Evidence of Extensive Interspecies Transfer of Integron-Mediated Antimicrobial Resistance Genes among Multidrug-Resistant Enterobacteriaceae in a Clinical Setting

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Multidrug resistance in gram-negative bacteria appears to be primarily the result of the acquisition of resistance genes by horizontal transfer. To what extent horizontal transfer may be responsible for the emergence of multidrug resistance in a clinical setting, however, has rarely been investigated. Therefore, the integron contents of isolates collected during a nosocomial outbreak of genotypically unrelated multidrug-resistant Enterobacteriaceae were characterized. The integron was chosen as a marker of transfer because of its association with multiresistance. Some genotypically identical isolates harbored different integrons. Grouping patients carrying the same integron yielded 6 epidemiologically linked clusters, with each cluster representing a different integron. Several patients carried multiple species harboring the same integron. Conjugation experiments with these strains resulted in the transfer of complete resistance patterns at high frequencies ($10^{-2}$ to $10^{-4}$). These findings provide strong evidence that the horizontal transfer of resistance genes contributed largely to the emergence of multidrug-resistant Enterobacteriaceae in this clinical setting.

Multidrug-resistant Enterobacteriaceae (MRE) are being isolated at an increasing rate in hospital settings and are having a significant impact on clinical practice and overall treatment costs [1–5]. This multiresistance may be mediated by chromosomally located resistance determinants or mutations in a resident gene; however, it may also develop through the acquisition of resistance genes or an array of resistance genes by horizontal transfer. This latter phenomenon is currently thought to play an increasing role in the development of multidrug resistance in Enterobacteriaceae [6]. Plasmids and transposons are known to be involved in the transfer of resistance genes from one cell to another. These elements can hunt as a pack by interacting with each other in a variety of ways that enhance their collective ability to transfer resistance genes. Some of the plasmids that carry multiple resistance genes also have transfer systems that enable them to transfer DNA between unrelated species (promiscuous or broad-host-range plasmids) [7].

In recent years, it has been shown that a substantial portion of the resistance genes present on plasmids and transposons in gram-negative bacilli is integrated in DNA elements called integrons [8]. These integrons are potentially mobile elements (namely, transposons or defective transposon derivatives) that comprise a site-specific recombination system capable of integrating and expressing the genes contained in cassette structures. The 3 essential components of an integron, located within the $5'$ conserved segment (CS) of the element, include (1) an integrase gene, IntI, which encodes a site-specific recombinase; (2) an adjacent $attB$ site, which is recognized by the integrase and acts as a receptor for gene cassettes; and (3) a promoter region, $P_{prom}$. There are 4 distinct classes of integrons, each encoding a distinct integrase gene. Class 1 integrons comprise most integrons found in clinical isolates and are strongly associated with the multiresistance seen in the hospital environment [9]. In total, ~50 different class 1 integrons and >60 different gene cassettes have been described to date. The currently known gene cassettes confer resistance to aminoglycosides, penicillins, cephalosporins, carbapenems, trimethoprim, chloramphenicol, rifampin, erythromycin, and quaternary ammonium compounds (disinfectants and antisepsics) [10]. Often, class 1 integrons also contain an additional resistance gene in the $3'$ CS, downstream from the gene cassettes, namely, $sulI$. This gene confers resistance to sulfamethoxazole [8].

In 1996, our hospital was confronted with a sudden increase in the incidence of MRE on the neurology and neurosurgery wards [5]. Genotyping showed that 17 (28%) of the 61 patients involved were either infected with or colonized by a single multidrug-resistant *Klebsiella oxytoca* strain. The isolates recovered from the other patients comprised 8 different bacterial species, and subsequent genotyping yielded a great variety of strains. The vast majority of these strains were resistant to at least 3 classes of antimicrobial agents, including trimethoprim-sulfamethoxa-
zole. The transfer of class 1 integron-mediated antimicrobial resistance genes, therefore, probably played an important role during this outbreak. Few attempts have been made to determine to what extent gene transfer occurs in nature [11, 12].

The objective of the present study was to investigate whether and to what extent the horizontal transfer of resistance genes contributed to the emergence of MRE on the neurology and neurosurgery wards. The integron was chosen as a marker of transfer because it is a well-defined transferable genetic determinant, and the variety of the contents of the gene cassettes made one specific integron an unlikely common trait for all of the isolates involved. The hypothesis was that maximal circumstantial evidence for horizontal transfer would be obtained if all of the following observations could be made: (1) Isolates with an identical genotype harbored different (combinations of) integrons; (2) different species or strains collected from the same patient harbored the same integron; and (3) different isolates of epidemiologically linked patients carried the same integron. Thus, the presence of integrons was determined among the outbreak strains, the contents of the integrons were characterized, and the extent to which integron-mediated transfer of antimicrobial resistance genes had occurred was assessed.

**Patients, Materials, and Methods**

**Setting and patients.** A sudden increase in the incidence of MRE in general and of multidrug-resistant *K. oxytoca* in particular was observed in March 1996 in the Neurodivision of the University Hospital Utrecht, Utrecht, The Netherlands, an 858-bed teaching and referral hospital [5]. The Neurodivision comprises the Departments of Neurology and Neurosurgery, which are located next to one another. The Department of Neurosurgery has a 7-bed medium-care area, 4 low-care private rooms, and 2 wards, one with 21 beds (3 private rooms, 3 2-bed rooms, and 3 4-bed rooms) and one with 19 beds (3 private rooms, 2 2-bed rooms, and 3 4-bed rooms). The Department of Neurology has an 8-bed medium-care area and 2 wards, one with 20 beds (4 private rooms, 4 2-bed rooms, and 2 4-bed rooms) and one with 31 beds (3 private rooms, 4 2-bed rooms, and 5 4-bed rooms). The private rooms are provided with negative air pressure in relation to their anteroom. During the 1-year period of study (week 46, 1995, through week 45, 1996), 2604 patients were admitted to the Neurodivision, with a mean length of stay of 17 days.

All patients who had had MRE isolated from clinical samples or screening samples during the 1-year period were included in the study (*n* = 61). Their mean length of stay in the hospital was 70 days (median length of stay, 50 days). Resistance to gentamicin was the sole criterion for MRE, because 95% of all gentamicin-resistant Enterobacteriaceae (GRE) are multidrug resistant (i.e., resistant to >1 agent belonging to >3 of the following antibiotic agent classes: aminoglycosides, broad-spectrum penicillins, cephalosporins, quinolones, and trimethoprim-sulfamethoxazole) and because no MRE were susceptible to gentamicin. The first and subsequent phenotypically different GRE isolates from almost all patients were stored at −20°C.

The first isolate from 27 of the 61 patients was obtained from specimens sent in by physicians for clinical reasons (clinical samples). Except for isolates from samples from 2 wounds, all isolates were recovered from urine and sputum samples, representing either colonization or infection. No deaths were attributable to MRE. The remaining 34 patients were identified as carriers of MRE on the basis of the results of an infection-control screening program to detect intestinal colonization (screening samples). Subsequent clinical samples from only 2 patients (6%) yielded GRE during these patients’ remaining hospital stays (median, 24 days). These samples were sent for screening 2 and 7 days after collection of the first positive screening samples. This low number was possibly the result of the lack of selective antibiotic pressure on these strains because of the implemented restrictive antibiotic policy followed at the Neurodivision at that time [5]. Environmental cultures were also obtained as part of the infection-control program. Multi-resistant Klebsiella pneumoniae and *K. oxytoca*, respectively, were cultured from a hand-directed soap dispenser in the staff refreshment room and from a brush used for cleaning the measuring glass needed for parenteral feeding. Eighty-one staff members practicing bedside care at the Neurodivision were screened for intestinal carriage. One neurologist was identified as a GRE carrier.

**Bacterial strains.** Isolates from 56 of 61 patients involved in the outbreak were available for study. Eighty-six of the isolates (29 *Escherichia coli*, 21 *K. oxytoca*, 20 *K. pneumoniae*, 10 *Citrobacter freundii*, 3 *Enterobacter cloacae*, 2 *Proteus mirabilis*, and 1 *Enterobacter aerogenes*) were selected. Criteria for selection were as follows: (1) at least 1 isolate per patient, (2) at least 1 isolate of each species per patient if multiple species were isolated from the patient, and (3) at least 1 isolate of each genotype per species per patient if multiple isolates of the same species were obtained from the patient. Also studied were 3 gentamicin-susceptible but sulfamethoxazole-trimethoprim-resistant isolates from 3 patients with GRE, the 2 environmental isolates, and the isolate from the neurologist.

**Identification and antimicrobial susceptibility.** Identification and susceptibility testing were done using an automated system (VITEK and AMS R09.1 software (both supplied by bioMérieux). Additional testing for susceptibility to sulfamethoxazole, streptomycin, aztreonam, erythromycin, and chloramphenicol was done using the agar diffusion method. The break points were those recommended by the National Committee for Clinical Laboratory Standards [13]. The production of extended-spectrum β-lactamases (ESBLs) was determined with the ESBL Etest (AB BIODISK) according to the guidelines of the manufacturer.

**Typing by random amplified polymorphic DNA (RAPD) analysis and pulsed-field gel electrophoresis (PFGE).** To determine whether the high prevalence of MRE was the result of an epidemic spread of a few strains or merely an increase in unrelated MRE, all isolates of the same species (except for *Proteus* species) were typed by RAPD analysis, as described elsewhere [5]. If isolates of the same species were shown to contain identical integrons, they were also typed by PFGE analysis. In brief, after the clinical isolates were grown overnight in Luria Bertani (LB) broth, the bacterial pellets were washed and suspended in 100 mM Tris-HCl (pH 8.0) plus 200 mM EDTA. Bacterial plugs were then prepared in solutions with a final concentration of 50 mM Tris-HCl (pH 8.0) and 100 mM EDTA (TE), 0.6% pulsed-field certified agarose (BioRad), 0.5% SDS, and 0.5 g/L proteinase K (Merck). After a 30-min incu-
cubation at 37°C in 50 mM Tris-HCl, 50 mM EDTA, 1% sarcosine, and 0.1 g/L proteinase K, the plugs were washed sequentially in aqua bidest and TE and were stored at 4°C until use. The bacterial DNA was digested with restriction endonuclease XbaI (Roche) according to the guidelines of the manufacturer. Restriction fragments were then separated by PFGE in a 1% pulsed-field-armed agarose gel (BioRad) in 45 mM Tris-borate (pH 8.3) and 1 mM EDTA with a CHEF-DRII drive module (Bio-Rad). The initial pulse time of 2.2 s was increased linearly to 54.2 s in 20 h at 6 V/cm at 14°C. The gels were then stained with ethidium bromide, and the restriction fragments were visualized under UV light. The banding patterns were compared visually and classified according to previously described criteria [14]. The strains were considered unique if the combination of the RAPD type and the PFGE type was not seen in any of the other isolates.

**Detection of integrons by polymerase chain reaction (PCR) amplification.** To identify the presence of an integron and to determine the size of any inserted gene cassette, a CS-PCR was performed according to the method of Lévesque et al. [15], with the exception that template DNA was isolated by use of Nucleobond AX columns (Machery-Nagel). Because the primers used in this PCR anneal specifically in the 5’- and 3’-CS of class 1 integrons, the amplification product contained inserted gene cassettes flanked on both sides with small parts of the CSs.

**Characterization of integrons by sequencing and restriction fragment–length polymorphism (RFLP) typing.** To determine whether different isolates carried identical integrons, the integron content of every isolate was characterized. That is, each CS-PCR amplification product that had a unique size (number of base pairs) was sequenced. The size of the CS-PCR product of each new strain was compared with the sizes of the already sequenced products. If a CS-PCR product of the same size had been sequenced before, both PCR products were compared by RFLP typing to discover whether they contained the same integron content. If the CS-PCR product of the unknown strain yielded the same RFLP pattern as the already sequenced CS-PCR product, the 2 integrons were considered to be identical. If the CS-PCR product contained a different RFLP pattern, the new CS-PCR product was sequenced as well. Amplicon sequencing reactions were performed on purified PCR products, using a PCR-cycle sequencing kit (Perkin-Elmer) and an automated sequencer (ABI 377; Applied Biosystems). Purification of single CS-PCR products was attained using MicroSpin G-25 columns (Pharmacia). If a strain contained multiple CS-PCR products, each product was cut from the agarose gel and purified, using the QIAquick Gel Extraction kit (Qiagen). For RFLP analysis, the purified CS-PCR amplicons were then digested. At least 2 different restriction endonucleases were chosen for each RFLP assay on the basis of the content of the sequenced product. Digestions were performed according to the manufacturer’s instructions.

**Conjugation experiments.** To obtain further evidence for in vivo horizontal transfer of resistance genes, conjugation experiments were done with at least 1 strain of clinical isolates from each patient who carried multiple species with the same (combination of) integrons and with a sulfamethoxazole-susceptible (Smz^S^), rifampicin-resistant (Rif^R^) *E. coli* K12 recipient strain. Liquid mating was performed in LB broth and incubated overnight at 37°C. Transconjugants were selected on LB agar plates containing both rifampicin (50 μg/mL) and sulfamethoxazole (512 μg/mL) or rifampicin (50 μg/mL) alone for counter selection. Five transconjugants were analyzed for each conjugation experiment. Donor strains and transconjugants were tested, using the VITEK (bioMérieux) automated system, for their susceptibility pattern and, depending on the integron contents, for sulfamethoxazole, streptomycin, aztreonam, erythromycin, and chloramphenicol, using the agar diffusion method. Tests for the presence and contents of the integrons were done as described above. Transfer frequencies (T_{trans}) were expressed as the ratio of the number of transconjugants to the number of recipients: T_{trans} = [N(Smz^R^Rif^R^)]/[N(Smz^S^Rif^S^)]

**Table 1.** Characteristics of integrons detected in isolates obtained during a hospital outbreak in 1996.

<table>
<thead>
<tr>
<th>CS-PCR length, bp</th>
<th>Gene cassettes</th>
<th>Resistance phenotype</th>
<th>Endonucleases</th>
<th>RFLP code</th>
<th>Source [reference]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>aadA2</td>
<td>Stm-Spm</td>
<td>Bgl, HindIII, HpaI</td>
<td>I</td>
<td>Cattle, swine [18]</td>
</tr>
<tr>
<td>1450</td>
<td>aadBisscatB3</td>
<td>Gm-Km-Tm, Chl</td>
<td>HpaII, NeI, Sau96I</td>
<td>II</td>
<td>Human [19]</td>
</tr>
<tr>
<td>2500</td>
<td>aacA7::oxa2</td>
<td>lauaadA8</td>
<td>Amik-Net-Tm, β-lactams, EcoRI, PvuI, XhoI</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>aadA1a</td>
<td>Stm-Spm</td>
<td>Bgl, HindIII, HpaI</td>
<td>I</td>
<td>V</td>
</tr>
<tr>
<td>2200</td>
<td>dfrA5::f cereA2</td>
<td>TMP, Em</td>
<td>EcoRI, MboII, XhoI</td>
<td>VI</td>
<td>Human, poultry, swine [15, 20–22]</td>
</tr>
<tr>
<td>1550</td>
<td>dfrA1::aadA1a</td>
<td>TMP, Stn-Spm</td>
<td>HpaII, Sau96I</td>
<td>VII</td>
<td>Human, swine [15, 20]</td>
</tr>
<tr>
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<td>lauaadA2</td>
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<td>Not done</td>
<td>VIII</td>
</tr>
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<td>EcoRI, PvuI, XhoI</td>
<td>X</td>
<td></td>
</tr>
<tr>
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<td>Unknown, Amik-Net-Tm, Chl</td>
<td>DraI, EcoRI, MboII, XhoI</td>
<td>XI</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.** Characteristics of integrons detected in isolates obtained during a hospital outbreak in 1996.

NOTE. Amik, amikacin; Chl, chloramphenicol; CS-PCR, conserved-segment polymerase chain reaction; Em, erythromycin; Gm, gentamicin; Km, kanamycin; Net, netilmicin; RFLP, restriction fragment–length polymorphism; Spm, spectinomycin; Stm, streptomycin; Tm, tobramycin; TMP, trimethoprim.

*The order of genes in the variable region reads from the 5’ CS to the 3’ CS of the integron. GenBank accession nos. for genes per RFLP code are as follows: I, X66227; II, U113880/U13888; III, U13880/M95287/A1F326210; V, X12870; VI, X12868/A1F326209; VII, X00926/X12870; VIII, Z21672/X68227; and XI, X66227/M55547/A2F27506.

1. Endonucleases used for RFLP typing.

2. Each unique restriction pattern obtained by RFLP typing is marked with a different Roman numeral.

3. Sources from which the integrons were previously isolated, based on the alignment of nucleotide sequences.
mon plasmid-determined β-lactamases found in the Enterobacteriaceae. To assess whether and to what extent plasmid-determined β-lactamases were cotransferred with integrons, we determined the presence of blaSHV and blaTEM genes in all the strains included in the conjugation experiments. Whole-cell DNA was prepared for amplification by boiling the cells for 5 min. The primers SHV-Nhe-F (5′-GAGCGGAAAGATCCACTATCG-3′) and SHV-Nhe-R (5′-GTATCCCCGAGATAATTCA-3′) were designed for the amplification of the 526-bp blaSHV gene–specific fragment (GenBank accession no. AF148850 [SHV1], bp 267–287 and bp 790–771, respectively). The PCR conditions were as follows: 35 cycles of 1 min at 94°C, 1 min at 55°C, and 30 s at 72°C. PCR amplification of the blaTEM gene was done as described by Arlet et al. [16].

Identification of blaSHV and blaTEM genes. The blaSHV and blaTEM genes were identified to determine whether they encoded for broad-spectrum β-lactamases or ESBLs. Tests for the identification of blaSHV and blaTEM genes were performed on all strains included in the conjugation experiments, except for the blaTEM genes of the transconjugants. To identify the SHV-encoded β-lactamases, PCR amplicons were digested with restriction endonuclease NheI. SHV genes containing an NheI restriction site encode for TEM genes. The TEM genes were performed on all strains included in the conjugation experiments, except for the blaTEM genes of the transconjugants. To identify the SHV-encoded β-lactamases, PCR amplicons were digested with restriction endonuclease NheI. SHV genes containing an NheI restriction site encode for TEM genes. The TEM genes. The TEM genes in all the strains included in the experiments. The remaining 40 isolates did not yield a CS-PCR product. These included isolates of 10 E. coli (10 genotypes), 8 K. pneumoniae (3 genotypes), 1 C. freundii, 1 E. cloacae, and 20 K. oxytoca. Of the 20 K. oxytoca isolates, 19 were found to have the same genotype as that identified on the brush used to clean the measuring glass needed for parenteral feeding.

Susceptibility testing of strains. Susceptibility testing of the 46 isolates containing an integron gave the following results: 96% of the isolates were resistant or intermediately susceptible to gentamicin, 83% to tobramycin, 87% to cotrimoxazole, 93% to aminoglycosides, and 46 isolates harboring at least 1 integron. These included isolates of 10 E. coli (17 genotypes), 12 K. pneumoniae (5 genotypes), 1 K. oxytoca, 9 C. freundii (3 genotypes), 2 P. mirabilis, 2 E. cloacae, and 1 E. aerogenes. The E. coli isolated from the staff member and the K. pneumoniae isolated from the soap dispenser also contained an integron. The remaining 40 isolates did not yield a CS-PCR product. These included isolates of 10 E. coli (10 genotypes), 8 K. pneumoniae (3 genotypes), 1 C. freundii, 1 E. cloacae, and 20 K. oxytoca. Of the 20 K. oxytoca isolates, 19 were found to have the same genotype as that identified on the brush used to clean the measuring glass needed for parenteral feeding.

Results

Presence of integrons. Eighty-six isolates were included in the study. Twenty-six of the 56 patients colonized or infected with MRE carried 46 isolates harboring at least 1 integron. These included 19 isolates of E. coli (17 genotypes), 12 K. pneumoniae (5 genotypes), 1 K. oxytoca, 9 C. freundii (3 genotypes), 2 P. mirabilis, 2 E. cloacae, and 1 E. aerogenes. The E. coli isolated from the staff member and the K. pneumoniae isolated from the soap dispenser also contained an integron. The remaining 40 isolates did not yield a CS-PCR product. These included isolates of 10 E. coli (10 genotypes), 8 K. pneumoniae (3 genotypes), 1 C. freundii, 1 E. cloacae, and 20 K. oxytoca. Of the 20 K. oxytoca isolates, 19 were found to have the same genotype as that identified on the brush used to clean the measuring glass needed for parenteral feeding.

Table 2. Distribution of integrons among multiresistant gram-negative bacilli isolated during a hospital outbreak in 1996.

<table>
<thead>
<tr>
<th>Patient or staff</th>
<th>Source</th>
<th>Escherichia coli</th>
<th>Klebsiella pneumoniae</th>
<th>Klebsiella oxytoca</th>
<th>Citrobacter freundii</th>
<th>Enterobacter aerogenes</th>
<th>Enterobacter cloacae</th>
<th>Proteus mirabilis</th>
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<tr>
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</tr>
<tr>
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<td>I + II + XI′</td>
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</tr>
<tr>
<td>26</td>
<td>Rectum</td>
<td>III′, I + II + XI′, II + XI′</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

NOTE. Roman numerals represent the unique restriction patterns obtained by restriction fragment–length polymorphism (RFLP) typing. GenBank accession nos. for genes per RFLP code are as follows: I, X68227; II, U13880/U13880; III, U13880/M95287/AF326210; V, X12870; VI, X12688/AF326209; VII, X00926/X12870; VIII, Z21672/X68227; and XI, X68227/M55547/AF227506. Isolates with identical genotypes are marked with the same superscript letter (a–g).
to ampicillin, 56% to piperacillin, 80% to amoxicillin-clavulanic acid, 49% to cephalotin, 49% to cefuroxime, 23% to ceftriaxone, 33% to ceftazidime, and 2% to ciprofloxacin. Moreover, 43% (9/21 strains tested) of the E. coli and Klebsiella species produced ESBLs. Susceptibility testing of the 40 isolates without a CS-PCR product gave similar results.

Characterization of integrons. Analysis of the amplification products revealed 10 different integrons. The content and order of the resistance genes integrated in the integrons are presented in table 1 [15, 18–25]. Fifteen different gene cassettes, encoding resistance to aminoglycosides, β-lactams, chloramphenicol, trimethoprim, and erythromycin, were detected. Two pairs of integrons shared identical gene cassettes: integrons VIII and I shared aadA2, and integrons V and VII shared aadA1a. Two CS-PCR amplification products were not sequenced. RFLP typing of these products, however, showed clear discriminatory patterns (RFLP codes IX and X).

Evidence of horizontal transfer and distribution of integrons among clinical isolates. The hypothesis was that maximal circumstantial evidence for horizontal transfer would be obtained if 3 observations could be made. The first observation was that isolates with an identical genotype harbor different (combinations of) integrons. Four unique strains harboring different (combinations of) integrons were found: E. coli<sup>b+c</sup> and K. pneumoniae<sup>d+e</sup> (table 2).

The second observation was that different species or strains collected from the same patient harbor the same integron. Seven patients (patients 12, 13, 20, 21, and 24–26) were colonized or infected with >1 species of GRE sharing identical (combinations of) integrons. Patients 25 and 26 also harbored strains of the same species with different PFGE types but with identical (combinations of) integrons. The strains isolated from patients 13, 20, and 24 were obtained from the same clinical site (urine obtained from a Foley catheter and sputum). The CS-PCR products of 8 consecutive isolates obtained from patient 26 during a 3.5-month hospital stay are shown in figure 1 as an example of horizontal transfer.

The third observation was that different isolates of epidemiologically linked patients carry the same integron. Integron VI was restricted to the strains isolated from patient 13, integron VIII was restricted to patient 15, and 1 integron combination (IX + X) was detected in a unique strain shared by 2 patients (patients 21 and 22). Analysis of the distribution of the remaining 6 integrons revealed 6 clusters of epidemiologically linked patients. Integrons III and XI, respectively, were found in 6 and 4 unique strains distributed over 3 and 4 species collected from 10 and 5 patients. Integron III was also isolated from a soap dispenser in the staff refreshment room. For both integrons, all patients could be linked to each other by their overlapping dates of hospitalization on the same wards. Integrons I and II, respectively, were found in 4 and 7 unique strains distributed over 3 and 4 species collected from 6 and 8 patients. All of these patients had overlapping dates of hospitalization, and most of them had been in the same ward. Integron V was found in 6 unique strains distributed over 4 species collected from 5 patients and the staff member. These isolates were obtained within a 4-month period in 3 different wards. Although these patients had overlapping dates of hospitalization, only 2 had been on the same ward at the same time. Integron VII was found in 3 different E. coli strains collected from 3 patients within a 2-month period. These patients stayed in different wards, but 2 shared a 6-week period of hospitalization.

Resistance transfer and susceptibility testing of transconjugants. Eleven strains sharing identical (combinations of) integrons, obtained from 7 patients colonized or infected with >1 species of GRE, were selected for the conjugation experiments. All of the strains included yielded transconjugants (table 3). The transfer frequencies varied from 1 to 3 × 10<sup>-2</sup> transconjugants per recipient cell for E. coli, K. oxytoca, and E. cloacae; from 4 to 8 × 10<sup>-3</sup> transconjugants per recipient cell for C. freundii; and from 10<sup>-3</sup> to 10<sup>-4</sup> transconjugants per recipient cell for K. pneumoniae. Resistance to sulfamethoxazole, ampicillin, cotrimoxazole, gentamicin, tobramycin, and streptomycin was transferred en bloc from all donor strains. Integrons were transferred in all but 2 experiments. In these 2 experiments, part of the transconjugants missed one of the multiple integrons present in the donor (transconjugants 11 and 13).

TEM genes were detected in 4 donor strains obtained from patients 13, 24, and 25. The TEM gene present in the E. coli from patient 13 was identified as a TEM-1 gene encoding broad-
Table 3. Transfer frequency of integrons and the resistance patterns of donor strains and transconjugants in a study of multidrug-resistant Enterobacteriaceae in a clinical setting.

<table>
<thead>
<tr>
<th>Patient, strain</th>
<th>Integron RFLP code</th>
<th>Transfer frequency</th>
<th>Smz</th>
<th>Cmz</th>
<th>Gm</th>
<th>Tm</th>
<th>Amp</th>
<th>Clv</th>
<th>Cfur</th>
<th>Ctri</th>
<th>Czid</th>
<th>Atm</th>
<th>Em</th>
<th>Chl</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>VI</td>
<td>3 × 10⁻³</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>NI</td>
<td>TEM</td>
</tr>
<tr>
<td>Transconjugant 1</td>
<td>VI</td>
<td>3 × 10⁻³</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>NI</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>III</td>
<td>4 × 10⁻³</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Transconjugant 2</td>
<td>III</td>
<td>4 × 10⁻³</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>III</td>
<td>1 × 10⁻³</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Transconjugant 3</td>
<td>III</td>
<td>1 × 10⁻³</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
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</table>

NOTE: The GenBank accession nos. for genes per restriction patterns obtained by restriction fragment–length polymorphism (RFLP) typing are as follows: I, X68227; II, U13880/U13880; III, U13880/M95287/AF336210; V, X12870; VI, X12868/AF32629; VII, X12868/X128672; and XI, X68227/AF326209. Isolates with identical genotypes are marked with the same superscript letter (d or f). TEM and SHV are the plasmid-determined spectrum β-lactamases. The TEM genes present in the other strains, however, have not been reported. Further investigation is needed to determine the character of the β-lactamases they encode. TEM genes were transferred in all of the conjugation experiments. SHV-ESBL genes were detected in 4 donor strains obtained from patients 25 and 26 and were transferred in nearly all the tests. Only transconjugants 4 and 6 missed the SHV-ESBL gene present in the donor strain. The non-transferred SHV genes detected in the *K. pneumoniae* isolates of patients 12 and 24 were probably chromosomally located SHV-1 genes [26].

### Discussion

The results of this study provide strong circumstantial evidence in support of the assumption that horizontal gene transfer contributed extensively to the high endemic level of MRE seen in the neurology and neurosurgery wards of our hospital. Isolates with an identical genotype were found to harbor different (combinations of) integrons at different collection times, indicating the acquisition or loss of integrons. Furthermore, 7 patients carried multiple species harboring identical integrons, very likely the result of the interspecies transfer of integrons within the patient. Of interest, the strains from 3 of the patients had been collected from the same clinical site (sputum or catheter urine), suggesting that the transfer of resistance genes may not take place just in the intestinal tract, as recently reported [11]. Furthermore, analysis of the distribution of the integrons revealed 6 clusters of patients carrying different strains with the same integron.
likely the result of clonal spread and horizontal transfer within the hospital. Patients harboring these integrons had overlapping dates of hospitalization in the same wards, and they often shared identical strains. In addition, neither integron has ever been isolated outside our hospital. With regard to the remaining 4 clusters, however, it cannot be totally excluded that some of the patients were carriers of the integrons prior to hospital admission. Because these latter integrons have been isolated previously from humans [4, 19, 23–25] and food-producing animals [18, 20–22], their presence in this study may reflect their prevalence in the community.

The results of the conjugation experiments are highly supportive of the hypothesis that horizontal transfer of resistance genes takes place in vivo. For example, resistance patterns that were very likely the result of horizontal transfer in vivo were shown to be transferred at very high frequency rates in vitro. Of interest, the complete resistance patterns (except for known chromosomally located resistance determinants), including those encoded by integrons and TEMs or SHVs, were transferred in their entirety in most of the conjugation experiments, suggesting a very efficient mechanism of transferring packages of resistance genes. The results from some experiments also show that part of the transconjugants missed one of the multiple integrons present in the donor. This implies that the various integrons of the donor strain were located on different mobile elements.

An unexpectedly high number (10) of different integrons were involved during this outbreak. Two pairs of integrons shared identical gene cassettes: integrons VIII and I shared aadA2, and integrons V and VII shared aadA1. It is possible, therefore, that integrons VIII and I or V and VII evolved from one another by the acquisition or deletion of genes. However, since all 4 integrons have been described before, these mutations may have occurred some time prior to our study. These findings support recent views that integrons are rather stable structures [27]. The predominant means of acquiring resistance, therefore, may be the acquisition of new integrons, often in association with other transferable resistance genes, rather than the acquisition of new resistance genes by resident integrons. This is supported by our observation that, over time, the integron content of K. pneumoniae from patient 26 changed from II + XI to II + III + XI, and the integron content of an E. coli strain from patient 18 changed from II to I + II.

Seven of 8 integrons harbored genes encoding resistance to either chloramphenicol or streptomycin and spectinomycin, antibiotics that were rarely, if ever, prescribed in our hospital in the last 2 decades. This finding supports the recent insight that withdrawal of a certain antimicrobial drug does not automatically lead to the disappearance of resistance to that agent [28, 29]. The persistence of these genes is most likely the result of their structural association with other resistance genes, such as the sulI gene, for which selective pressure has continued over the past years. Other factors, however, may also contribute to the persistence of these genes (e.g., the administration of these antimicrobial agents to food-producing animals) [20, 28].

The results of this study also illustrate the limitations of conventional genotyping in the management of an outbreak of MRE. The results of RAPD typing in 1996 and the subsequent PFGE typing performed in this study indicate that most isolates were unrelated. Analysis of the integron contents of the same strains, however, suggests the opposite in many cases. Furthermore, as part of the strategy to control the high level of MRE in 1996, rectal swab samples from the medical staff were cultured in search of environmental sources. The culture from a neurologist yielded a gentamicin-resistant E. coli. Not much attention was paid to that strain at the time, because it possessed a unique RAPD type not seen in any of the patients. Later analysis of the integron content of the strain, however, identified this person as a potential source for one cluster of integrons.

In conclusion, this study has shown that the horizontal transfer of antimicrobial resistance genes can occur very efficiently and at a high rate among Enterobacteriaceae in a nosocomial setting. This study has also demonstrated that integron typing can be a useful tool for studying the dissemination of resistance genes among gram-negative bacteria. Further studies are needed, however, to determine whether antibiotic policies or other measures can halt or lower the amount of horizontal transfer that occurs in a hospital.

References


