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Emergence of OXA-48-type carbapenemase-producing *Enterobacteriaceae* in German hospitals

Yvonne Pfeifer[1]*, Kathrin Schlatterer[2], Elisabeth Engelmann[3], Reinhold A. Schiller[4], Hans Reiner Frangenberg[5], Doris Stiewe[6], Martin Holfelder[7], Wolfgang Witte[1], Patrice Nordmann,[8] and Laurent Poirel[8]

Robert Koch Institute, Nosocomial Infections, Wernigerode, Germany[1]; Ernst-Moritz-Arndt University, Dept. Clin. Chemistry and Laboratory Medicine, Greifswald, Germany [2]; Sankt Gertrauden-Hospital, Central Laboratory, Berlin, Germany[3]; Charite Berlin, Institute of Microbiology and Hygiene, Berlin, Germany[4]; Evangelisches Krankenhaus Oberhausen, Institute of Laboratory Medicin and Clinical Microbiology, Oberhausen, Germany[5]; Medical Laboratory Wahl, Lüdenscheid, Germany[6]; Limbach Laboratory, Heidelberg, Germany[7]; Dept. of Microbiology, Hôpital de Bicêtre, 94275 Le Kremlin de Bicêtre, France[8]

* Corresponding author: Dr. Yvonne Pfeifer, Robert Koch Institute, FG13 Nosocomial Infections, Burgstraße 37, 38855 Wernigerode, Germany, Tel: +49 (0)3943 679 337, Fax: +49 (0)3943 679 317, E-mail: pfeifery@rki.de

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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 that is a point mutant 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 $bla_{\text{OXA-48/-162}}$ genes in all isolates. PFGE typing indicated a 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 up-regulation 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 the 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 been also identified from 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 possess poor activity against broad-spectrum cephalosporins. Multidrug-resistance in OXA-48 producing strains is often resulting from co-production 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 have been recovered in Germany between 2008 and 2010 and sent to the Robert Koch Institute, Wernigerode, for further characterization. Nine isolates, being *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 phenotypical tests for production of metallo-β-lactamases or KPC enzyme production (MBL-Etest, bioMérieux, Nürtingen, Germany; KPC+MBL Confirm ID Kit, Alere GmbH, Switzerland).

In April and May 2008, two *E. coli* isolates were isolated from wound swab and secretion from tracheal cannula (colonization) in two hospitals in Berlin (hospitals A and B). One patient developed sepsis but recovered. The second patient exhibiting several co-morbidity factors developed sepsis and ventilator-associated pneumonia and was treated with various

antibiotics (tigecycline, piperacillin/sulbactam, meropenem). In addition, one *R. ornithinolytica* recovered from blood culture and one *C. freundii* recovered from broncho-alveolar lavage 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* were sent in from intensive care units of two hospitals (hospitals C and D) located within distance of 40 km in the federal state of North Rhine-Westphalia. The strains had been isolated from urine cultures or tracheal aspirations 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 South 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) from Libya.

Antimicrobial susceptibility testing of ten antibiotics (ampicillin, cefoxitin, cefotaxime, ceftazidime, gentamicin, kanamycin, chloramphenicol, tetracycline, ciprofloxacin, and sulfamethoxazole/trimethoprim) was determined 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}, $bla_{\text{CTX-M}}$, bla_{OXA}) and several carbapenemase genes like bla_{VIM}, bla_{IMP}, $bla_{\text{NDM-1}}$, bla_{KPC}, and $bla_{\text{OXA-48}}$ (6, 13, 14). Identification of *qnr*-like genes encoding plasmid-mediated quinolone resistance determinants was performed as described (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 using the Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany). Southern hybridization of the plasmids using DIG-labelled $bla_{\text{OXA-48}}$-specific probes and signal detection using CDP-*Star* were performed following the manufacturer's

guidelines (Roche Diagnostics Ltd, West Sussex, UK). 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 of intermediate susceptibility to imipenem and meropenem. Co-resistances 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, R. ornithinolytica*) harbored the $bla_{\text{OXA-162}}$ gene whereas the $bla_{\text{OXA-48}}$ 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 $bla_{\text{TEM-1}}$ gene was identified in eight out of the nine isolates, and the $bla_{\text{SHV-11}}$ and $bla_{\text{OXA-9}}$ genes were identified in all the *K. pneumoniae* isolates. Furthermore, genes encoding ESBLs SHV-5 or CTX-M-15 were found in isolates being resistant to ceftazidime and cefotaxime (Table 1). The *qnrB1* gene was additionally identified from the *E. cloacae* isolate.

Conjugation assays were successful for all isolates and allowed to identify $bla_{\text{OXA-162}}$- and $bla_{\text{OXA-48}}$-carrying plasmids which size being of ca. 60 kb in all isolates transferred into *E. coli* recipients (Figure 1). No other resistance genes were co-transferred on these plasmids. PCR-based typing targeting genes identified from other $bla_{\text{OXA-48}}$-bearing plasmids as recently described (15) showed that the genes $bla_{\text{OXA-48}}$ and $bla_{\text{OXA-162}}$ identified in the present study corresponded to IncL/M-type plasmids, further reinforcing the hypothesis that the current spread of the $bla_{\text{OXA-48}}$-like genes in different strain backgrounds and different countries is mainly the consequence of the diffusion of an epidemic plasmid. Analysis of the upstream-located genetic environment of the $bla_{\text{OXA-48}}$ and $bla_{\text{OXA-162}}$ genes (1, 2) revealed the presence

112 of insertion sequence IS*1999* in the four *K. pneumoniae* isolates, although IS*1999* was
113 truncated by insertion sequence IS*1R* in all other isolates as described previously (2).

114 The antibiotic resistance pattern and β-lactamase content of the four *K. pneumoniae*
115 isolates recovered from two different hospitals were identical. Additional sequencing of outer
116 membrane protein genes *ompK35* and *ompK36* performed as described (11) revealed the
117 disruption of *ompK36* by an IS-insertion in all four *K. pneumoniae*, therefore resulting in
118 porin loss and increased MIC values for carbapenems, as previously described (11). The
119 higher MIC values for carbapenems observed for the *E. coli* and *E. cloacae* clinical isolates
120 compared to their respective transconjugants may likely be attributed to permeability defects
121 for the clinical isolates, related to porin loss or efflux mechanisms. By PFGE typing, identical
122 restriction patterns were observed for all four isolates, indicating a clonal spread of a
123 multidrug-resistant *K. pneumoniae* strain. No link between the four patients from the two
124 hospitals located at 40 km distance could be evidenced.

125 The present study showed the emergence of OXA-48 and OXA-162 producers
126 among enterobacterial isolates in Germany. Although spread of OXA-48-producers has been
127 recently identified in different countries from the Mediterranean area and Western Europe (2,
128 8), it is noteworthy that Turkey represents a main reservoir. Considering the high frequency of
129 population exchanges between Germany and Turkey, we speculate that at least some of the
130 isolates currently emerging in Germany could originate from Turkey. We identified the novel
131 OXA-162 enzyme which is a point mutant derivative of OXA-48, and that has been identified
132 also recently in Turkey according to the GenBank databases (Accession numbers HM015773
133 and GU197550). Identification of a same $bla_{\text{OXA-162}}$-carrying plasmid in *R. ornithinolytica* and
134 *C. freundii* isolated from one patient may have resulted from horizontal gene transfer. We
135 further detected loss of porin OmpK36 in *K. pneumoniae* as a combined mechanism of
136 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 co-produce ESBL and other plasmid-mediated resistance determinants like Qnr. We observed dissemination of $bla_{\text{OXA-48-like}}$ genes by conjugative plasmid transfer as well as the regional spread and 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 characterisation of OXA-48 producers are needed to shed up light upon all transmission ways in Germany and over continents. Taking in 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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Figure 1: Plasmid preparations from OXA-carbapenemase producing clinical strains and transconjugants (Tc). A), native plasmid preparation of clinical strains and transconjugants in agarose gel; B), Southern hybridisation of plasmids of clinical strains and transconjugants on nylon membrane with a DIG-labelled $bla_{\text{OXA-48}}$-probe; C), native plasmid preparation of clinical strains isolated in 2010 and transconjugants in agarose gel; M, plasmid marker *E. coli* K12J53 V517 (53.000 bp plasmid); N, plasmid marker *E. coli* K12J53 V517 + *E. coli* K12J53 R222 (53.000 bp and 90.000 bp plasmid); S, DIG-labelled Molecular Weight Marker II (Roche Diagnostics Ltd, West Sussex, UK); 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 hybridisation signals are framed. Hybridisation signals less than 50 kb result from plasmid residues and linear plasmid DNA, respectively.

233 Table 1. Phenotypical and genotypical characteristics of OXA-carbapenemase producing clinical isolates and transconjugants

234

No.	Species	Hospital	Year	β-lactamases	Antimicrobial resistances	MIC_{IPM} [mg/L][4]	MIC_{MPM} [mg/L][4]	PFGE type
84/08	*E. coli*	A	2008	OXA-162 TEM-1	AMP FOX GEN CMP OTE CIP SXT	8	16	1
215/09[1]	*R. ornithinolytica*	A	2009	OXA-162 TEM-1 OXA-1 SHV-5	AMP CTX CAZ KAN CMP CIP	4	4	2
216/09[1]	*C. freundii*	A	2009	OXA-162 SHV-5	AMP FOX CTX CAZ GEN CMP	2	2	3
131/08	*E. coli*	B	2008	OXA-48 TEM-1 OXA-1	AMP GEN CMP OTE SXT	32	32	4
229/09	*K. pneumoniae*	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	*K. pneumoniae*	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	*K. pneumoniae*	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	*K. pneumoniae*	D	2010	OXA-48 TEM-1 OXA-9 SHV-11 CTX-M-15	AMP FOX CTX CAZ GEN KAN AMK CIP	32	>32	5
1/10	*E. cloacae*	E	2010	OXA-48 TEM-1 CTX-M-15	AMP FOX CTX CAZ GEN KAN CMP CIP SXT	4	8	6
Tc 84/08	*E. c. J53*	-	-	OXA-162 TEM-1	AMP GEN OTE SXT	0.25	1	-
Tc 131/08	*E. c. J53*	-	-	OXA-48 TEM-1	AMP GEN CMP	0.25	0.5	-
Tc[2]	*E. c. J53*	-	-	OXA-48 or OXA-162	AMP	1	1	-
Rc[3]	*E. c. J53*	-	-	-	-	≤0.063	≤0.063	-

235

236 MIC, minimum inhibitory concentration; Tc, transconjugant; [1], isolates from the same patient; [2], characteristics of transconjugants Tc 215/09, Tc 216/09, Tc 229/09, Tc 238/09, Tc 239/09, Tc 16/10, Tc 1/10; [3], recipient *E.*
237 *coli* J53 resistant to sodium azide; [4], determined by Etest; AMP, ampicillin; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; GEN, gentamicin; KAN, kanamycin; AMK, amikacin; CMP, chloramphenicol; OTE,
238 oxytetracycline, CIP, ciprofloxacin; SXT, sulfamethoxazole-trimethoprim;; IPM; imipenem; MPM, meropenem.

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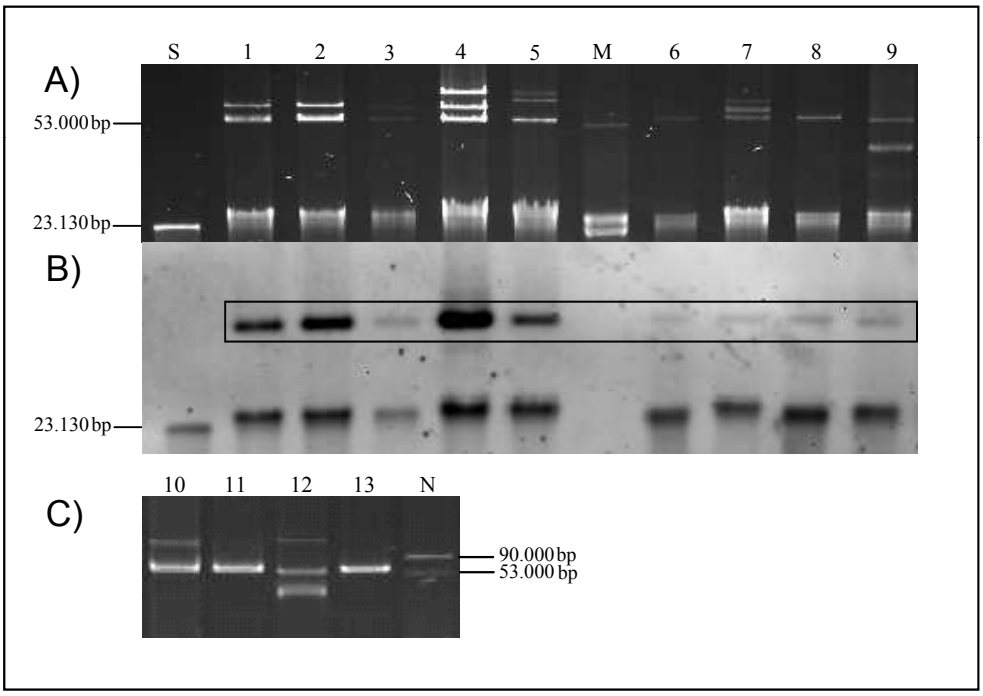

Figure 1: Plasmid preparations from OXA-carbapenemase producing clinical strains and transconjugants (Tc). A), native plasmid preparation of strains 1-9 in agarose gel; B), Southern hybridisation of plasmids (strains 1-9) on nylon membrane with a DIG-labelled *bla*OXA-48-probe; C), native plasmid preparation of strains 10-13 in agarose gel; M, plasmid marker *E. coli* K12J53 V512; N, plasmid marker *E. coli* K12J53 V512 + *E. coli* K12J53 R222; S, DIG-labelled Molecular Weight Marker II (Roche Diagnostics Ltd, West Sussex, UK); 1, *E. coli* 131/08; 2, Tc 131/08; 3, *E. coli* 84/08*;* 4, Tc 84/08; 5, *K. oxytoca* 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 hybridisation signals are framed.