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Changes in *aac(6′)-Ib-cr* Prevalence and Fluoroquinolone Resistance in Nosocomial Isolates of *Escherichia coli* Collected from 1991 through 2005▽

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Clinical isolates of *Escherichia coli* collected from 1991 through 2005 at a tertiary-care center were studied for *qnr* and *aac(6′)-Ib-cr* genes. Isolates bearing *aac(6′)-Ib-cr* emerged in 1998, coinciding with an increase in ciprofloxacin resistance. The presence of *aac(6′)-Ib-cr* was multiclonal and was associated with the presence of extended-spectrum β-lactamases.

Plasmid-mediated *qnr* and *aac(6′)-Ib-cr* genes confer reduced quinolone susceptibility, facilitating the selection of chromosomal mutations that confer high-level resistance (7, 8, 15). Qnr and Aac(6′)-Ib-cr have therefore been hypothesized as potential contributors to the increase in prevalence of quinolone resistance among gram-negative bacteria. Epidemiological surveys have found *qnr* genes in various *Enterobacteriaceae* (14). Findings from a recent study suggest that *qnrA* and *qnrB* may have contributed to the emergence of fluoroquinolone resistance among *Klebsiella pneumoniae* and *Enterobacter* spp. (17). This survey also observed an association between ceftazidime (CAZ) resistance and the presence of *qnr* genes, suggesting that in some species, the frequent clinical association of cephalosporin resistance with fluoroquinolone resistance might be due to the genetic linkage of these two elements on plasmids.

The *cr* variant of *aac(6′)-Ib* encodes an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin by the N-acetylation of its piperazinyl amine (15). Aac(6′)-Ib-cr has two amino acid changes, Trp102Arg and Asp179Tyr, which together are necessary and sufficient for the enzyme's ability to acetylate ciprofloxacin. Several prior surveys of *aac(6′)-Ib-cr* prevalence (5, 10, 12) were limited to cephalosporin- and/or ciprofloxacin-resistant isolates, which were collected over relatively short time frames. Hence, little is known about the epidemiological patterns of *aac(6′)-Ib-cr* in population-representative clinical isolates over time. Interestingly, in two of these earlier studies *aac(6′)-Ib-cr* was found predominantly in *Escherichia coli*. We therefore surveyed bloodstream isolates of *E. coli* collected over a 15-year period for *aac(6′)-Ib-cr* and *qnr* genes in order to more broadly characterize the changes over time in the prevalence of these resistance elements in a large collection of clinical isolates. Additionally, we examined the isolates for evidence of an association between the genes of interest and CAZ resistance.

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Bacterial strains. Since 1990 all patient-unique bloodstream isolates at Hadassah Ein-Kerem Hospital in Jerusalem, Israel, have been preserved at −70°C. For this study, we screened all available nosocomial *E. coli* isolates (i.e., isolated from cultures taken >3 days after admission) from 1991 through 2005. Certain isolates were unavailable due to nonsystematic clerical errors.

Susceptibility testing. Disk susceptibility testing and clavulanic acid enhancement of cephalosporin susceptibility for the detection of extended-spectrum ß-lactamases (ESBLs) followed the recommendations of the CLSI by using unsupplemented Mueller-Hinton agar and incubation at 37°C for 16 to 20 h (3).

Screening for plasmid-mediated quinolone resistance. Screening for *qnr* was carried out by multiplex PCR amplification of *qnrA*, *qnrB*, and *qnrS* as previously described (16, 17).

Because Aac(6′)-Ib-cr confers resistance to kanamycin, albeit at a level lower than that of Aac(6′)-Ib (15), screening for *aac(6′)-Ib-cr* was carried out in two steps; strains were screened for kanamycin susceptibility (Sigma Chemical Co., Rehovot, Israel) at a concentration of 32 μg/ml, followed by direct screening by gap-ligase chain reaction (LCR) for G535T, one of the two defining mutations of *aac(6′)-Ib-cr* (15). Gap-LCR is a powerful method for detecting single base changes (1). For this technique, two same-directional primers, separated by a gap of several nucleotides, are chosen. These hybridize to complementary strands of target DNA and will be extended by DNA polymerase and subsequently ligated into a single long oligonucleotide when the mutation of interest, which corresponds to the 3′ end of the first primer, is present. This oligonucleotide can then be amplified. We used as forward primers aac-glcr-F1 (5′-AGGTACCGTAACCACCC CAT) and aac-glcr-F2-P (5′/5Phos/GTCCAGCCGTGTACAT GG), matching positions 516 to 535 and 539 to 556, respectively, with respect to the Aac(6′)-Ib-cr translational starting point. Our reverse primers were aac-glcr-R3 (5′-CCA TGTACACGGCTGGACC) and aac-glcr-R4-P (5′/5Phos/TGG

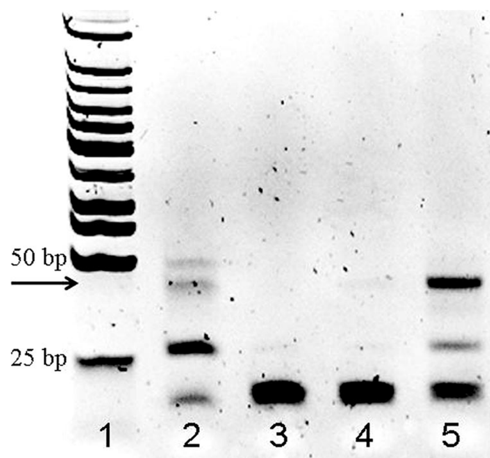

FIG. 1. Polyacrylamide gel analysis of gap-LCR assay. Lane 1, low-molecular-weight DNA ladder (New England Biolabs, Beverly, MA); lane 2, pBAD24-*aac(6′)-Ib-cr*; lane 3, pBAD24-*aac(6′)-Ib*; lane 4, *aac(6′)-Ib-cr*-negative *E. coli*; lane 5, *aac(6′)-Ib-cr*-positive *E. coli*. Arrow points to the putative reaction product.

GGTGGTTACGGTACCT) and were designed to amplify a 41-bp fragment corresponding to the oligonucleotide formed by the ligation of the forward primers. Bacterial DNA was prepared with a DNA isolation kit (Genekam Biotechnology AG, Duisburg, Germany). The four primers, at a concentration of 0.033 μM each, were added to the template DNA. Gap-LCR amplification was performed with AmpliTaq DNA polymerase Stoffel fragment (Applied Biosystems, Foster City, CA) at 0.033 U/μl, *Taq* DNA ligase (New England Biolabs, Beverly, MA) at 0.4 U/μl, and dATP, dTTP, and dGTP, each at 200 μM, in a final volume of 30 μl containing 1× *Taq* DNA ligase reaction buffer. The template concentration can play a major role in the specificity of the gap-LCR amplification procedures. By optimizing the assay conditions, we were able to use unquantified extracts of whole-cell DNA and maintain specificity. The gap-LCR conditions were 94°C for 30 s, 50°C for 30 s, 72°C for 5 s, and 60°C for 4 min, with a cycle number of 20. The amplification products were provisionally identified from their sizes in ethidium bromide-stained 16% polyacrylamide gels (Fig. 1). All strains that tested positive by gap-LCR were sequenced to confirm the presence of the G535T mutation and the T304C mutation. The gap-LCR assay was validated by assaying 10 strains known by sequencing to carry the *aac(6′)-Ib-cr* gene, as well as 8 negative isolates, among which were isolates carrying the wild-type *aac(6′)-Ib* gene. Gap-LCR gave correct results for all 18 isolates.

PFGE. Pulsed-field gel electrophoresis (PFGE) after restriction with XbaI was performed according to a standardized protocol (13) on all *aac(6′)-Ib-cr*$^+$ isolates. The band patterns were analyzed according to accepted criteria (18).

Detection of ESBL genes by PCR. Amplification of the major ESBL resistance determinants was performed for all strains exhibiting an ESBL phenotype using previously validated primers for the detection of bla_{TEM} (9), bla_{SHV} (6), and two sets of primers for $bla_{\mathrm{CTX\text{-}M}}$ genes (11).

Statistical methods. Fisher's exact test (http://www.langsrud.com/fisher.htm) was used to compare the prevalence of ciprofloxacin resistance (defined as a MIC of ≥2 μg/ml) (3), *aac(6′)-Ib-cr*, and *qnr* before and after 1 January 1998. The confidence intervals for the risk ratios were calculated at http://www.cebm.utoronto.ca/practise/ca/statscal/.

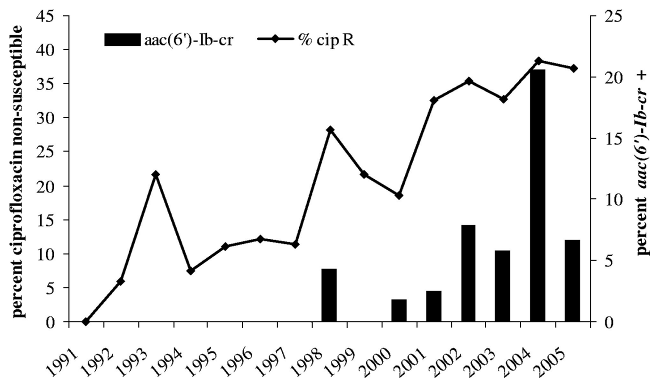

FIG. 2. Percentage of clinical *E. coli* isolates (n = 718) harboring resistance to ciprofloxacin (MIC ≥ 2 μg/ml) and harboring *aac(6′)-Ib-cr*. No *aac(6′)-Ib-cr* genes were found before 1998.

Results. From 1991 through 2005, there were 904 episodes of nosocomial *E. coli* bloodstream infection and 718 isolates were available for analysis. From 1991 through 1997, 27 of 265 *E. coli* (11.3%) isolates were ciprofloxacin intermediate or resistant, compared with 144 of 453 (46.6%) from 1998 through 2005 ($P < 0.001$) (Fig. 2). From 1991 through 1997, no isolate had *aac(6′)-Ib-cr*, whereas from 1998 onwards, 32 of 453 (7.1%) of the isolates had *aac(6′)-Ib-cr* ($P < 0.001$) (Fig. 2). Among the *aac(6′)-Ib-cr*$^+$ isolates, 30 of 32 (93.8%) were not susceptible to ciprofloxacin. PFGE of all the *aac(6′)-Ib-cr*$^+$ strains demonstrated a cluster of five indistinguishable isolates and an identical pair with a different pattern, while the remaining 25 isolates were unrelated (Fig. 3). A single isolate from

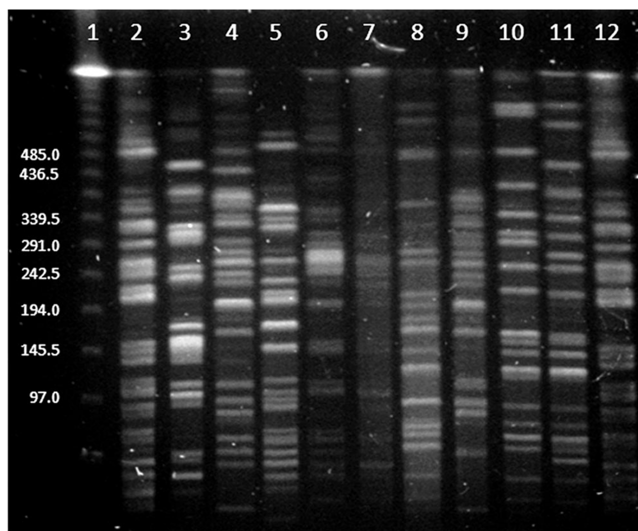

FIG. 3. PFGE of 10 *E. coli* isolates from 2004 and *E. coli* KL-16 (serving as the control strain) after restriction with XbaI. Lane 1, phage lambda ladder (size marks in Kb); lanes 2 and 12, *E. coli* KL-16; lanes 3 to 11, *E. coli* isolates from 2004 (in lane 4, one of the five PFGE-identical isolates).

TABLE 1. Distribution of ciprofloxacin and ceftazidime susceptibilities among *aac(6')-Ib-cr*$^{+}$ and *aac(6')-Ib-cr* *E. coli* isolates[a]

Ciprofloxacin susceptibility	No. of isolates with indicated CAZ susceptibility				Risk ratio	95% CI
	aac(6')-Ib-cr positive		*aac(6')-Ib-cr* deficient			
	CAZ-R	CAZ-S	CAZ-R	CAZ-S		
S + I/R	18	14	67	619	5.8	3.9–8.4
S	1	2	28	517	6.5	1.2–33.4
I/R	17	12	39	102	2.1	1.4–3.2

[a] S, susceptible; R, resistant; I, intermediate; CI, confidence interval.

2002 harbored *qnrA*. The genes *qnrB* and *qnrS* were not found. Thus, *aac(6')-Ib-cr* was the predominant plasmid-mediated quinolone resistance gene in *E. coli*, and its polyclonal emergence coincided with the rise in ciprofloxacin resistance. Unlike *qnr* genes in other *Enterobacteriaceae* (17), the presence of the *aac(6')-Ib-cr* gene was strongly associated with ciprofloxacin resistance defined by CLSI criteria.

The presence of *aac(6')-Ib-cr* was also associated with CAZ resistance (risk ratio, 5.8 [95% confidence interval, 3.9 to 8.4]). To adjust for the potential confounding effect of an association between fluoroquinolone resistance and CAZ resistance independent of the *aac(6')-Ib-cr* gene, isolates were stratified by quinolone susceptibility (Table 1). The association between CAZ resistance and *aac(6')-Ib-cr* was present in both fluoroquinolone-susceptible and -resistant *E. coli*.

A total of 25 of 32 (78%) *aac(6')-Ib-cr*$^{+}$ *E. coli* manifested an ESBL phenotype. Of these, one isolate harbored both bla_{SHV-12} and $bla_{CTX-M-15}$ genes. Two additional isolates contained bla_{SHV-12}. Two groups of bla_{CTX-M} genes were identified in 22 strains. One group consisted of two strains identified as CTX-M-25; sequence analysis of five nonrelated isolates (by PFGE) that belonged to the second group revealed that all were CTX-M-15. Seventeen isolates also harbored bla_{TEM} that was further identified in two strains as TEM-1. Thus, although *aac(6')-Ib-cr* appeared in different clones it was associated with a limited number of ESBL-encoding genes.

Discussion. The prevalence of quinolone resistance among nosocomial *Enterobacteriaceae* increased, at a different rate for each genus, following the introduction of ciprofloxacin into the Hadassah hospitals at the end of 1989. We recently showed that this increase coincided with the entry of the *qnr* genes into the *K. pneumoniae* and *Enterobacter* spp. populations (17). Using gap-LCR, an inexpensive technique better suited to large-scale epidemiologic surveys than previous surveillance methods (2, 12), we demonstrated a similar pattern of penetration of *aac(6')-Ib-cr* into multiple clinical *E. coli* clones, coincident with a rise in fluoroquinolone resistance from 11% in 1997 to 37% in 2005. A caveat regarding any observational study of this kind is that correlation does not prove causation. Also, further studies are needed to determine whether these observations hold up globally.

Of note, we also demonstrated an epidemiologic link between *aac(6')-Ib-cr* and CAZ resistance and found that a majority of *aac(6')-Ib-cr*$^{+}$ isolates (22 out of 32 [68.8%]) harbored a CTX-M ESBL. To ascertain that these genes in our collection are located on the same plasmid was beyond the scope of this work. However, our findings support earlier suggestions of a linkage between *aac(6')-Ib-cr* and CTX-M ESBLs (4, 12) and raise the possibility that the use of ciprofloxacin—a widely prescribed fluoroquinolone in the world—is a driver of both fluoroquinolone resistance and the emergence of CTX-M ESBLs.

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