm AST results, but there is no national requirement to submit carbapenemase-producing isolates. Thus, unusually resistant isolates are more likely to be sent to the CDC and included in this study. Also, no prevalence rates of Enterobacteriaceae with bla_{OXA-48}–like genes in the United States can be inferred because there is not an evaluable denominator. In addition, almost all the isolates we studied were clinical isolates; colonizing isolates might have different phenotypic characteristics.

Another limitation is that the 10 isolates selected for the transformation experiment and the 3 isolates selected for Pacific Biosciences sequencing might not have been representative of the other isolates in this collection. Ideally, all isolates would have been sequenced by using Pacific Biosciences technology and been a part of the transformation experiment, but this testing was not performed because of limited resources. In addition, the decisions regarding which isolates to select for transformation experiments and sequencing by using Pacific Biosciences technology were made before WGS was complete. In retrospect, it would have been better to select bla_{OXA-181} isolates that were hypothesized to be on a plasmid for the transformation experiment; instead, chromosomal bla_{OXA-181} isolates were selected. Thus, the bla_{OXA-181} loci for the isolates in this study are inconclusive.

In summary, the continued increase of CRE in the United States is a major problem, and the increasing prevalence of OXA-48–like carbapenemases is also concerning. We found Enterobacteriaceae in the United States with bla_{OXA-48}–like genes on similar mobile genetic elements to those described elsewhere and that displayed relatively resistant AST profiles. The first step in continued detection of CRE producing these and other carbapenemases is identifying all carbapenem resistance among Enterobacteriaceae, including resistance to ertapenem. Future prospective investigations are needed to determine the true prevalence of OXA-48–like carbapenemases in the United States.

This study was supported by the Advanced Molecular Detection Program at CDC.

About the Author
Dr. Lutgring is an assistant professor of medicine at Emory University School of Medicine, Atlanta, GA. His primary research interest is the molecular mechanisms of antimicrobial resistance in gram-negative bacteria.

References


In 1965, the transferability of ampicillin resistance was reported, and the plasmid-encoded mechanism of resistance for 2 Salmonella sp. isolates from the United Kingdom and 1 Escherichia coli isolate from Greece was determined. Resistance (R) factors from Salmonella sp. isolates were designated R1818 and R7268 (R7268 encoding the current TEM-1). The E. coli isolate and its plasmid were named TEM (encoding the current TEM-2) because the isolate was recovered from a feces culture of an Athenian patient named Tennoniera in 1963.

β-lactam resistance is a problem worldwide; >2,000 β-lactamases are currently identified. Of these β-lactamases, >200 enzymes are classified within TEM family, including extended-spectrum β-lactamases (ESBLs). However, the original TEM-1 and TEM-2 hydrolyze only penicillin derivatives.

**Sources**


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Phenotypic and Genotypic Characterization of Enterobacteriaceae Producing Oxacillinase-48–Like Carbapenemases, United States

Technical Appendix

Additional Materials and Methods

To assess clonality of plasmid sequences in our dataset, we mapped Illumina (San Diego, CA) reads from each β-lactamase oxacillinase-48–like (blaOXA-48-like) isolate to the appropriate internal reference isolate sequenced by using Pacific Biosciences (Menlo Park, CA, USA) long-read technology by using the lyve-SET V1.0 pipeline (https://github.com/lskatz/lyve-SET). Single-nucleotide polymorphisms (SNPs) with 100% consensus and >5× sequencing depth were reported (1). We measured and visualized the number of SNPs in each pairwise comparison of a mapped isolate and the reference plasmid (Pacific Biosciences), as well as the total number of read-covered nucleotides on each reference plasmid assembly (depth of coverage ≥5×), in a scatter plot by using R 3.2.3 statistical software (https://www.r-project.org/).

All Illumina contigs that harbored blaOXA-48-like genes were extracted from each genome assembly and aligned against the GenBank database by means of BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch) to estimate the genomic location of the blaOXA-48-like gene (either plasmid or chromosome) and were also screened for plasmid replicon sequences by using the PlasmidFinder database (http://www.genomicepidemiology.org/).

A phylogenetic tree was inferred from whole-genome SNP data of 7 sequence type 34 Klebsiella pneumoniae isolates by means of the Lyve-Set1.1.4f pipeline (1). First, phage genes present in the genome sequence (Pacific Biosciences) of isolate 1 (K. pneumoniae sequence 34 isolate) were identified by screening against the PHAST database (2), and trimmed Illumina reads were subsequently mapped to unmasked regions of the reference by using SMALT (3).
SNPs with ≥95% consensus and ≥20× sequencing depth were reported as high-quality SNPs. To deal with possible regions of homologous recombination, leading to high-density SNP regions, we accepted 1 SNP/5 bp, a flanking distance close to the average recombination cassette length (4). A maximum-likelihood phylogeny was inferred from high-quality SNPs by using RAxML version 8 (5) and a generalized time reversible substitution model and a gamma model of rate heterogeneity.

Additional Results

The number of core plasmid SNPs between isolate 11 (pColKP3_DHQP1300920) (sequenced by using Illumina and Pacific Biosciences technology) and other isolates with \textit{bla}_{OXA-232} (sequenced by using Illumina technology) ranged from 0 to 1 and covered 5,400–6,100 bp (Technical Appendix Figure 1). Among the isolates with \textit{bla}_{OXA-48}, the number of core SNPs between the plasmid sequence from reference isolate 23 (Pacific Biosciences) (pIncL_M_DHQP_1400954) and those from other OXA-48 isolates (sequenced by using Illumina technology) ranged from 371 to 430 and covered 56,000 and 62,000 bp (Technical Appendix Figure 2).

Alignments of Illumina contigs harboring \textit{bla}_{OXA-48}-like run against the GenBank database showed that all contigs mapped to plasmid sequences. Alignments of Illumina contigs with \textit{bla}_{OXA-181} run against the GenBank database estimated 9 isolates to have a chromosomal location and isolates 8, 13, 20, and 24 to have a plasmid location. In 3 instances (isolates 8, 13, and 24), \textit{bla}_{OXA-181} was found on the same contig as a plasmid replicon gene (2 ColKP3 replicons and 1 IncHI1B replicon), further suggesting that \textit{bla}_{OXA-181} was located on a plasmid in those isolates.

References


**Technical Appendix Figure 1.** Genetic diversity among ColKP3 plasmids that harbor β-lactamase oxacillinase-232. SNPs were identified by mapping trimmed Illumina (San Diego, CA, USA) sequencing reads to a fully closed PacBio ColKP3 plasmid assembly (isolate 11). The y-axis shows the amount of Pacific Biosciences (Menlo Park, CA, USA) reference nucleotides covered by Illumina reads of each sample (minimum of 5× coverage), and the x-axis shows the quantity of SNPs observed between the Pacific Biosciences reference and each isolate. Boxplot indicates 25th–75th interquartile ranges and relatedness among these type of oxacillinase-232–carrying plasmids in terms of shared nucleotide positions between isolates and the reference. Horizontal line in box indicates median. Outermost line (error bar) indicates 1.5× interquartile range. Only 2 isolates are considered outliers and thus more distantly related. SNP count variation ranges from 0 to 1 and indicates relatedness between isolates and the reference. SNP, single-nucleotide polymorphism.
Technical Appendix Figure 2. Genetic diversity among IncL/M plasmids that harbor β-lactamase oxacillinase-48. SNPs were identified by mapping trimmed Illumina sequencing reads to a fully closed (Pacific Biosciences, Menlo Park, CA, USA) IncL/M plasmid assembly (isolate 23). The y-axis shows the amount of Pacific Biosciences reference nucleotides covered by Illumina reads of each sample (minimum of 5× coverage), and the x-axis shows the quantity of SNPs observed between the Pacific Biosciences reference and each isolate. Boxplots indicate 25th–75th interquartile ranges and show that 100% coverage and 0 SNPs (indicating 100% sequence similarity) are considered features of an outlier and therefore rare in this dataset. Horizontal lines indicate medians. Outermost lines (error bars) indicate 1.5× interquartile ranges. Most IncL/M plasmids are distantly related to the Pacific Biosciences plasmid reference, indicating substantial heterogeneity of plasmids within this dataset. SNP, single-nucleotide polymorphism.