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 Enterobacter 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 Enterobacter 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 Enterobacter cloacae clusters III and IV, Enterobacter 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 Enterobacter hormaechei subsp. steigerwaltii, 50% of Enterobacter cloacae cluster III, 40% of Enterobacter hormaechei subsp. oharae, 33% belonging to Enterobacter cloacae cluster IV and 20% of Enterobacter hormaechei subsp. hormaechei were int11-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 adenyltransferases responsible for resistance to aminoglycosides, and dfr cassettes conferring resistance to trimethoprim. Integrons of the Enterobacter 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.

INTRODUCTION

Bacteria of the Enterobacter cloacae complex are Gram-negative, chemo-organotrophic, facultatively anaerobic rods or cocccobacilli. Recently, the importance of members of the Enterobacter 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 Enterobacter. The complex is, however, heterogeneous. According to the sequence of the hsp60 gene, it has been divided into 12 genetic clusters: Enterobacter asburiae, Enterobacter cloacae subsp. cloacae, Enterobacter cloacae subsp. dissolvens, Enterobacter cloacae cluster III, Enterobacter cloacae cluster IV, Enterobacter cloacae cluster IX, Enterobacter hormaechei subsp. hormaechei, Enterobacter hormaechei subsp. oharae, Enterobacter hormaechei subsp. steigerwaltii, Enterobacter kobei, Enterobacter ludwigii and Enterobacter nimipressuralis (Hoffmann & Roggenkamp, 2003). The members of the Enterobacter cloacae complex differ in pathogenicity towards humans. Enterobacter hormaechei subsp. steigerwaltii, Enterobacter hormaechei subsp. oharae and Enterobacter cloacae cluster III are the species most frequently recovered from clinical specimens (Hoffmann et al., 2005; Morand et al., 2009). Moreover, Enterobacter 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, cephalothin 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 recombination 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. ludwigi* EN-119^T^, *E. hormaechei* subsp. *ehratae* 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 cowani* CIP 107300^T^, *Enterobacter gergoviae* ATCC 33028^T^, *Enterobacter pyrus* ATCC 49851^T^ and *Enterobacter sakazakii* ATCC 29544^T^ (Hoffmann & Roggenkamp, 2003; Morand et al., 2009).

**Enterobacterial repetitive intergen consensucis (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, ticaricillin, ciprofloxacin, tetracycline, cefotaxime, cefadiazide, sulfamethoxazole, co-trimoxazol, 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 (Tsakis 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 μl volume with 5 μl 10 × PCR buffer with NH₄₂(SO₄)₂, 0.6 μM each primer, 200 μM dNTP mix, 2.5 mM MgCl₂, 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 (Levesque 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 (RifR). 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 χ² 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 (53.1 %), 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*...
(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 1. Association between resistance to antibiotics and the presence of integrons in strains of the *E. cloacae* complex

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>No. of resistant isolates</th>
<th>No. of sensitive isolates</th>
<th>P value ($\chi^2$ test)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (%)</td>
<td>Integron positive (%) of resistant strains</td>
<td>Total (%)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>39 (56.5)</td>
<td>35 (92.3)</td>
<td>30 (43.5)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>38 (55.1)</td>
<td>37 (97.4)</td>
<td>31 (44.9)</td>
</tr>
<tr>
<td>Netilmicin</td>
<td>38 (55.1)</td>
<td>38 (100)</td>
<td>31 (44.9)</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>39 (56.5)</td>
<td>37 (94.9)</td>
<td>30 (43.5)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>36 (52.2)</td>
<td>26 (72.2)</td>
<td>33 (47.8)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>10 (14.5)</td>
<td>6 (60.0)</td>
<td>59 (85.5)</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>36 (52.2)</td>
<td>31 (86.1)</td>
<td>33 (47.8)</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>44 (63.8)</td>
<td>33 (75)</td>
<td>25 (36.2)</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>36 (52.2)</td>
<td>29 (80.6)</td>
<td>33 (47.8)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>41 (59.4)</td>
<td>33 (80.5)</td>
<td>28 (40.6)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>39 (56.5)</td>
<td>33 (84.6)</td>
<td>30 (43.5)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>43 (62.3)</td>
<td>38 (88.4)</td>
<td>26 (37.7)</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>54 (78.3)</td>
<td>36 (66.7)</td>
<td>15 (21.7)</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>38 (55.1)</td>
<td>36 (94.7)</td>
<td>31 (44.9)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>43 (62.3)</td>
<td>33 (76.7)</td>
<td>26 (37.7)</td>
</tr>
</tbody>
</table>

*Significant values are in bold.

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

<table>
<thead>
<tr>
<th>Type of gene cassette array</th>
<th>Size of integron variable region (kb)</th>
<th>Nomenspecies and no. of strains with gene cassette array</th>
</tr>
</thead>
<tbody>
<tr>
<td>aadA1</td>
<td>1.0</td>
<td>‘<em>E. hormaechei</em> subsp. <em>oharae</em>’ (1), <em>E. cloacae</em> cluster III (1)</td>
</tr>
<tr>
<td>aadB-aadA2</td>
<td>1.6</td>
<td>‘<em>E. hormaechei</em> subsp. <em>steigerwaltii</em>’ (3), ‘<em>E. hormaechei</em> subsp. <em>hormaechei</em>’ (1), <em>E. cloacae</em> cluster III (1)</td>
</tr>
<tr>
<td>dfrA1-aadA1</td>
<td>1.6</td>
<td>‘<em>E. hormaechei</em> subsp. <em>steigerwaltii</em>’ (2), <em>E. cloacae</em> cluster III (1)</td>
</tr>
<tr>
<td>aacA4-aadA1</td>
<td>1.6</td>
<td>‘<em>E. hormaechei</em> subsp. <em>steigerwaltii</em>’ (1)</td>
</tr>
<tr>
<td>dfrA12-orfF-aadA2</td>
<td>1.9</td>
<td>‘<em>E. hormaechei</em> subsp. <em>steigerwaltii</em>’ (1)</td>
</tr>
<tr>
<td>dfrA1-aadA1, dfrA12-orfF-aadA2</td>
<td>1.6/1.9</td>
<td>‘<em>E. hormaechei</em> subsp. <em>steigerwaltii</em>’ (6)</td>
</tr>
<tr>
<td>aadB-aadA2, dfrA12-orfF-aadA2</td>
<td>1.6/1.9</td>
<td>‘<em>E. hormaechei</em> subsp. <em>steigerwaltii</em>’ (3)</td>
</tr>
</tbody>
</table>
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 (RifR) 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. cloaca 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 (Paton, 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, cefazidime, co-trimoxazole, piperacillin, piperacillin + tazobactam and chloramphenicol. In the study performed under the European SENTRY programme, in reports from 2003, 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
integrons of class 1 are 100% resistant to more than one antibiotic. Moreover, horizontal gene transfer of integrons often occurs and is very efficient in a hospital environment, and the intra- and interspecies transfer of integrons 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 integrons 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; Sabatè et al., 2002).

Analyses of the variable part of the integrons 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, almost all integrons, 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 integrons 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 blaNDM 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.

REFERENCES


