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Prevalence in the United States of *aac(6′)-Ib-cr* Encoding a Ciprofloxacin-Modifying Enzyme▽

Chi Hye Park,[1] Ari Robicsek,[2] George A. Jacoby,[3] Daniel Sahm,[4] and David C. Hooper[1]*

Massachusetts General Hospital, Boston, Massachusetts[1]; Evanston Northwestern Healthcare, Evanston, Illinois[2]; Lahey Clinic, Burlington, Massachusetts[3]; and Focus BioInova, Herndon, Virginia[4]

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Among 313 *Enterobacteriaceae* from the United States with a ciprofloxacin MIC of ≥0.25 μg/ml and reduced susceptibility to ceftazidime, *aac(6′)-Ib* was present in 50.5% of isolates, and of these, 28% carried the *cr* variant responsible for low-level ciprofloxacin resistance. *aac(6′)-Ib-cr* was geographically widespread, stable over time, most common in *Escherichia coli*, equally prevalent in ciprofloxacin-susceptible and -resistant strains, and not associated with *qnr* genes.

Quinolone resistance is traditionally mediated by chromosomal mutations in bacterial topoisomerase genes, genes regulating expression of efflux pumps, or both (4, 5). In addition, *qnr* genes responsible for plasmid-borne quinolone resistance have been found in clinical isolates of *Enterobacteriaceae* (7). These genes encode pentapeptide repeat proteins that block the action of ciprofloxacin on bacterial DNA gyrase and topoisomerase IV (11). Recently, a new mechanism of transferable quinolone resistance was reported: enzymatic inactivation of certain quinolones. The *cr* variant of *aac(6′)-Ib* encodes an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin by *N*-acetylation of its piperazinyl amine (9). 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. When both *qnrA* and *aac(6′)-Ib-cr* are present in the same cell, the level of resistance is increased fourfold more than that conferred by *qnrA* alone, with an MIC of ciprofloxacin of 1.0 μg/ml, a value near the clinical breakpoint for susceptibility. In addition, the presence of *aac(6′)-Ib-cr* alone increased substantially the frequency of selection of chromosomal mutants upon exposure to ciprofloxacin (9).

The three known *qnr* genes, *qnrA*, *qnrB* (6), and *qnrS* (3), and their variants have widely penetrated clinical isolates of *Enterobacteriaceae* from the United States and are present in a substantial minority of ceftazidime-resistant organisms (10). Among clinical *Escherichia coli* isolates collected in Shanghai, China, in 2000 to 2001, 51% had the *cr* variant of *aac(6′)-Ib* (9). No previous survey, however, has evaluated clinical isolates in the United States for the presence of *aac(6′)-Ib-cr*.

Test isolates were drawn from the Focus Diagnostics collection of *Enterobacteriaceae* from the years 1999, 2000, 2001, and 2004. This same strain set was previously surveyed for *qnr* genes (10), allowing a direct comparison of the distribution of *qnr* genes and *aac(6′)-Ib-cr*. No isolates were available from 2003, and only an incomplete set was available from 2002. Between January and March of each study year, participating clinical microbiology laboratories from each of the nine continental U.S. census regions provided 6,979 nonrepeat clinical isolates of requested enterobacterial genera without regard to antibiotic resistance phenotype. All *Klebsiella pneumoniae*, *Enterobacter*, and *E. coli* isolates with a ceftazidime MIC of ≥16 μg/ml and a ciprofloxacin MIC of ≥0.25 μg/ml were selected from this collection for study. Of 323 such isolates, 313 (97%) were available. Thirty-one (29%) of 106 *K. pneumoniae* isolates, 54 (34%) of 160 *Enterobacter* isolates, and none of 47 (0%) *E. coli* isolates were ciprofloxacin susceptible (MIC ≤ 1.0 μg/ml).

aac(6′)-Ib was amplified by PCR with primers 5′-TTGCGATGCTCTATGAGTGGCTA and 5′-CTCGAATGCCTGGCGTGTTT to produce a 482-bp product. Primers were chosen to amplify all known *aac(6′)-Ib* variants. PCR conditions were 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s for 34 cycles. Strains positive and negative for *aac(6′)-Ib* were included as

TABLE 1. Prevalence of *aac*(6′)-*Ib-cr* and *qnr* genes in *Enterobacteriaceae* from the United States

Year	Variant	No. of isolates with *aac*(6′)-*Ib* variant/total no. of isolates (%)		
		K. pneumoniae	*Enterobacter* spp.	*E. coli*
1999	*cr*	5/21 (23.8)	9/45 (20.0)	3/9 (33.3)
	Any	9/21 (42.9)	28/45 (62.2)	4/9 (44.4)
2000	*cr*	4/33 (12.1)	1/38 (2.6)	4/8 (50.0)
	Any	19/33 (57.6)	15/38 (39.5)	7/8 (87.5)
2001	*cr*	5/20 (25.0)	0/39 (0.0)	2/5 (40.0)
	Any	13/20 (65.0)	12/39 (30.8)	3/5 (60.0)
2004	*cr*	3/32 (9.3)	2/38 (5.2)	6/25 (24.0)
	Any	23/32 (71.9)	13/38 (34.2)	12/25 (48.0)
Total	*cr*	17/106 (16.0)	12/160 (7.5)	15/47 (31.9)
No. of isolates with *qnr*/total no. of isolates (%)		21/106 (20)	50/160 (31)	2/47 (4)

* Corresponding author. Mailing address: Division of Infectious Diseases, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114-2696. Phone: (617) 643-3856. Fax: (617) 726-7416. E-mail: dhooper@partners.org.

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TABLE 2. Geographic distribution of *aac(6′)-Ib-cr*-positive isolates

Region	No. of isolates with *aac(6′)-Ib-cr*/total no. of isolates (%)		
	K. pneumoniae	*Enterobacter* spp.	*E. coli*
East North Central	2/12 (16.7)	1/22 (4.5)	0/3 (0.0)
East South Central	2/6 (33.3)	0/27 (0.0)	1/4 (25.0)
Middle Atlantic	7/56 (12.5)	3/30 (10.0)	6/16 (37.5)
Mountain	0/1 (0.0)	0/9 (0.0)	1/6 (16.7)
New England	2/11 (18.2)	4/14 (28.6)	0/2 (0.0)
Pacific	0/4 (0.0)	2/21 (9.5)	5/7 (71.4)
South Atlantic	2/6 (33.3)	2/21 (9.5)	2/7 (28.6)
West North Central	0/0 (0.0)	0/6 (0.0)	0/0 (0.0)
West South Central	2/10 (20.0)	0/10 (0.0)	0/2 (0.0)

controls. All positives were further analyzed by digestion with BstF5I (New England Biolabs, Ipswich, MA) and/or direct sequencing of the PCR products with primer 5′ CGTCACTC CATACATTGCAA to identify *aac(6′)-Ib-cr*, which lacks the BstF5I restriction site present in the wild-type gene. Screening for *qnrA*, *qnrB*, and *qnrS* was carried out by multiplex PCR amplification as previously described (10).

MICs were determined by a broth microdilution method for ceftazidime, ciprofloxacin, trimethoprim-sulfamethoxazole, and gentamicin. Susceptibility interpretations were defined according to the Clinical and Laboratory Standards Institute (2). Statistical analysis used SPSS 14.0 software package (SPSS, Chicago, IL). A Mantel-Haenszel χ^2 test was used for the comparison of dichotomous variables and trend analysis.

aac(6′)-Ib-cr was detected in 15 (32%) of 47 *E. coli* isolates, 17 (16%) of 106 *K. pneumoniae* isolates, and 12 (7.5%) of 160 *Enterobacter* isolates collected during the study period (Table 1). There was no statistically significant change in the overall prevalence of *aac(6′)-Ib-cr* over time, although *aac(6′)-Ib-cr* was detected in a slightly smaller proportion of isolates in 2004 than in the prior years studied.

The Middle Atlantic region provided the largest number of isolates meeting inclusion criteria and the largest number of *aac(6′)-Ib-cr*-positive *K. pneumoniae* and *E. coli* isolates (Table 2). The Pacific region had the highest prevalence of *aac(6′)-Ib-cr* (22%) overall, but *aac(6′)-Ib-cr*-positive isolates of *K. pneumoniae* were found in all but three census regions, and isolates of *Enterobacter* and *E. coli* were found in all but four. Overall, there was no geographic clustering of *aac(6′)-Ib-cr*, but in comparison to other regions, the prevalence of *Enterobacter* isolates was higher in New England (4/14, 29% versus 8/146, 5.5%) ($P = 0.005$), and the prevalence of *E. coli* was higher in the Pacific region (5/7, 72% versus 10/40, 25%) ($P = 0.03$).

There was no relationship between *aac(6′)-Ib-cr* prevalence and patient age, patient gender, or inpatient status. There was also no relationship between the presence of *qnrA*, *-B*, or *-S* genes and *aac(6′)-Ib-cr. qnr* genes were present in 7 of 44 (15.9%) *aac(6′)-Ib-cr*-positive strains compared to 66 of 269 (24.5%) *aac(6′)-Ib-cr*-negative strains ($P = 0.26$ by a two-tailed Fisher's exact test), indicating that the *qnr* genes and *aac(6′)-Ib-cr* can circulate independently (Table 3).

We analyzed the relationship between *aac(6′)-Ib-cr* and susceptibility to ciprofloxacin, gentamicin, and trimethoprim-sulfamethoxazole. Among all isolates, the *aac(6′)-Ib-cr* allele was present in almost equal proportions of ciprofloxacin-susceptible (MIC, 0.25 to 1.0 μg/ml) and -resistant (MIC ≥ 2.0 μg/ml) isolates, and neither ciprofloxacin nor trimethoprim-sulfamethoxazole resistance was associated with *aac(6′)-Ib-cr* prevalence. In *E. coli* isolates, however, the risk of harboring

TABLE 3. Characteristics of *aac(6′)-Ib-cr*-positive and *aac(6′)-Ib-cr*-negative isolates

Characteristic[a]	No. of isolates with characteristic/total no. of isolates with *aac(6′)-Ib-cr* result (%)		Odds ratio (95% confidence interval)	*P* value[d]
	Positive (%)	Negative (%)		
Patient characteristics				
Age ≥ 65 years	17/44 (38.6)	105/269 (39.0)	0.9 (0.5–1.9)	0.96
Male[b]	21/44 (47.7)	137/267 (51.3)	0.9 (0.5–1.6)	0.66
Inpatient[c]	21/44 (47.7)	158/262 (60.3)	0.6 (0.3–1.1)	0.12
qnr positive	7/44 (15.9)	66/269 (24.5)	0.6 (0.3–1.5)	0.26
Strain characteristics				
K. pneumoniae				
CIP-R	12/17 (70.6)	63/89 (70.8)	1.0 (0.3–3.1)	0.99
GEN-R	7/17 (41.2)	55/89 (61.8)	0.4 (0.2–1.2)	0.12
SXT-R	12/17 (70.6)	72/89 (80.9)	0.6 (0.2–1.8)	0.34
Enterobacter spp.				
CIP-R	8/12 (66.7)	98/148 (66.2)	1.0 (0.3–3.6)	0.98
GEN-R	7/12 (58.3)	74/148 (50.0)	1.4 (0.4–4.6)	0.58
SXT-R	10/12 (83.3)	83/148 (56.1)	3.9 (0.8–18.5)	0.09
E. coli				
CIP-R	15/15 (100)	32/32 (100)		
GEN-R	13/15 (86.7)	18/32 (56.3)	5.8 (1.1–29.6)	0.04
SXT-R	9/15 (60.0)	25/32 (78.1)	0.4 (0.1–1.6)	0.20

[a] CIP, ciprofloxacin; GEN, gentamicin; SXT, trimethoprim-sulfamethoxazole; R, resistant.
[b] Information on sex was not available for two patients.
[c] Information on inpatient status was not available for seven patients.
[d] *P* values were determined by a two-tailed Fisher's exact test.

aac(6′)-Ib-cr was significantly associated with gentamicin resistance (odds ratio, 5.78; 95% confidence interval, 1.1 to 29.6). Thirteen (42%) of 31 gentamicin-resistant isolates harbored *aac(6′)-Ib-cr*, in contrast to 2 (12%) of 16 gentamicin-susceptible isolates (Table 3), an unexpected association since *aac(6′)-Ib-cr* confers resistance to kanamycin but not to gentamicin (10).

Among ceftazidime-nonsusceptible enteric bacteria collected between 2000 and 2004 from across the United States, the prevalence of *aac(6′)-Ib-cr* was 44/313 (14%). The distribution of this variant, however, differed among species. It was detected most often in *E. coli* isolates, next most often in *K. pneumoniae* isolates, and least often in *Enterobacter* isolates (Table 1), a relative prevalence that is the reverse of that for the *qnr* genes (12). The reason for these differences is not yet understood, since it is known that some plasmids can carry both *aac(6′)-Ib-cr* and *qnrA* (10).

The most striking finding of our study was the wide penetration of the *aac(6′)-Ib-cr* allele. In nearly one-third of cases in which an allele of *aac(6′)-Ib* was identified, it was the *cr* variant. Although this variant gene was not reported until 2006, it was already present in more than half of multidrug-resistant *E. coli* isolates in Shanghai, China, in 2000 to 2001 (13), and it is now present in the majority of census regions of the United States. The various antibiograms and the range of species of the isolates studied suggest that the dissemination of *aac(6′)-Ib-cr* does not occur through clonal spread or the spread of a single plasmid. The diversity of plasmids on which this gene circulates is not yet known, but its presence as part of an integron cassette (1, 9) suggests that it could be widely mobile among plasmids.

Our analysis also indicated that there was no association between the *aac(6′)-Ib-cr* gene and *qnr* genes. This result is consistent with our previous results showing that *E. coli* strains from Shanghai carrying *aac(6′)-Ib-cr* are substantially more prevalent than those carrying *qnrA*, although a few strains carried both genes on plasmids that transferred higher levels of quinolone resistance than plasmids carrying either gene alone (9, 13).

The overall proportion of isolates harboring *aac(6′)-Ib-cr* was stable over time. Although the *cr* variant of *aac(6′)-Ib* encodes an enzyme that had slightly reduced efficiency in acetylation of kanamycin (9), it is not yet known whether there are any additional biological costs of this variant over other variants of *aac(6′)-Ib*. Selection pressures from the use of aminoglycosides that are enzyme substrates (kanamycin, tobramycin, and amikacin) and the use of quinolones with a piperazinyl amine that is subject to *N*-acetylation by the *cr* variant enzyme would be predicted to promote gene prevalence but have not yet been studied in clinical settings. It is interesting to speculate that future shifts in the choice of quinolones used to those that are not substrates for Aac(6′)-Ib-cr might reduce selection pressures for this variant but not for the *qnr* genes.

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