Resistance patterns and integron cassette arrays of *Enterobacter cloacae* complex strains of human origin

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The aim of this research was to analyse the resistance patterns and characterize the distribution and genetic content of resistance integrons within *Enterobacter cloacae* complex strains originating from hospitalized patients. The strains were included in the *E. cloacae* complex study following sequence analysis of the *hsp60* gene. The determination of resistance towards eight classes of antimicrobials was followed by PCR detection of integrons and analyses of the size and sequences of their variable parts. The majority of 69 clinical strains of the *E. cloacae* complex were identified as *Enterobacter hormaechei*. They were isolated from a variety of samples, including urine, wounds, blood and stools. The remaining isolates belonged to *E. cloacae* clusters III and IV, *E. cloacae* subsp. *cloacae* and *Enterobacter kobei*. Fifty-two isolates (75.4 %) were resistant to more than three unrelated antibiotics. The resistance for each antibiotic, except imipenem, was significantly associated with the presence of integrons. Class 1 integrons were detected in 55 % of isolates: 63.3 % of '*E. hormaechei* subsp. *steigerwaltii*', 50 % of *E. cloacae* cluster III, 40 % of '*E. hormaechei* subsp. *oharae*', 33 % belonging to *E. cloacae* cluster IV and 20 % of '*E. hormaechei* subsp. *hormaechei*' were *intl1*-positive. All of the integrons were located on transferable genetic elements. The transferred resistance primarily included that to aminoglycosides, ticarcillin, piperacillin, sulfamethoxazole, trimethoprim and tetracycline. Sequence analysis of the variable regions of integrons identified two groups of genes: those encoding aminoglycoside adenylotransferases responsible for resistance to aminoglycosides, and *dfr* cassettes conferring resistance to trimethoprim. Integrons of the *E. cloacae* complex showed limited variability of genes encoding resistance to therapeutics and were stable in structure with the following cassette arrays: *dfrA12-orfF-aadA2*, *aadB-aadA2*, *dfrA1-aadA1* and *aacA4-aadA1*. Hospital-dependent differences in type and arrays of gene cassettes were observed, which seemed to be conserved and not liable to changes.

Received 23 November 2010

Accepted 15 February 2011

INTRODUCTION

Bacteria of the *Enterobacter cloacae* complex are Gram-negative, chemo-organotrophic, facultatively anaerobic rods or coccobacilli. Recently, the importance of members of the *E. cloacae* complex as nosocomial pathogens has been highlighted, and strains belonging to this species are encountered among the most-often-isolated strains able to cause severe opportunistic infections in hospitalized and debilitated patients, especially in intensive care units. They cause pneumonia and urinary tract, wound, skin and soft tissue, ophthalmic and bloodstream infections (Fraser *et al.*,

2008). By using commercial biochemical kits, they are routinely identified as '*E. cloacae*'. The complex is, however, heterogeneous. According to the sequence of the *hsp60* gene, it has been divided into 12 genetic clusters: *Enterobacter asburiae*, *E. cloacae* subsp. *cloacae*, *E. cloacae* subsp. *dissolvens*, *E. cloacae* cluster III, *E. cloacae* cluster IV, *E. cloacae* cluster IX, '*Enterobacter hormaechei* subsp. *hormaechei*', '*E. hormaechei* subsp. *oharae*', '*E. hormaechei* subsp. *steigerwaltii*', *Enterobacter kobei*, *Enterobacter ludwigii* and *Enterobacter nimipressuralis* (Hoffmann & Roggenkamp, 2003). The members of the *E. cloacae* complex differ in pathogenicity towards humans. '*E. hormaechei* subsp. *steigerwaltii*', '*E. hormaechei* subsp. *oharae*' and *E. cloacae* cluster III are the species most frequently recovered from clinical specimens (Hoffmann *et al.*, 2005; Morand *et al.*, 2009). Moreover, *E. hormaechei* was reported to have caused an epidemic outbreak (Paauw *et al.*, 2009).

Abbreviations: CS, conserved segment; ERIC, enterobacterial repetitive intergenic consensus.

A dendrogram showing the genetic relatedness of the 69 clinical strains of the *E. cloacae* complex is available with the online version of this paper.

Bacteria of the *E. cloacae* complex are becoming increasingly prevalent nosocomial pathogens, probably due to higher levels of resistance to disinfectants and antimicrobial agents in comparison with other members of the *Enterobacteriaceae* (Paauw *et al.*, 2008). *E. cloacae* is naturally resistant to ampicillin, cephalotin and other older cephalosporins, and cefoxitin. A growing number of resistant *Enterobacter* species isolates, particularly those resistant to broad-spectrum cephalosporins, has been observed worldwide (Kang *et al.*, 2004). Therapy for *Enterobacter* species infections is often complicated by multiple antibiotic resistance, a phenomenon that leads to higher morbidity and mortality in infections caused by strains of *Enterobacteriaceae* and substantially increases treatment costs (Daikos *et al.*, 2007; Nijssen *et al.*, 2005; Norrby, 2005). Antimicrobial resistance may develop through mutations in chromosomal DNA or horizontal gene transfer including acquisition of plasmids or transposons carrying resistance determinants often assembled in platforms called integrons (Hall & Collis, 1995). The integron consists of an integrase gene of the tyrosine recombinase family, a primary recombination site called *attI* and a P_C promoter that directs transcription of the captured genes. Five classes of resistance integrons have been defined based on the polymorphism of the integrase genes. Three classes of integrons are responsible for multidrug resistance, with class 1 being most ubiquitous among resistant bacteria, and are considered to play the main role in the emergence and wide dissemination of resistance genes (Fluit & Schmitz, 2004; Mazel, 2006). Moreover, integron-mediated resistance can be silenced in a bacterial population at no biological cost, which means that resistant bacteria persist even if the selective pressure imposed by antibiotic usage is reduced (Andersson & Hughes, 2010).

The prevalence of multidrug resistance among isolates of the *E. cloacae* complex in human infections is rising. Therefore, the mechanisms of resistance, role of appropriate antimicrobial therapy and effect of specific antimicrobial usage must be taken into consideration.

The aim of this research was to analyse the resistance patterns and characterize the distribution and genetic content of resistance integrons within the *E. cloacae* complex originating from hospitalized patients.

METHODS

Clinical specimens. Sixty-nine unique strains of *E. cloacae* were collected from December 2006 to May 2008. They were obtained from specimens of inpatients at two Polish hospitals located 300 km away from each other. The organisms were grown on MacConkey Agar No. 3 (Oxoid) and identified using API 20E (bioMérieux) as *E. cloacae*. They were isolated from urine (30 strains), blood (ten strains), wounds (seven strains), catheters (three strains), anus swabs (four strains), aspirate (four strains), peritoneal cavity fluid (two strains), ulceration (two strains) and bronchoalveolar lavage, abscess, ear, eye, throat, gall bladder and faeces (a single strain from each).

Molecular identification of strains by *hsp60* sequence analysis.

The strains were further identified by sequence analysis of the *hsp60* gene. Bacterial DNA was isolated using a Novabeads Bacterial Genomic DNA kit (Novazym). Hi-Fi *Taq* polymerase and other PCR reagents were also purchased from Novazym. Primers Hsp60-F and Hsp60-R were used to amplify a portion of *hsp60* by PCR according to Morand *et al.* (2009). The PCR products were purified using ExoSAP-IT (Affymetrix) and sequenced in a 3130xl Genetic Analyzer (Applied Biosystems). A 272 nt fragment of the sequence was compared with the sequences of type and reference strains using CLUSTAL W and the neighbour-joining method (Morand *et al.*, 2009). The *hsp60* sequences of the following type and reference strains were used: *E. cloacae* subsp. *cloacae* ATCC 13047^T, *E. cloacae* subsp. *dissolvens* ATCC 23373^T, *E. cloacae* cluster III EN114, *E. cloacae* cluster IV EN117, *E. cloacae* cluster IX EN25, *E. asburiae* ATCC 35953^T, *E. kobei* ATCC BAA-260^T, *E. ludwigii* EN-119^T, '*E. hormaechei* subsp. *oharae*' EN-314^T, '*E. hormaechei* subsp. *hormaechei*' ATCC 49162^T, '*E. hormaechei* subsp. *steigerwaltii*' CIP 108489^T, *E. nimipressuralis* ATCC 9912^T, *Enterobacter cancerogenus* ATCC 33241^T, *Enterobacter amnigenus* ATCC 33072^T, *Enterobacter cowanii* CIP 107300^T, *Enterobacter gergoviae* ATCC 33028^T, *Enterobacter pyrinus* ATCC 49851^T and *Enterobacter sakazakii* ATCC 29544^T (Hoffmann & Roggenkamp, 2003; Morand *et al.*, 2009).

Enterobacterial repetitive intergenic consensus (ERIC)-PCR typing.

The ERIC-PCR method utilizes primers complementary to enterobacterial repetitive intergenic consensus sequences of genomic DNA. A PCR using primers ERIC 1 and ERIC 2 was carried out according to Versalovic *et al.* (1991). Computer analysis of electrophoretic patterns was carried out using GelCompar II version 3.5 software (Applied Maths): similarities between fingerprints were calculated with the Dice coefficient and cluster analysis was performed by UPGMA.

Antimicrobial susceptibility.

The susceptibility to 15 antibiotics representing eight classes was determined according to the standard disc diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2009). The antimicrobials were: amikacin, tobramycin, netilmicin, gentamicin, ticarcillin, ciprofloxacin, tetracycline, cefotaxime, ceftazidime, sulfamethoxazole, co-trimoxazole, piperacillin, piperacillin + tazobactam, chloramphenicol and imipenem. Production of extended-spectrum β -lactamases was checked by a double-disc synergy test with ceftazidime, cefotaxime and amoxicillin/clavulanic acid. The production of AmpC β -lactamases was checked by a three-dimensional assay according to Coudron *et al.* (2000). *Klebsiella pneumoniae* carbapenemases were detected by disc test with phenylboronic acid and imipenem (Tsakris *et al.*, 2009). Metallo- β -lactamase production was checked by an EDTA-disc synergy test (Lee *et al.*, 2001). All antibiotic discs were provided by Oxoid.

Multiplex PCR analyses for identification of integron integrase genes.

PCR amplifications were performed in a 50 μ l volume with 5 μ l 10 $\times$ PCR buffer with $\text{NH}_4(\text{SO}_4)_2$, 0.6 μ M each primer, 200 μ M dNTP mix, 2.5 mM MgCl_2 , 2 U Hi-Fi *Taq* polymerase and 200 ng genomic DNA. Amplification involved initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min and extension at 72 °C for 1 min, with a final extension step at 72 °C for 8 min. The sequences of the primers targeting the *intI1*, *intI2* and *intI3* genes were those recommended by Dillon *et al.* (2005).

Analysis of the variable region of class 1 integron by conserved segment (CS)-PCR.

Sequences of primers complementary to the 5'- and 3'-conserved regions of class 1 integrons have been published elsewhere (Lévesque *et al.*, 1995). PCR amplification was conducted as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles

of 94 °C for 1 min, 59 °C for 1 min and 72 °C for 5 min, with a final elongation at 72 °C for 8 min.

All PCRs were performed in a C1000 Thermal Cycler (Bio-Rad) and the products were separated in a 1.5% agarose gel (Prona). The molecular masses of the PCR products were determined by Bio-Capt version 99.04 software (Vilber Lourmat). All experiments were carried out in triplicate.

CS-PCR products were purified with ExoSAP-IT and sequenced. When two amplicons were present, the PCR products were separated by electrophoresis and purified using a QIAquick Gel Extraction kit (Qiagen). Some of the products were cloned using the pGEM-T Easy vector (Promega). Sequence data were analysed with DNA Baser (Heracle Software) and aligned with available GenBank data using BLAST.

Conjugation assay. Strains positive for the *intI* gene were examined for the ability to transfer resistance by conjugation. The recipient strain was *Escherichia coli* J53 (Rif^R). Briefly, the donor and recipient strains were grown in Luria–Bertani medium overnight at 30 °C, mixed in equal volumes, grown to mid-exponential phase (20 h), plated on Mueller–Hinton agar plates containing rifampicin (100 µg ml⁻¹) and streptomycin (20 µg ml⁻¹) and incubated for 24 h at 30 °C. The transconjugants were identified phenotypically by API 20E and tested for the presence and size of the variable part of the transferred integrons by PCR analyses. MIC values of streptomycin and rifampicin of donor strains were determined according to a standard method.

Statistical analysis. The statistical significance between the susceptibility and number of integron-positive and integron-negative isolates was calculated using Pearson's χ^2 test.

RESULTS

Molecular identification of strains by *hsp60* sequence analysis

PCR amplification of part of the *hsp60* heat-shock protein gene resulted in a 341 bp product for all strains. The PCR products were sequenced and 272 nt sequences were compared with the sequences of type and reference strains of *Enterobacter* species. All strains phenotypically identified as *E. cloacae* were clustered within the *E. cloacae* complex. The majority of the strains were identified as *E. hormaechei* (84%), with '*E. hormaechei* subsp. *steigerwaltii*' being the most frequent subspecies (69.6%). Five strains (7.2%) were identified as '*E. hormaechei* subsp. *hormaechei*'. Five isolates (7.2%) belonged to '*E. hormaechei* subsp. *oharae*', six (8.7%) to *E. cloacae* cluster III and three to *E. cloacae* cluster IV. One strain was identified as *E. cloacae* subsp. *cloacae* and one as *E. kobei*. ERIC-PCR analysis showed that the strains were genetically unrelated (see Supplementary Fig. S1, available in JMM Online).

Antimicrobial susceptibility

We analysed the profiles of resistance to 15 antibiotics belonging to eight groups (Table 1). Five strains of '*E. hormaechei* subsp. *steigerwaltii*' and two strains of '*E. hormaechei* subsp. *oharae*' produced extended-spectrum

β -lactamases. Fifty-two isolates (75.4%) were resistant to more than three unrelated antibiotics. Four strains (5.8%), belonging to '*E. hormaechei* subsp. *steigerwaltii*' (three) and *E. cloacae* cluster IV (one), were resistant to all of the 15 antibiotics used in the test. Two of these strains were isolated from blood and two from urine. Altogether, 71.4% strains of '*E. hormaechei* subsp. *steigerwaltii*' were multi-resistant, 67% of *E. cloacae* cluster IV, 50% of *E. cloacae* cluster III and 40% of '*E. hormaechei* subsp. *hormaechei*' and '*E. hormaechei* subsp. *oharae*'. The percentage of multiresistant strains in hospital no. 1 was 64%, whereas in hospital no. 2 it reached 54.5%. The highest resistance percentages were found towards sulfamethoxazole (78.3%), ticarcillin (63.8%), ciprofloxacin (64.6%), tetracycline (62.3%), cefotaxime (59.4%), amikacin (56.5%), tobramycin (56.5%), ceftazidime (55.7%), netilmicin, gentamicin and co-trimoxazole (55.1%), followed by piperacillin, piperacillin + tazobactam and chloramphenicol (52.2%) and imipenem (14.5%) (Table 1). The resistance to imipenem in all cases was conferred by an AmpC-mediated mechanism.

Multiplex PCR analysis for identification of integron integrase genes

Class 1 integrons were detected in 38 isolates (55.1%). No *intI2* and *intI3* genes were found. Thirty-one strains (63.3%) of '*E. hormaechei* subsp. *steigerwaltii*' were *intI1*-positive, three strains (50%) of *E. cloacae* cluster III, two strains (40%) of '*E. hormaechei* subsp. *oharae*', one (33.3%) belonging to *E. cloacae* cluster IV and one strain (20%) of '*E. hormaechei* subsp. *hormaechei*'. The resistance to each of the antibiotics, except imipenem, was significantly associated with the presence of integrons ($P < 0.01$) (Table 1). Moreover, of the 52 strains (75.4%) resistant to more than three antibiotics, 76% carried an integron, whereas none of the susceptible strains or those resistant to only one of the antimicrobial agents revealed the presence of the *intI* gene. All of the 38 integron-positive strains were resistant to more than three antibiotics; in fact, 92.1% (35 isolates) of the integron-positive strains were resistant to more than 10 antibiotics.

Analysis of the variable region of class 1 integrons

We detected and identified gene cassettes in 37/38 *intI1*-positive strains. The variable part of the class 1 integron was amplified using primers complementary to the conserved regions of the platform flanking the inserted resistance genes. The variable region of 180 bp present in the integron of an *E. cloacae* cluster IV strain indicated a structure with no incorporated cassettes. The other amplicon sizes were 1.0, 1.6 and 1.9 kb (Table 2). A 1.0 kb product was present in two integrons found in strains belonging to '*E. hormaechei* subsp. *oharae*' and *E. cloacae* cluster III. A single amplicon of 1.6 kb was found in 10 strains belonging to '*E. hormaechei* subsp. *steigerwaltii*'

Table 1. Association between resistance to antibiotics and the presence of integrons in strains of the *E. cloacae* complex

Antibiotic	No. of resistant isolates		No. of sensitive isolates		P value (χ^2 test)*
	Total (%)	Integron positive (% of resistant strains)	Total (%)	Integron positive (% of sensitive strains)	
Amikacin	39 (56.5)	35 (92.3)	30 (43.5)	3 (10)	<0.001
Gentamicin	38 (55.1)	37 (97.4)	31 (44.9)	1 (3.2)	<0.001
Netilmicin	38 (55.1)	38 (100)	31 (44.9)	0	<0.001
Tobramycin	39 (56.5)	37 (94.9)	30 (43.5)	1 (3.4)	<0.001
Chloramphenicol	36 (52.2)	26 (72.2)	33 (47.8)	12 (36.4)	0.0028
Imipenem	10 (14.5)	6 (60.0)	59 (85.5)	32 (54.2)	0.7348
Piperacillin	36 (52.2)	31 (86.1)	33 (47.8)	7 (21.2)	<0.001
Ticarcillin	44 (63.8)	33 (75)	25 (36.2)	5 (20)	<0.001
Piperacillin/tazobactam	36 (52.2)	29 (80.6)	33 (47.8)	9 (27.3)	<0.001
Cefotaxime	41 (59.4)	33 (80.5)	28 (40.6)	5 (17.8)	<0.001
Ceftazidime	39 (56.5)	33 (84.6)	30 (43.5)	5 (16.7)	<0.001
Ciprofloxacin	43 (62.3)	38 (88.4)	26 (37.7)	0	<0.001
Sulfamethoxazole	54 (78.3)	36 (66.7)	15 (21.7)	2 (13.4)	<0.001
Co-trimoxazole	38 (55.1)	36 (94.7)	31 (44.9)	2 (6.4)	<0.001
Tetracycline	43 (62.3)	33 (76.7)	26 (37.7)	5 (19.2)	<0.001

*Significant values are in bold.

(six), *E. cloacae* cluster III (two), '*E. hormaechei* subsp. *hormaechei*' (one) and '*E. hormaechei* subsp. *oharae*' (one). An amplicon of 1.9 kb was obtained for 16 strains of '*E. hormaechei* subsp. *steigerwaltii*'. Two amplicons of 1.6 and 1.9 kb were found exclusively in nine strains identified as '*E. hormaechei* subsp. *steigerwaltii*', eight of which were isolated from urine and one from a catheter.

The sequence analysis identified two groups of genes: those encoding aminoglycoside adenylotransferase responsible for resistance to aminoglycosides, and *dfr* cassettes conferring resistance to trimethoprim. Genes encoding aminoglycoside adenylotransferase represented 58.2 % of all cassettes found and included *aadA2* (31.6 % of all

cassettes), *aadA1* (15.2 %), both conferring resistance to streptomycin and spectinomycin, and *aadB* (10.1 %) encoding aminoglycoside-2"-O-nucleotidyltransferase that confers resistance to amikacin, gentamicin, tobramycin, dibekacin, sisomicin, netilmicin and kanamycin. One strain of '*E. hormaechei* subsp. *steigerwaltii*' had an *aacA4* cassette (1.3 % of all cassettes) responsible for resistance to tobramycin and amikacin. The *dfr* cassettes, representing 41.8 % of all cassettes detected, included *dfrA12* (30.4 %) and *dfrA1* (11.4 %).

The cassette arrays were as follow: *dfrA12-orfF-aadA2* for the 1.9 kb CS-PCR product and *aadB-aadA2*, *dfrA1-aadA1* and *aacA4-aadA1* for the 1.6 kb CS-PCR products (Table 2).

Table 2. Distribution of gene cassette arrays among *intI*-positive strains of the *E. cloacae* complex within hospitals

Type of gene cassette array	Size of integron variable region (kb)	Nomenclatures and no. of strains with gene cassette array	
		Hospital 1	Hospital 2
<i>aadA1</i>	1.0	' <i>E. hormaechei</i> subsp. <i>oharae</i> ' (1), <i>E. cloacae</i> cluster III (1)	
<i>aadB-aadA2</i>	1.6	' <i>E. hormaechei</i> subsp. <i>steigerwaltii</i> ' (3), ' <i>E. hormaechei</i> subsp. <i>hormaechei</i> ' (1), <i>E. hormaechei</i> subsp. <i>oharae</i> ' (1), <i>E. cloacae</i> cluster III (1)	
<i>dfrA1-aadA1</i>	1.6	' <i>E. hormaechei</i> subsp. <i>steigerwaltii</i> ' (2), <i>E. cloacae</i> cluster III (1)	
<i>aacA4-aadA1</i>	1.6	' <i>E. hormaechei</i> subsp. <i>steigerwaltii</i> ' (1)	
<i>dfrA12-orfF-aadA2</i>	1.9	' <i>E. hormaechei</i> subsp. <i>steigerwaltii</i> ' (1)	
<i>dfrA1-aadA1</i> , <i>dfrA12-orfF-aadA2</i>	1.6/1.9	' <i>E. hormaechei</i> subsp. <i>steigerwaltii</i> ' (6)	
<i>aadB-aadA2</i> , <i>dfrA12-orfF-aadA2</i>	1.6/1.9	' <i>E. hormaechei</i> subsp. <i>steigerwaltii</i> ' (3)	

The *dfrA12-orfF-aadA2* array was present in strains from both hospitals. Hospital-dependent differences were observed for the *dfrA1-aadA1* array, which was found exclusively in the genomes of strains from the first hospital, and for the *aadB-aadA2* array, identified only in strains from the other hospital. The cassette arrays of integrons identified in the *E. cloacae* complex together with the hospital affiliations are listed in Table 2. The gene cassette array found most often was *dfrA12-orfF-aadA2* in the 1.9 kb variable region present exclusively in '*E. hormaechei* subsp. *steigerwaltii*'. The CLUSTAL W-based comparison of the sequences showed them to be identical for >95 %, regardless of whether they were the only integron in the bacterial genome or a second one. This cassette array was found in strains from both hospitals.

Conjugation assay

Integrons were transferred to *E. coli* J-53 (Rif^R) in a conjugation assay. Thirty-seven transconjugants were selected on media complemented with antibiotics and screened for the presence of *intI1* and variable region size. All of the integrons were on transferable genetic elements, as we managed to produce transconjugants with integron platforms. Transconjugants were analysed for the acquired resistance. The transferred resistance primarily included that to aminoglycosides, ticarcillin, piperacillin, sulfamethoxazole, trimethoprim and tetracycline. Additionally, for selected strains with different integrons and resistance patterns, we performed plasmid analyses of donor and transconjugant isolates to check whether the integrons were embedded in transferable plasmids. We found plasmids present in both donor and transconjugant isolates.

DISCUSSION

The phenotypic similarity of strains belonging to different species of the *E. cloacae* complex creates many problems with their identification. We used sequencing of the *hsp60* gene, which shows good discriminatory power, to identify species and subspecies within the *E. cloacae* complex (Paauw *et al.*, 2008). The majority of clinical strains were identified as *E. hormaechei*, with '*E. hormaechei* subsp. *steigerwaltii*' as a predominant group. *E. hormaechei* strains were isolated from a variety of samples, including urine, wounds, blood and stools. However, strains of *E. cloacae* III and IV and *E. cloacae* subsp. *cloacae* clusters can also be pathogenic to humans, as they were isolated from nine infections.

Among the published reports, the resistance of *E. cloacae* strains varies depending on geographical location and year of testing (Sanders & Sanders, 1997). We noted higher levels of resistance, described as a percentage of resistant strains, than those reported earlier (Paterson, 2006; Turner, 2008; Vakulenko & Mobashery, 2003). Over 60 % of strains were resistant to sulfamethoxazole, ticarcillin, ciprofloxacin and tetracycline, and more than 50 % were resistant to

aminoglycosides, cefotaxime, ceftazidime, co-trimoxazole, piperacillin, piperacillin + tazobactam and chloramphenicol. In the study performed under the European SENTRY programme, in reports from 2003, including over 7000 clinical isolates of four *Enterobacteriaceae* genera, the susceptibility to amikacin, gentamicin and tobramycin among *Enterobacter* species was 95.8, 90.1 and 82.1 %, respectively (Vakulenko & Mobashery, 2003). In the same report, analysis of more than 4000 strains of *Enterobacteriaceae* in the USA revealed that *Enterobacter* species together with other enterobacterial genera are quite susceptible to aminoglycosides: 96–100 % to amikacin, 90–96 % to gentamicin and 94–97 % to tobramycin. Turner (2008) reported the results of the SENTRY programme from 2006 and its comparison with results from 2002. All antibiotics demonstrated a reduction in activity against *Enterobacter* species in 2006 in comparison with the results from 2002: the percentage of sensitive strains was 89.3 % for gentamicin (94.5 % in 2002), 86.7 % (90.4 %) for tobramycin, 86.0 % (91.9 %) for ciprofloxacin, 75.2 % (76.4 %) for ceftazidime, 74.3 % (78 %) for piperacillin + tazobactam and 98.3 % (98.7 %) for imipenem.

The percentage of *E. cloacae* complex strains possessing integrons was also higher (53.6 %) in comparison with earlier studies (Daikos *et al.*, 2007; Schmitz *et al.*, 2001). All *int*-positive strains were multiresistant, i.e. resistant to more than three unrelated antimicrobials. Schmitz *et al.* (2001) compared the frequency of *intI1*-positive *E. cloacae* strains, which ranged from 9.5 % in 1993 to 36.4 % in 1999, with the sizes of the variable part of the integron, ranging from 0.8 kb in 1993 to 1.5 kb in 1999. Daikos *et al.* (2007) found 44.4 % of *intI1*-positive *Enterobacter* species isolated from bloodstream infections with variable regions of 1.0, 1.6, 1.7 and 3.2 kb. In our research, most of the integrons (76.3 %) were carried by '*E. hormaechei* subsp. *steigerwaltii*', a predominant group in infections caused by the *E. cloacae* complex. Moreover only '*E. hormaechei* subsp. *steigerwaltii*' strains harboured two integrons and those with the largest variable region (1.9 kb).

Integron-positive strains demonstrated a greater tendency towards multiple antibiotic resistance than integron-negative isolates ($P < 0.01$). The resistance for each of the antibiotics, except for imipenem, was significantly associated with the presence of integrons ($P < 0.01$) (Table 1). Moreover, of the 61 isolates (88.4 %) resistant to more than three antibiotics, 60.7 % carried an integron, whereas none of the strains that were susceptible or resistant to one of the antimicrobial agents revealed the presence of the *intI* gene. All of the 38 integron-positive strains were resistant to more than three antibiotics; in fact, 94.6 % of them (35 isolates) were resistant to more than 10 antibiotics, indicating that the presence of integrons in the *E. cloacae* complex strains is associated with multidrug resistance regardless of the gene cassette content ($P < 0.01$). Studies by Leverstein-van Hall *et al.* (2002b, 2003) showed that resistance is associated with the presence of integrons in members of the *Enterobacteriaceae* and that strains with

integrations of class 1 are 100 % resistant to more than one antibiotic. Moreover, horizontal gene transfer of integrations often occurs and is very efficient in a hospital environment, and the intra- and interspecies transfer of integrations plays a dominant role in development of multiresistance in the *Enterobacteriaceae* (Leverstein-van Hall *et al.*, 2002a). We noted a high rate of integron-containing-element transfer *in vitro* and showed that the integrations were embedded in transferable plasmids. The transferred resistance was not limited to aminoglycosides, trimethoprim and sulfamethoxazole; it also included resistance to other classes of antimicrobials, e.g. β -lactams. Resistance genes were present on the same plasmid and possibly were part of an extended integron in which additional resistance genes are located after *orf513* (Paauw *et al.*, 2006; Sabaté *et al.*, 2002).

Analyses of the variable part of the integrations showed only a limited diversity of gene cassettes. We noticed location-dependent differences in the type of gene cassette content; namely, *dfrA1-aadA1* was found exclusively in the genomes of strains from the first hospital, whereas *aadB-aadA2* was found from the second hospital, regardless of whether they were the only one or the second integron in the genome. The gene cassettes identified conferred resistance to older antimicrobials seldom used therapeutically nowadays, namely trimethoprim and aminoglycosides such as spectinomycin and streptomycin. In the first hospital, in almost all integrations, the *dfr* cassettes were nearer the 5'-end of the integron, so they were expressed more efficiently. This also suggests that selection for cassettes carrying *dfr* genes might have occurred in this population.

Comparison of sequences of *dfrA12-orfF-aadA2* cassette arrays with those available in GenBank showed that they were >99 % identical to integrations described in the *Enterobacteriaceae*, and also in non-fermenting rods and staphylococci isolated from both human and animal samples (Leverstein-van Hall *et al.*, 2002a; Kadlec & Schwarz, 2008).

The *dfr1-aadA1* cassette array was present as a single integron in two strains of '*E. hormaechei* subsp. *steigerwaltii*' isolated from blood and *E. cloacae* cluster III originating from aspirate, and, as a second platform, in six isolates of '*E. hormaechei* subsp. *steigerwaltii*'. Previously, this cassette has been shown in other members of the *Enterobacteriaceae* and in *E. cloacae* within a larger integron associated with *bla_{VIM}* genes (Kang *et al.*, 2004; Leverstein-van Hall *et al.*, 2002a). The *aadB-aadA2* array was found in nine strains: as a single integron in three '*E. hormaechei* subsp. *steigerwaltii*', one '*E. hormaechei* subsp. *hormaechei*', one '*E. hormaechei* subsp. *oharae*' and one *E. cloacae* cluster III, and as a second platform in three '*E. hormaechei* subsp. *steigerwaltii*'; they had a similar sequence (98 % similarity) to that of *E. cloacae* isolated in Nanfang Hospital in China (GenBank accession no. GU906259.1).

One strain of '*E. hormaechei* subsp. *steigerwaltii*' had an *aacA4-aadA1* cassette array with 97 % identity to an *Aeromonas caviae* partial class 1 integron sequence containing the *oxa10*, *aacA4* and *aadA1* genes (GenBank accession no. FM207629.1).

In summary, we found a high frequency of multidrug resistance and integron carriage among the *E. cloacae* complex strains of human origin, especially '*E. hormaechei* subsp. *steigerwaltii*'. The types of gene cassettes and the gene arrays in the *E. cloacae* complex seemed to be protected and not liable to changes. Our assumption is that there is a tendency to gather the whole integron rather than to incorporate new gene cassettes. It seems that horizontal gene transfer is a phenomenon that is more prevalent than integration of gene cassettes by integron integrase.

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