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Emergence of OXA-48-Type Carbapenemase-Producing *Enterobacteriaceae* in German Hospitals

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Nine carbapenem-resistant *Enterobacteriaceae* isolates collected from eight patients in five German hospitals were investigated. Six isolates produced the OXA-48 carbapenemase, and three isolates produced OXA-162, which is a point mutant form of OXA-48. Both carbapenemase genes were located on IncL/M-type conjugative plasmids. Insertion sequence IS*1999* (truncated or not by IS*1R*) was located upstream of the bla_{OXA-48} and $bla_{OXA-162}$ genes in all of the isolates. Pulsed-field gel electrophoresis typing indicated the clonal transmission of an OXA-48-producing *Klebsiella pneumoniae* strain in two hospitals.

Carbapenem resistance in *Enterobacteriaceae* is based on various mechanisms that may involve upregulation of efflux pumps or loss of porins. Most prevalent is the acquisition of carbapenem-hydrolyzing enzymes, or carbapenemases. Some commonly identified carbapenemases are KPC-, NDM-, and OXA-48-type enzymes whose respective genes are located on plasmids that enable their transfer between different enterobacterial species (19). The OXA-48 carbapenemase was first described in *Klebsiella pneumoniae* epidemic isolates from Turkey and then in several European countries, such as France and Belgium. Recently, it has also been identified in enterobacterial isolates recovered from non-European countries, such as Lebanon, Tunisia, Senegal, Morocco, Israel, and India (2, 5, 9, 10, 12, 18). In addition to *K. pneumoniae*, OXA-48 has been identified in *Escherichia coli*, *Enterobacter cloacae*, *Citrobacter freundii*, and *Providencia rettgeri* (2). This enzyme is able to hydrolyze penicillins and carbapenems but possesses poor activity against broad-spectrum cephalosporins. Multidrug resistance in OXA-48-producing strains often results from the coproduction of various resistance mechanisms, in particular, extended-spectrum β-lactamases (ESBLs) and other resistance determinants.

Here we report on the molecular analysis of carbapenem-resistant *Enterobacteriaceae* isolates that were recovered in Germany between 2008 and 2010 and sent to the Robert Koch Institute, Wernigerode, for further characterization. Nine isolates, including *E. coli* ($n = 2$), *K. pneumoniae* ($n = 4$), *Raoultella ornithinolytica* ($n = 1$), *C. freundii* ($n = 1$), and *E. cloacae* ($n = 1$), were selected since they gave negative phenotypic test results for the production of metallo-β-lactamases or KPC enzyme production (MBL-Etest [bioMérieux, Nürtingen, Germany] and KPC+MBL Confirm ID Kit [Alere GmbH, Zug, Switzerland]).

In April and May 2008, two *E. coli* isolates were isolated from a wound swab and secretion from a tracheal cannula (colonization) in two hospitals in Berlin (hospitals A and B). One patient developed sepsis but recovered. The second patient, exhibiting several comorbidity factors, developed sepsis and ventilator-associated pneumonia and was treated with various antibiotics (tigecycline, piperacillin-sulbactam, and meropenem). In addition, one *R. ornithinolytica* strain recovered from a blood culture and one *C. freundii* strain recovered from bronchoalveolar lavage fluid were isolated from a 67-year-old patient in hospital A in September 2009.

Between November 2009 and January 2010, four multidrug-resistant *K. pneumoniae* isolates were sent in from intensive care units of two hospitals (hospitals C and D) located within 40 km of each other in the federal state of North Rhine-Westphalia. The strains had been isolated from urine cultures or tracheal aspirates of four different patients. These patients all presented with underlying diseases (myocardial infarction, congestive heart failure, plasmacytoma), and two patients had previously received meropenem. Additionally, an *E. cloacae* strain was isolated in 2009 from a drainage swab in hospital E, which is located in southern Germany. None of the patients reported any link with Turkey, one patient (*E. coli*, hospital B) came from Syria, and another patient (*E. cloacae*, hospital E) was from Libya.

Antimicrobial susceptibility testing of 10 antibiotics (ampicillin, cefoxitin, cefotaxime, ceftazidime, gentamicin, kanamycin, chloramphenicol, tetracycline, ciprofloxacin, and sulfamethoxazole-trimethoprim) was done by broth microdilution according to the CLSI guidelines (3). MIC determinations for carbapenems (imipenem, meropenem) were performed by Etest (bioMérieux). Occurrence of β-lactamases was detected by PCR amplification and sequencing of ESBL genes (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA}) and several carbapenemase genes (bla_{VIM}, bla_{IMP}, bla_{NDM-1}, bla_{KPC}, and bla_{OXA-48}) (6, 13, 14). Identification of *qnr*-like genes encoding plasmid-mediated quinolone resistance determinants was performed as described previously (13). Transfer of resistance was performed by broth mating assays using a sodium azide-resistant *E. coli* J53 recipient (4). Plasmid DNA of clinical isolates and transconjugants was isolated

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TABLE 1 Phenotypic and genotypic characteristics of OXA-type carbapenemase-producing clinical isolates and transconjugants

Strain[a]	Hospital	Yr	β-Lactamase(s)	Antimicrobial resistance profile	MIC (mg/liter)[e]		PFGE type
					IPM	MPM	
E. coli 84/08	A	2008	OXA-162, TEM-1	AMP FOX GEN CMP OTE CIP SXT	8	16	1
R. ornithinolytica 215/09[b]	A	2009	OXA-162, TEM-1, OXA-1, SHV-5	AMP CTX CAZ KAN CMP CIP	4	4	2
C. freundii 216/09[b]	A	2009	OXA-162, SHV-5	AMP FOX CTX CAZ GEN CMP	2	2	3
E. coli 131/08	B	2008	OXA-48, TEM-1, OXA-1	AMP GEN CMP OTE SXT	32	32	4
K. pneumoniae							
229/09	C	2009	OXA-48, TEM-1, OXA-9, SHV-11, CTX-M-15	AMP FOX CTX CAZ GEN KAN AMK CIP	>32	>32	5
238/09	C	2009	OXA-48, TEM-1, OXA-9, SHV-11, CTX-M-15	AMP FOX CTX CAZ GEN KAN AMK CIP	>32	>32	5
239/09	C	2009	OXA-48, TEM-1, OXA-9, SHV-11, CTX-M-15	AMP FOX CTX CAZ GEN KAN AMK CIP	>32	>32	5
16/10	D	2010	OXA-48, TEM-1, OXA-9, SHV-11, CTX-M-15	AMP FOX CTX CAZ GEN KAN AMK CIP	32	>32	5
E. cloacae 1/10	E	2010	OXA-48, TEM-1, CTX-M-15	AMP FOX CTX CAZ GEN KAN CMP CIP SXT	4	8	6
E. coli J53							
Tc 84/08			OXA-162, TEM-1	AMP GEN OTE SXT	0.25	1	
Tc 131/08			OXA-48, TEM-1	AMP GEN CMP	0.25	0.5	
Tc[c]			OXA-48 or OXA-162	AMP	1	1	
Rc[d]					≤0.063	≤0.063	

[a] Tc, transconjugant.
[b] Isolates from the same patient.
[c] Characteristics of transconjugants Tc 215/09, Tc 216/09, Tc 229/09, Tc 238/09, Tc 239/09, Tc 16/10, and Tc 1/10.
[d] Recipient *E. coli* J53 resistant to sodium azide.
[e] Determined by Etest. AMP, ampicillin; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; GEN, gentamicin; KAN, kanamycin; AMK, amikacin; CMP, chloramphenicol; OTE, oxytetracycline, CIP, ciprofloxacin; SXT, sulfamethoxazole-trimethoprim; IPM; imipenem; MPM, meropenem.

using the Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany). Southern hybridization of the plasmids using digoxigenin (DIG)-labeled, bla_{OXA-48}-specific probes and signal detection using CDP-*Star* were performed by following the manufacturer's guidelines (Roche Diagnostics Ltd., West Sussex, United Kingdom). In addition, all nine isolates were typed by pulsed-field gel electrophoresis (PFGE) using XbaI-restricted whole genomic DNA.

Both *E. coli* isolates were resistant to carbapenems but remained susceptible to expanded-spectrum cephalosporins. All other isolates were resistant to cefotaxime and ceftazidime and either resistant (*K. pneumoniae* isolates) or intermediately susceptible to imipenem and meropenem. Coresistances to fluoroquinolones (seven isolates), aminoglycosides (nine isolates), and sulfamethoxazole-trimethoprim (three isolates) were frequently observed (Table 1).

PCR and sequencing analysis revealed that the three isolates from hospital A (*E. coli*, *C. freundii*, and *R. ornithinolytica*) harbored the $bla_{OXA-162}$ gene, whereas the bla_{OXA-48} gene was detected in *E. cloacae* isolates and the four *K. pneumoniae* isolates (Table 1). OXA-162 is a recently identified OXA-48-type variant, differing from OXA-48 by a Thr-to-Ala substitution at position 224 (DBL numbering; 17). Additionally, the bla_{TEM-1} gene was identified in eight out of the nine isolates, and the bla_{SHV-11} and bla_{OXA-9} genes were identified in all of the *K. pneumoniae* isolates. Furthermore, genes encoding ESBL SHV-5 or CTX-M-15 were found in isolates resistant to ceftazidime and cefotaxime (Table 1). The *qnrB1* gene was additionally identified in the *E. cloacae* isolate.

Conjugation assays were successful for all isolates and allowed the identification of $bla_{OXA-162}$- and bla_{OXA-48}-carrying plasmids with a size of ca. 60 kb in all isolates transferred into *E. coli* recipients (Fig. 1). No other resistance genes were cotransferred on these plasmids. PCR-based typing targeting genes identified from other bla_{OXA-48}-bearing plasmids as recently described (15) showed that the genes bla_{OXA-48} and $bla_{OXA-162}$ identified in the present study corresponded to IncL/M-type plasmids, further reinforcing the hypothesis that the current spread of the bla_{OXA-48}-like genes in different strain backgrounds and different countries is mainly the consequence of the diffusion of an epidemic plasmid. Analysis of the genetic environment located upstream of the bla_{OXA-48} and $bla_{OXA-162}$ genes (1, 2) revealed the presence of insertion sequence IS*1999* in the four *K. pneumoniae* isolates, although IS*1999* was truncated by insertion sequence IS*1R* in all of the other isolates, as described previously (2).

The antibiotic resistance patterns and β-lactamase contents of the four *K. pneumoniae* isolates recovered from two different hospitals were identical. Additional sequencing of outer membrane protein genes *ompK35* and *ompK36*, performed as described previously (11), revealed the disruption of *ompK36* by an insertion sequence in all four *K. pneumoniae*, resulting in porin loss and increased carbapenem MICs, as previously described (11). The higher carbapenem MICs observed for the *E. coli* and *E. cloacae* clinical isolates than those for the respective transconjugants may likely be attributable to permeability defects in the clinical isolates, related to porin loss or efflux mechanisms. By PFGE typing, identical restriction patterns were observed for all four isolates, indicating the clonal spread of a multidrug-resistant *K. pneumoniae*

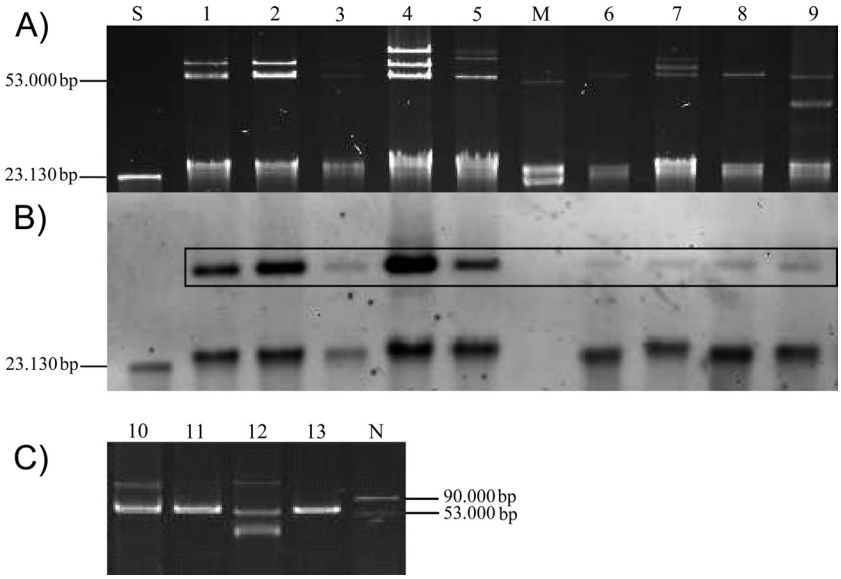

FIG 1 Plasmid preparations from OXA-type carbapenemase-producing clinical strains and transconjugants (Tc). (A) Native plasmid preparation of clinical strains and transconjugants in agarose gel. (B) Southern hybridization of plasmids of clinical strains and transconjugants on nylon membrane with a DIG-labeled bla_{OXA-48} probe. (C) Native plasmid preparation of clinical strains isolated in 2010 and transconjugants in agarose gel. Lanes: M, plasmid marker *E. coli* K12J53 V517 (53,000-bp plasmid); N, plasmid marker *E. coli* K12J53 V517 plus *E. coli* K12J53 R222 (53,000-bp and 90,000-bp plasmids); S, DIG-labeled Molecular Weight Marker II (Roche Diagnostics Ltd., West Sussex, United Kingdom); 1, *E. coli* 131/08; 2, Tc 131/08; 3, *E. coli* 84/08; 4, Tc 84/08; 5, *R. ornithinolytica* 215/09; 6, Tc 215/09; 7, *C. freundii* 216/09; 8, Tc 216/09; 9, *K. pneumoniae* 229/09; 10, *E. cloacae* 1/10; 11, Tc 1/10; 12, *K. pneumoniae* 16/10; 13, Tc 16/10. Positive hybridization signals are framed. Hybridization signals of less than 50 kb result from plasmid residues and linear plasmid DNA, respectively.

strain. No links among the four patients from the two hospitals located 40 km apart could be evidenced.

The present study showed the emergence of OXA-48 and OXA-162 producers among enterobacterial isolates in Germany. Although the spread of OXA-48 producers has been recently identified in different countries in the Mediterranean area and western Europe (2, 8), it is noteworthy that Turkey represents a main reservoir. Considering the high frequency of population exchanges between Germany and Turkey, we speculate that at least some of the isolates currently emerging in Germany could be from Turkey. We identified the novel OXA-162 enzyme, which is a point mutant derivative of OXA-48 and has also been identified recently in Turkey according to the GenBank database (accession numbers HM015773 and GU197550). Identification of the same $bla_{OXA-162}$-carrying plasmid in *R. ornithinolytica* and *C. freundii* isolated from one patient may have resulted from horizontal gene transfer. We further detected loss of the porin OmpK36 in *K. pneumoniae* as a combined mechanism of carbapenem resistance, as identified in *K. pneumoniae* 11978 (7, 16).

Here we identified carbapenemases OXA-48 and OXA-162 in different multidrug-resistant *Enterobacteriaceae* species that coproduce ESBLs and other plasmid-mediated resistance determinants like Qnr. We observed the dissemination of bla_{OXA-48}-like genes by conjugative plasmid transfer, as well as the regional spread of a multidrug-resistant, OXA-48-producing *K. pneumoniae* clone. Because of limited therapeutic options and higher mortality caused by these carbapenem-resistant *Enterobacteriaceae*, continuous surveillance and molecular characterization of OXA-48 producers are needed to shed light upon all of the transmission pathways in Germany and over continents. Taking into account the relationships between Germany and many countries located in North Africa and the Middle East, this study underlines the need to detect OXA-48 producers as early as possible.

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