MAJOR ARTICLE

Multidrug Resistance among Enterobacteriaceae Is Strongly Associated with the Presence of Integrons and Is Independent of Species or Isolate Origin

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This study investigated the extent to which multidrug resistance (MDR) among Enterobacteriaceae is related to DNA elements called "integrons," whether the relationship is species dependent or origin dependent, and which resistance patterns are associated with integrons. Analysis of 867 nonrepeat isolates comprising 8 species and originating from the community and 23 European hospitals showed a significant relation between MDR and integrons, independent of species or origin. Although resistance to each tested antimicrobial agent was significantly associated with integrons, only resistance to sulfamethoxazole, cotrimoxazole, gentamicin, tobramycin, ampicillin, piperacillin, and cefuroxime predicted the presence of integrons. Combined resistance to both ampicillin and sulfamethoxazole-trimethoprim was the starting point for the development of resistance to additional β-lactams, aminoglycosides, cephalosporins, and ciprofloxacin, a development paralleled by an increasing prevalence of integrons. The acquisition of resistance genes is not random, and the transfer of integron-carrying elements plays a dominant role in the development of MDR by Enterobacteriaceae.

Nosocomial infections caused by multidrug-resistant Enterobacteriaceae are an increasing problem worldwide. Multidrug resistance (MDR) 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. Plasmids and transposons are involved in the transfer of resistance genes from one cell to another. In recent years, it has been shown that a substantial portion of the resistance genes present on the plasmids and transposons of gram-negative bacilli are integrated into DNA elements called "integrons" [1]. These integrons are potentially mobile elements (namely, transposons or defective transposon derivatives) that constitute a site-specific recombination system capable of integrating and expressing the genes in cassette structures.

Integrons comprise three essential components located within the 5′ conserved segment (CS): an integrase gene, *IntI1,* which encodes a site-specific recombinase; an adjacent *attI1* site, which is recognized by the integrase and acts as a receptor for gene cassettes; and a promoter region, P_c. There are four distinct classes of multiresistant integrons, each encoding a distinct integrase gene *(IntI1, IntI2, IntI3,* and *IntI9).* Class 1 integrons, located on plasmids and transposons, make up the majority of the integrons found in clinical isolates and are associated with the MDR seen in the hospital environment [2]. The known gene cassettes confer re-

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sistance to antimicrobial agents and to quaternary ammonium compounds (disinfectants and antiseptics). Frequently, class 1 integrons contain an additional resistance gene, *sulI,* in the 3′ CS, downstream from the gene cassettes. This gene confers resistance to sulfamethoxazole [1, 3].

Our goals were to determine to what extent MDR is integron related in Enterobacteriaceae, whether the presence of integrons is species dependent, and whether there are differences between integrons from bacteria isolated in hospitals and those isolated in the community. We also investigated which resistance patterns are associated with the presence of integrons.

MATERIALS AND METHODS

Bacterial Strains

We studied 867 Enterobacteriaceae isolates (8 species). The isolates were from 3 different sources (a Dutch university hospital, hospitals throughout Europe, and the community; table 1).

Group I (University Medical Centre Utrecht* [*UMCU*]*). In 1996, a sudden increase in the prevalence of gentamicin-resistant Enterobacteriaceae was observed in the neurology and neurosurgery wards of UMCU [4]. In total, 57 strains (28 *Escherichia coli,* 14 *Klebsiella pneumoniae,* 2 *Klebsiella oxytoca,* 6 *Citrobacter freundii,* 3 *Proteus mirabilis,* 3 *Enterobacter cloacae,* 1 *Enterobacter aerogenes*) with a unique genotype, determined by random-amplified polymorphic DNA analysis [4], were isolated and included in this study. An additional 90 clinical isolates of MDR Enterobacteriaceae (40 *E. coli,* 19 *K. pneumoniae,* 13 *K. oxytoca,* 9 *C. freundii,* 2 *P. mirabilis,* 5 *E. cloacae,* 2 *E. aerogenes*), isolated from 90 patients on other wards in the hospital during 1994–2000, were randomly selected from the hospital database. To include susceptible isolates in the study, we selected 122 blood culture isolates (48 *E. coli,* 16 *K. pneumoniae,* 15 *K. oxytoca,* 6 *C. freundii,* 10 *P. mirabilis,* 9 *E. cloacae,* 10 *E. aerogenes,* and 8 *Serratia marcescens*) from 122 patients hospitalized at UMCU during 1994–2000. These were either intermediate susceptible or resistant to 0, 1, or 2 antimicrobial agents.

Group II (Europe). A second group included 514 clinical isolates originating in 23 European hospitals (Athens, Greece; Ankara and Istanbul, Turkey; Tirana, Albania; Dusseldorf and Freiburg, Germany; Cracow and Warsaw, Poland; Coimbria, Portugal; Linz, Austria; Barcelona, Seville, and Madrid, Spain; Lille, Lyon, and Paris, France; Brussels, Belgium; Lausanne, Switzerland; Rome and Genoa, Italy; London and Birmingham, United Kingdom, and Utrecht, The Netherlands) and collected during 1997 and 1998. All of the strains resistant to ⩾1 antimicrobial agent were sorted in the database, first by species and then by the number of antimicrobial classes to which resistance was expressed.

Resistance to the following 8 antimicrobial classes (combinations) was noted: ampicillin or piperacillin; amoxicillin/clavulanate or piperacillin/tazobactam; gentamicin or tobramycin; amikacin; cefuroxime; ceftriaxone; ciprofloxacin; and meropenem. No susceptibility testing for sulfamethoxazole and cotrimoxazole was done. Isolates from each cluster expressing resistance to the same number of antimicrobial agents were selected in such a manner that the different origins and years of collection were evenly represented. The number of isolates selected from each cluster was dependent on the availability of the isolates but was not >30.

Group III (community). To obtain strains present in the community, rectal swabs were taken from patients on the day of their admission to the Neurodivision of UMCU, during 2000. Patients who had been admitted to any hospital or long-term care facility during the preceding 3 months were excluded. In total, we cultured 84 strains from 53 patients [3].

Identification and Antimicrobial Susceptibility Testing

Strains were identified by the VITEK 1 system with AMS R09.1 software (Biomerieux). The MIC values were determined, in our laboratory, by the broth-microdilution method described

Table 1. Distribution of species in relation to origin.

Microorganism	Origin of isolates			
	UMCU	Europe	Community	Total
Escherichia coli	116 (43)	148 (29)	72 (86)	336 (39)
Klebsiella pneumoniae	49 (18)	184 (36)	5 (6)	238 (27)
Proteus mirabilis	15 (6)	101 (20)	—	116 (13)
Klebsiella oxytoca	30 (11)	42 (8)	3 (4)	75 (9)
Enterobacter cloacae	17 (6)	15 (3)	3 (4)	35 (4)
Enterobacter aerogenes	13 (5)	13 (2)	—	26 (3)
Citrobacter freundii	21 (8)	11 (2)	1 (1)	33 (4)
Serratia marcescens	8 (3)	—	—	8 (1)
Total	269 (100)	514 (100)	84 (100)	867 (100)

NOTE. Data are no. (%). UMCU, University Medical Centre Utrecht.

Table 2. Association between resistance to antibiotics and presence of integron.

Antibiotic	No. tested	No. resistant (% integron positive)	No. sensitive (% integron positive)
Tobramycin	829	279 (82.1)	550 (39.6)
Gentamicin	867	370 (81.6)	497 (29.2)
Cotrimoxazole	867	444 (84.7)	423 (16.8)
Sulfamethoxazole	867	568 (78.7)	299 (0.0)
Ciprofloxacin	867	197 (75.6)	670 (44.5)
Amikacin	833	77 (75.3)	756 (51.3)
Cefuroxime	765[a]	300 (73.7)	465 (40.2)
Ceftriaxone	758[a]	225 (76.9)	533 (43.2)
Piperacillin/tazobactam	671[a]	225 (72.9)	446 (40.1)
Amoxicillin/clavulanate	758[a]	382 (68.3)	376 (38.8)
Piperacillin	452[b]	279 (64.9)	173 (17.9)
Ampicillin	452[b]	324 (61.7)	128 (9.4)

NOTE. Associations for all antibiotics were $P < .0001$ (χ^2 test).

[a] *Serratia, Enterobacter,* and *Citrobacter* species were excluded.

[b] *Serratia, Enterobacte, Citrobacter,* and *Klebsiella* species were excluded.

by the National Committee for Clinical Laboratory Standards (NCCLS) [5]. Susceptibility testing for sulfamethoxazole and cotrimoxazole was by the agar-diffusion method, according to NCCLS criteria. In the subsequent analyses, all intermediate-susceptible strains were grouped together with the populations of resistant strains.

Polymerase Chain Reaction (PCR) Amplification for Detection of Integrons

Class 1 integrons were detected by PCR amplification of a class 1 integrase-specific fragment of the *IntI1* gene (GenBank accession number M73819). The primer sequences were 5′-TCT CGG GTA ACA TCA AGG-3′ (*IntI1*-F) and 5′-AGG AGA TCC GAA GAC CTC-3′ (*IntI1*-R). The PCR amplifications were performed in a total volume of 25 μL containing 10 m*M* Tris-HCl (pH 9.0), 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.1% Triton X-100, 100 μM of each dNTP (Pharmacia Biotech), 50 pmol of each primer, 2.5 U of Super*Taq* polymerase (HT-Biotechnology), and 1 μL of DNA, which included the whole-cell bacterial lysate. Amplification in a Perkin-Elmer GeneAmp 9600 thermal cycler was as follows: 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 30 s at 72°C. The PCR products were separated by agarose gel electrophoresis through a 1.5% gel, were stained with ethidium bromide, and were visualized under UV light.

Statistical Analysis

All statistical analyses were performed by the Statistical Package for the Social Sciences software (version 10.0; SPSS). χ^2 Tests, Mantel-Haenszel procedures, and multivariate logistic regression models were used.

RESULTS

Resistance phenotypes associated with the presence of an integron. There was a significant association between the presence of an integron and the phenotypic resistance to each antimicrobial agent tested (table 2). For a clear assessment of the relation between resistance and integrons, resistance determinants not associated with integrons were excluded from the analysis. This was applicable to (1) the chromosomally located *AmpC* genes—in *Serratia, Enterobacter,* and *Citrobacter* species—conferring resistance to all β-lactams and (2) the chromosomally located LEN and potential chromosomally located SHV genes—in *Klebsiella* species—conferring resistance to ampicillin and piperacillin.

To determine which resistance phenotypes were independent of other resistance phenotypes associated with the presence of an integron, a logistic regression analysis was performed on all 867 isolates, with regard to antimicrobial agents other than β-lactams. This was the case for resistance to sulfamethoxazole ($P < .0001$), cotrimoxazole ($P < .0001$), and gentamicin and/or tobramycin ($P < .0001$). In a model that used these 4 antimicrobial agents, resistance to either amikacin or ciprofloxacin had no further predictive value with regard to the presence of an integron.

A second analysis was performed on all *E. coli* and *P. mirabilis* isolates ($n = 452$), to identify which β-lactam resistance had predictive value with regard to the presence of an integron. In a model that used sulfamethoxazole, cotrimoxazole, and either gentamicin and/or tobramycin, concurrent resistance to ampicillin and/or piperacillin ($P = .05$) and to cefuroxime ($P = .003$) had predictive value with regard to the presence of an integron. Resistance to amoxicillin/clavulanate and/or pi-

peracillin/tazobactam and to ceftriaxone did not have additional predictive value. Resistance to amoxicillin/clavulanate and/or piperacillin/tazobactam had predictive value ($P = .05$) only in a model that used *Klebsiella* species ($n = 313$) and in which the ampicillin and/or piperacillin variable was excluded from the analysis.

Association between MDR and integrons. MDR was defined as resistance to ⩾2 of the following (combinations of) antimicrobial agents: sulfamethoxazole and/or cotrimoxazole; gentamicin and/or tobramycin; amikacin, ciprofloxacin, ampicillin and/or piperacillin; amoxicillin/clavulanate and/or piperacillin/tazobactam; or cefuroxime and/or ceftriaxone. No resistance to meropenem was observed. Of the 619 isolates resistant to ⩾2 antimicrobial agents, 71% carried an integron, in contrast to just 3% of the isolates resistant to 0 or 1 antimicrobial agent (relative risk, 23.7; 95% confidence interval, 10.0–56.4). No integrons were present in the 108 completely susceptible strains.

Association between MDR and integrons in relation to origin and species. When corrected for isolate origin or species, identical results were obtained by the Mantel-Haenszel procedure ($P < .0001$). This procedure uses stratified analysis of contingency tables, leading to the estimation of the common odds ratio. Homogeneity tests were used to assess the assumption that the odds ratios in all strata were indeed equal. The association between MDR and integrons, therefore, was independent of the origin (community, UMCU, or Europe) of the isolates and of the species *(E. coli, K. pneumoniae, K. oxytoca, C. freundii, P. mirabilis, E. cloacae, E. aerogenes,* and *S. marcescens)* investigated.

Observed resistance patterns and their association with integrons. Multivariate analysis revealed that only resistance to sulfamethoxazole, cotrimoxazole, gentamicin and/or tobramycin and, to a lesser extent, ampicillin and/or piperacillin and cefuroxime had predictive value with regard to the presence of an integron. Univariate analysis showed a highly significant association between resistance to all antimicrobial agents tested and the presence of integrons. To explain this apparent contradistinction, we next determined how and to what extent the resistances to the group of antimicrobials without predictive value with regard to the presence of integrons were linked to the resistances to the group of antimicrobials with this predictive value.

To set up a test panel representative of resistant Enterobacteriaceae in European hospitals, we excluded all UMCU-outbreak strains, all UMCU MDR group I strains, and all completely susceptible isolates. The resultant panel comprised 192 *E. coli,* 95 *P. mirabilis,* 41 *K. oxytoca,* and 183 *K. pneumoniae* isolates. The most prevalent resistance patterns encountered by these isolates were placed in flow diagrams representing 80% of the *E. coli* and *P. mirabilis* isolates and 92% of the *Klebsiella* isolates. For clarity, the remaining isolates (20% and 8%, respectively) were not included, because they had resistance patterns represented by just 1 or a few strains. Since no differences were found between the resistance patterns expressed by the *E. coli* and *P. mirabilis* isolates, they were considered together (figure 1). The same approach also was applied to the *K. oxytoca* and *K. pneumoniae* isolates (figure 2). A number of points can be deduced from the 2 figures.

First, the acquisition of resistance determinants is not a random process. We observed a surprisingly low number of combinations of resistance phenotypes, and the great majority of isolates (55% of the *E. coli* and *P. mirabilis* isolates and 76% of the *Klebsiella* isolates) expressing resistance to ⩾1 antimicrobial agent followed the same main pathway (the trunk), starting with the combined resistance to sulfamethoxazole-(trimethoprim) and ampicillin and/or piperacillin. Second, 80% of the *E. coli* and *P. mirabilis* isolates and 92% of the *Klebsiella* isolates are represented in the flow diagrams, indicating that the acquisition of resistance determinants by *E. coli* and *P. mirabilis* is slightly more scattered than that by *Klebsiella* species. Third, the combined resistance to sulfamethoxazole-(trimethoprim) and ampicillin and/or piperacillin is the starting point for further resistance development and is associated with an integron prevalence of ⩾70%. Fourth, except for the acquisition of ciprofloxacin resistance, which occurs earlier in the development of multiresistance by *E. coli* and, especially, by *P. mirabilis* (data not shown), the sequence of antimicrobial agents for which resistance was expressed is identical for *E. coli, P. mirabilis,* and *Klebsiella* species. Fifth, the increasing number of antimicrobial agents against which resistance is expressed parallels an increasing prevalence of integrons. Class 1 integrons, however, are not a prerequisite for the development of MDR. Finally, resistance to ciprofloxacin, amikacin, gentamicin, or tobramycin rarely occurs alone. In the total test panel of 867 isolates, ciprofloxacin resistance occurred only in combination with other resistance phenotypes (e.g., with 51% ceftriaxone resistance in *E. coli, P. mirabilis,* and *Klebsiella* species). Also, resistance to amikacin never occurred alone; in fact, 93% of the amikacin-resistant isolates expressed resistance to ⩾3 other antimicrobial classes. Resistance to just gentamicin occurred only once (in a community strain), and 97% of the gentamicin-resistant isolates expressed resistance to ⩾3 other antimicrobial classes.

DISCUSSION

Our results show that class 1 integrons are widespread among Enterobacteriaceae, in both clinical isolates and the community. Moreover, their prevalence increases with the number of antibiotic classes for which resistance is expressed, confirming the results of a study by Martinez et al. [2]. In contrast to the

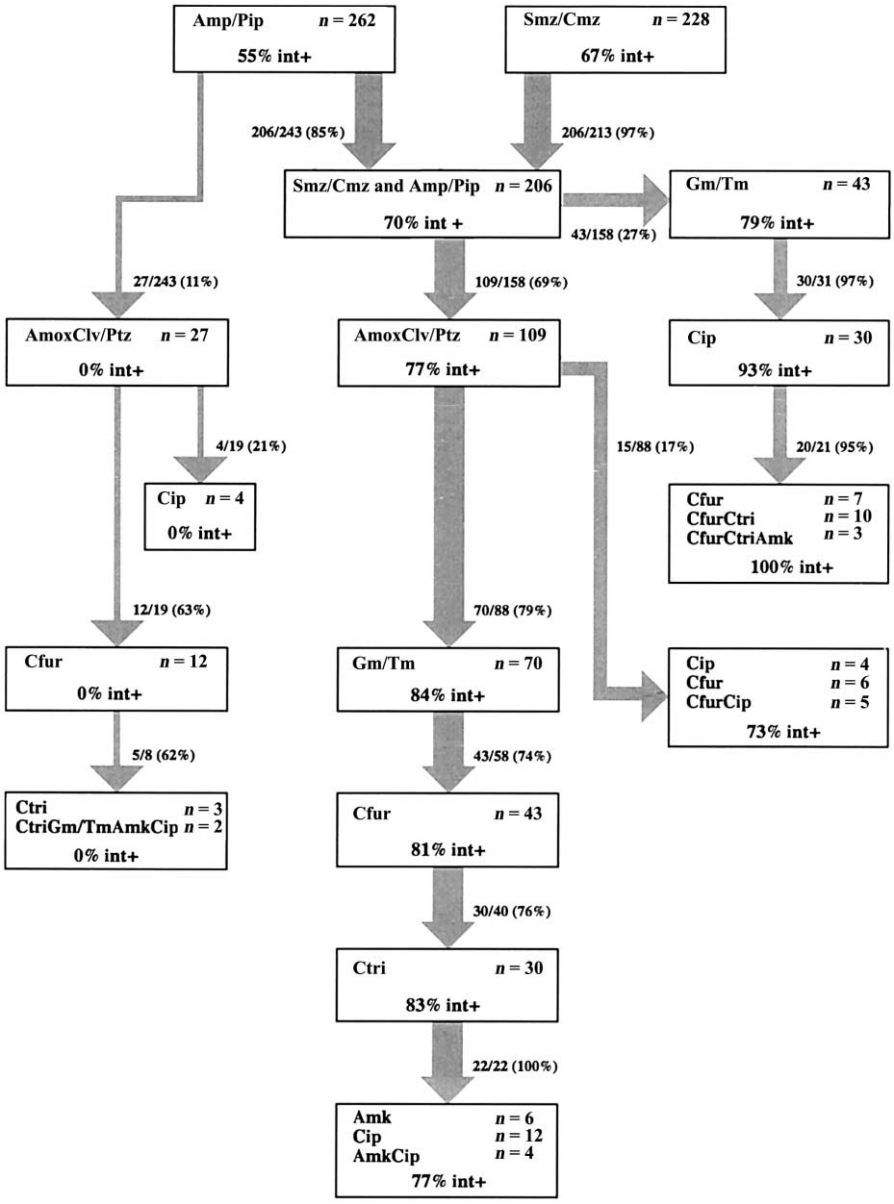

Figure 1. Resistance patterns observed in *Escherichia coli* and *Proteus mirabilis*. Each box includes the number of isolates expressing resistance to at least the antimicrobial agents included in that box and to all antimicrobial agents included in preceding, or "upstream," boxes. The prevalence of integrons in these strains is also shown. Fractions next to arrows connecting the boxes are number of isolates after the arrow (numerator) (in addition to the antimicrobial agents included in upstream boxes; these isolates are also resistant to at least the antimicrobial agent in the subsequent, or "downstream," box)/total number of isolates that leave the box (denominator) (no. of strains that are resistant to more antimicrobial agents than are included in upstream boxes); this fraction therefore is the proportion of isolates downstream of the arrow. For example, 206 *E. coli* and *P. mirabilis* isolates were resistant to Smz/Cmz and to Amp/Pip. Of these 206 isolates, 158 were resistant to ⩾1 additional antimicrobial agent (of these 158 isolates, 109 [69%] were also resistant to AmoxClv/Ptz, 43 [27%] were also resistant to Gm/Tm but not resistant to AmoxClv/Ptz, and 6 [4%] had a unique, additional resistance phenotype not shown in the figure). Amk, amikacin; AmoxClv, amoxicillin-clavulanate; Amp, ampicillin; Cfur, cefuroxime; Cip, ciprofloxacin; Cmz, cotrimoxazole; Ctri, ceftriaxone; Gm, gentamicin; int+, integron positive; Pip, piperacillin; Ptz, piperacillin/tazobactam; Smz, sulfamethoxazole; Tm, tobramycin; / (as connector of antimicrobial agents), and/or.

Martinez et al. study, however, we found that 100% of the integron-carrying strains express resistance to ⩾1 antibiotic. This can be explained by the inclusion of sulfamethoxazole and cotrimoxazole in our susceptibility panel, and, as has recently been reported, the presence of an integron is in 100% of the isolates associated with the expression of sulfamethoxazole resistance [3].

We also found a significant ($P < .0001$) relation between mul-

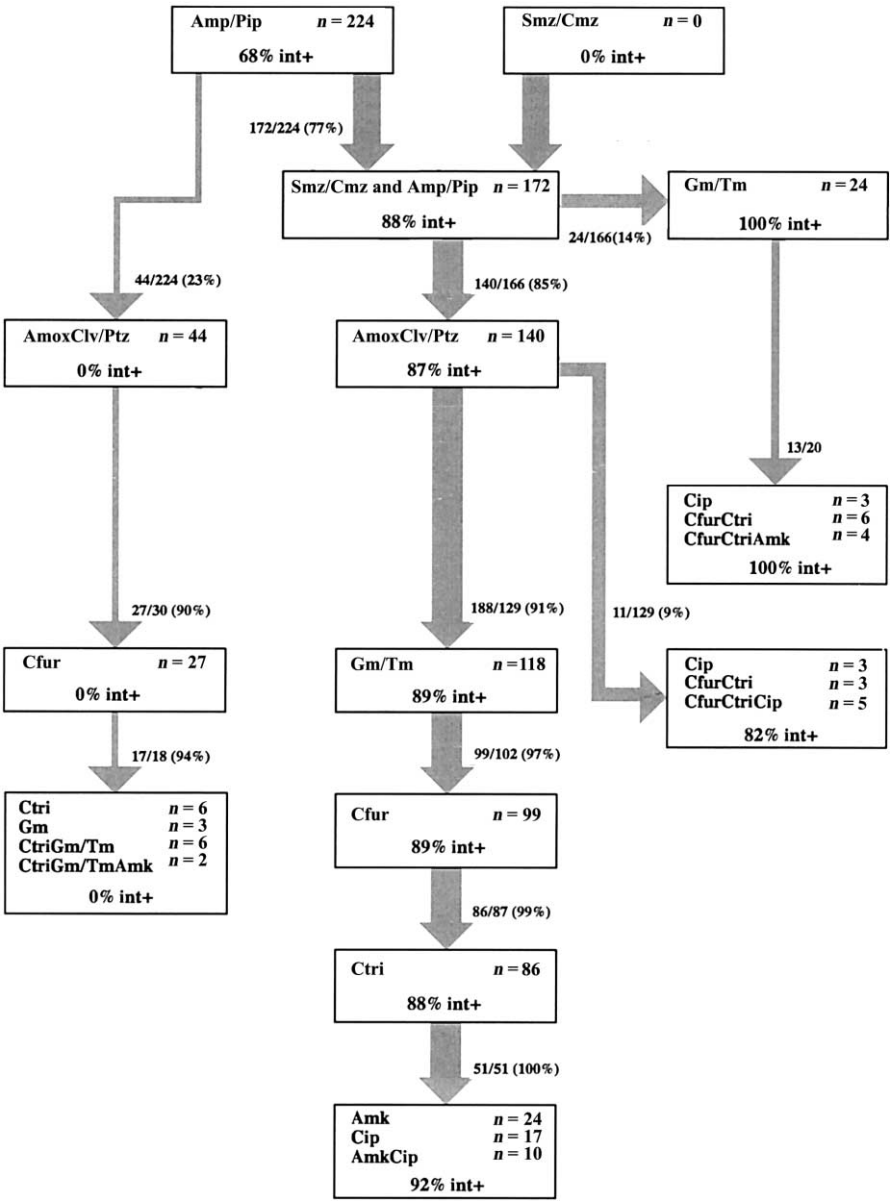

Figure 2. Resistance patterns observed in *Klebsiella* species. Each box includes the number of isolates expressing resistance to at least the antimicrobial agents included in that box and to all antimicrobial agents included in preceding, or "upstream," boxes. The prevalence of integrons in these strains is also shown. Fractions next to arrows connecting the boxes are number of isolates after the arrow (numerator) (in addition to the antimicrobial agents included in upstream boxes; these isolates are also resistant to at least the antimicrobial agent in the subsequent, or "downstream," box)/total number of isolates that leave the box (denominator) (no. of strains that are resistant to more antimicrobial agents than are included in upstream boxes); this fraction therefore is the proportion of isolates downstream of the arrow. For example, 172 *Klebsiella* isolates were resistant to Smz/Cmz and to Amp/Pip. Of these 172 isolates, 166 were resistant to ⩾1 additional antimicrobial agent (of these 166 isolates, 140 [85%] were also resistant to AmoxClv/Ptz, 24 [14%] were also resistant to Gm/Tm but not resistant to AmoxClv/Ptz, and 2 [1%] had a unique, additional resistance phenotype not shown in the figure). Amk, amikacin; AmoxClv, amoxicillin-clavulanate; Amp, ampicillin; Cfur, cefuroxime; Cip, ciprofloxacin; Cmz, cotrimoxazole; Ctri, ceftriaxone; Gm, gentamicin; int+, integron positive; Pip, piperacillin; Ptz, piperacillin/tazobactam; Smz, sulfamethoxazole; Tm, tobramycin; / (as connector of antimicrobial agents), and/or.

tiresistance and integrons, whereas Martinez et al. [2] described only a tendency toward multiresistance. This difference can be explained by the inclusion of sulfamethoxazole and cotrimoxazole in the test panel, the inclusion of many more isolates (867 vs. 170) in our study than in that by Martinez et al., and the use of different methods for integron detection. We used an integrase PCR whereas Martinez et al. used a CS-PCR.

The results of the multivariate analysis in the present study reveal that the spectrum of resistance phenotypes predictive of the presence of an integron is limited. Only resistances to sul-

famethoxazole, cotrimoxazole, gentamicin, and/or tobramycin were found to be independent of other resistances highly significantly associated with the presence of an integron. The association between sulfamethoxazole and integrons can be explained by the presence of the *sulI* gene in >90% of the class 1 integrons [3]. The acquisition of resistance to trimethoprim, gentamicin, and/or tobramycin is likely to be, in part, the result of the integration of these gene cassettes into the integron. Previous studies characterizing integrons in clinical isolates showed that inserted gene cassettes predominantly confer resistance to trimethoprim and aminoglycosides, in addition to resistance to spectinomycin, chloramphenicol, and erythromycin. Of note, the most prevalent gene cassettes confer resistance to the older aminoglycosides, such as streptomycin and kanamycin [6–9].

The multivariate analysis also revealed an independent association between integrons and ampicillin and/or piperacillin and cefuroxime. This association, although less significant than the association between integrons and the previously mentioned antibiotics, was unexpected.

The β-lactam resistance seen in *E. coli* and *P. mirabilis* isolates is predominantly the result of plasmid-determined TEM and SHV β-lactamases and, with increasing frequency, *AmpC* β-lactamases [10,11], all of which are β-lactamases for which the encoding genes have not been detected in integrons. Several studies, however, have shown the presence of both integrons and TEM or SHV on the same plasmid [12–15]. Results of conjugation studies investigating the transfer of integrons have also shown that integrons and these β-lactamase–encoding genes are located either on the same plasmid or on different plasmids that are being cotransferred [9]. This common location may explain the predictive value of ampicillin resistance with regard to the presence of an integron.

Resistance to penicillin-inhibitor combinations and ceftriaxone did not have further predictive value with regard to the presence of an integron. This is to be expected, because such resistance is either the result of mutations in the genes that encode ampicillin resistance or the result of an increased level of expression of these same genes. Similarly, resistance to ciprofloxacin did not have further predictive value with regard to the presence of an integron. This is in line with the general belief that plasmid-mediated resistance to ciprofloxacin is exceptional, because it has been reported just once [16, 17]. It is intriguing, therefore, that resistance to ciprofloxacin was never observed without resistance to ⩾1 plasmid-encoded antimicrobial agent. In fact, in 90% of the cases, ciprofloxacin resistance was associated with resistance to ⩾3 different antibiotic classes. For example, 51% of the ciprofloxacin-resistant *E. coli, P. mirabilis,* and *Klebsiella* isolates were also resistant to ceftriaxone.

These results are in accordance with a recent study that found evidence of extended-spectrum β-lactamase (ESBL) production by 60% of *K. pneumoniae* blood-culture isolates resistant to ciprofloxacin [18]. This observed linkage may, to some extent, result from the practice of prescribing ciprofloxacin only for infections caused by bacteria resistant to all other available antibiotics. However, since ciprofloxacin is also a generally accepted empiric drug of choice for urinary-tract infections and gastrointestinal infections and is widely used prophylactically for selective digestive decontamination in hematology wards, this is unlikely to be the complete explanation. The strong linkage may also be the result of selective antimicrobial pressure in the hospital; both ciprofloxacin-resistant and R-plasmid–carrying strains persist in the hospital environment, resulting in the acquisition of R-plasmids by the ciprofloxacin-resistant strains, through horizontal transfer.

Alternatively, the presence of plasmids facilitates the development of ciprofloxacin resistance by chromosomal mutation, as has been described, by Ashraf et al. [19], for nalidixic acid. The one R-plasmid encoding ciprofloxacin resistance also facilitates the selection of high-level (chromosomally encoded) quinolone resistance [16]. The biochemical mechanism by which the plasmid increases the chromosomal mutation frequency to (higher) quinolone resistance is unknown. Our observation of ciprofloxacin resistance only in association with plasmid-mediated resistance favors the latter explanation. These alternative explanations, however, are not mutually exclusive.

The flow diagrams in which nearly 90% of the randomly selected *E. coli* and *Klebsiella* and *P. mirabilis* isolates are included show that the acquisition of MDR is not a random process. The combined resistance to ampicillin and/or piperacillin and sulfamethoxazole(-trimethoprim) has been shown to be the starting point for the progressive development of resistance to other β-lactams, aminoglycosides, cephalosporins, and ciprofloxacin. This development is associated with an increasing prevalence of integrons and indicates that the integron-carrying strains from the total pool of isolates resistant to ampicillin and/or pipericillin and sulfamethoxazole(-trimethoprim) are more likely to acquire additional resistance genes than are the strains with the same resistance pattern but without an integron. This suggests that integron-carrying elements facilitate the acquisition of additional resistance genes, which may be the result of factors, present on these elements, that either facilitate the insertion of resistance genes in the genetic elements (e.g., integrases, recombinases, and IS elements [19]–[21]) or increase the strain survival in the hospital environment. For example, encoded on these plasmids may be other virulence factors that provide the host cell with additional selective advantages [22]–25]. The resultant persistence of these strains under the antimicrobial selective pressure in the hospital environment will then increase both the chance of an advanta-

geous mutation (e.g., from β-lactamase to ESBL) and the chance to acquire additional resistance genes.

The sequence of the expressed resistance in the flow diagrams downstream of the combination of sulfamethoxazole-(trimethoprim) and ampicillin probably reflects the relative use of various antimicrobial agents over the past decades in European hospitals. For example, we found that ciprofloxacin resistance was more prevalent in the *E. coli/P. mirabilis* diagram than in the *Klebsiella* diagram. This is mainly due to the relatively high level of ciprofloxacin resistance found in *P. mirabilis. P. mirabilis* is predominantly found in urinary-tract infections, for which ciprofloxacin is often prescribed. Theoretically, the sequence may also be influenced by the availability of the resistance genes in the environment and by the ease with which these genes are incorporated into the plasmids. To our knowledge, no data are yet available on these matters.

The observation that resistance to ciprofloxacin, amikacin, gentamicin, or tobramycin either did not or rarely occurred alone but was found in association with resistance to ⩾3 other antimicrobial classes in >90% of the resistant isolates indicates that the acquisition of new resistance genes does not imply the loss of others. Furthermore, this observation indicates that the burden of resistance genes does not necessarily lead to a clinically relevant cost of fitness for the host bacterium, as is shown by the high number of strains with resistance to >6 of the antimicrobial classes that were available for this study.

The strong link between the resistances to different antimicrobial classes has a great impact on the rationale for using antimicrobial policies to reduce MDR in the hospital environment and even in the community. The restriction of a single antibiotic class may have a limited effect if the use of other antibiotics for which resistance is encoded is continued or switched to. The idea of cycling as a strategy to reduce resistance is therefore disputable and may even increase MDR, unless the only antimicrobial classes used are those for which no resistance is expressed in the study population. We recently have shown the success of the latter policy in combination with strict hospital control measures [4]. Furthermore, the pivotal role of integron-associated sulfonamide resistance in the increasing development of MDR strongly suggests that policies aimed at the reduction of sulfonamide use in the hospital—and, probably of more importance, in food animals—should be seriously considered. For example, in 1998 80 tons of cotrimoxazole were sold for use in food-producing animals in the United Kingdom [26].

In summary, our study results show a very strong association between MDR and the presence of integrons in Enterobacteriaceae, independent of species or strain origin. The acquisition of MDR is not a random process and the resistance patterns present in *E. coli, P. mirabilis,* and *Klebsiella* species are nearly identical, suggesting that these different species have the same mechanisms for acquisition of (multi)resistance. Considering these results in combination with our earlier finding of the extremely efficient interspecies transfer of integron-carrying elements, we conclude that horizontal transfer of integron-carrying elements plays a dominant role in the development of multiresistance by Enterobacteriaceae, independent of species or origin.

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