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Antimicrobial Surveillance of *Haemophilus influenzae* in the United States during 2000–2001 Leads to Detection of Clonal Dissemination of a β-Lactamase-Negative and Ampicillin-Resistant Strain

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A 2000–2001 U.S. *Haemophilus influenzae* surveillance study (n = 1,434) detected nine (0.6%) β-lactamase-negative and ampicillin-resistant (BLNAR) isolates collected from two different hospitals. The MICs of ampicillin for all nine isolates were 4 μg/ml, with results being reproducible; and all nine isolates were susceptible to amoxicillin-clavulanate, cefuroxime, cefprozil, macrolides, and fluoroquinolones. Pulsed-field gel electrophoresis of genomic DNA following *Sma*I digestion demonstrated identical patterns for each of the nine isolates, suggesting intra- and interhospital dissemination of a BLNAR clone.

Clinical microbiology laboratories frequently isolate *Haemophilus influenzae* from respiratory tract specimens of patients with acute exacerbations of chronic bronchitis, otitis media, sinusitis, and pneumonia (8). Among clinical isolates of *H. influenzae*, resistance has arisen predominantly to ampicillin (amoxicillin) but also, to a lesser extent, to oral cephalosporins such as cefaclor, cefprozil, and loracarbef, as well as trimethoprim-sulfamethoxazole and clarithromycin (7, 9, 11, 22). Isolates of *H. influenzae* resistant to ampicillin were first reported in the 1970s (23), and their prevalence increased markedly during the 1980s and early 1990s in the United States (3, 5, 7, 22). Hydrolysis by two β-lactamases, TEM-1 and ROB-1, has been reported to account for almost all decreased susceptibility to ampicillin (8). The widespread dissemination of TEM-1 (15) and, to a lesser extent, ROB-1 (2) among clinical isolates of *H. influenzae* has effectively removed the aminopenicillins (without the addition of a β-lactamase inhibitor) and certain cephalosporins as options for empirical treatment of infections in which this organism is a suspected pathogen. Current North American studies now suggest that increases in the proportions of isolates that produce β-lactamases may have plateaued at 30 to 40% (3, 6, 7, 21, 22).

β-Lactamase-negative and ampicillin-resistant (BLNAR) isolates of *H. influenzae* were first reported in 1980 (17) and generally continue to be isolated at low frequencies (0.04 to 2.5%) (3, 7, 8, 22). However, recent surveillance studies performed in Spain (5.5%) and Japan (3.4%) have reported higher proportions of BLNAR isolates among β-lactamase-negative isolates (16, 20). β-Lactamase-positive and amoxicillin-clavulanate-resistant (BLPACR) isolates of *H. influenzae* have also been reported (3, 14), but MIC results for such isolates appear to be difficult to reproduce and may depend upon testing variables such as inoculum, medium, spheroplast production, and clavulanate content (11). This report describes a recent surveillance study that detected nine BLNAR isolates of *H. influenzae*; each isolate was shown to be clonally related to the others by a standard pulsed-field gel electrophoresis (PFGE) method. This represents the first description of the apparent intrahospital clonal dissemination of a U.S. BLNAR strain of *H. influenzae*.

Prospective isolates of *H. influenzae* (n = 1,434) were collected in 2000–2001 from 65 clinical microbiology laboratories distributed across the United States. Isolates were transported to a central laboratory (Focus Technologies, Herndon, Va.), where each was subcultured for purity on chocolate agar and its identity was confirmed by standard laboratory methods (1). β-Lactamase production was detected with the chromogenic substrate nitrocefin (BBL DrySlide Nitrocefin; Becton Dickinson, Sparks, Md.). Isolate susceptibilities to ampicillin, amoxicillin-clavulanate, cefuroxime, cefprozil, azithromycin, clarithromycin, levofloxacin, and gatifloxacin were assessed by the NCCLS broth microdilution method (18) with dried microdilution panels prepared by TREK Diagnostics (Westlake, Ohio). The MICs for *H. influenzae* were interpreted by using the recommendations in NCCLS standard M100-S11 (19).

PFGE was performed with the nine BLNAR isolates and three non-BLNAR control isolates. For each isolate, agarose-embedded bacterial DNA was prepared from 50 μl of an *H. influenzae* culture grown to an optical density at 450 nm of approximately 1.5. Each cell suspension was lysed for 30 min at 39°C with 10 μl (50 mg/ml) of lysozyme and was subsequently added to 50 μl of melted 1.2% SeaKem gold agarose (FMC BioProducts, Rockland, Maine) and 0.4 μl of proteinase K (25 mg/ml). After solidification, the DNA-containing agarose plug was incubated in 2 ml of cell lysis buffer for 1 h at 50°C, followed by digestion with 100 U of *Sma*I (20,000 U/ml) for 2 h at 25°C. Electrophoresis was performed with a CHEF-DR III electrophoresis system (Bio-Rad, Hercules, Calif.). The running parameters were set at an initial switch time of 1 s, a final switch time of 25 s, a voltage of 6 V/cm, and a temperature of 120°C for 20 h. The gel was stained with ethidium bromide and photographed. A 50- to 1,000-kb bacteriophage lambda ladder

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TABLE 1. Susceptibilities of 1,434 *H. influenzae* isolates to all antimicrobials according to β-lactamase status[a]

Antimicrobial	Isolates	MIC (μg/ml)				MIC interpretation		
		Range	50%	90%	Mode	% Susceptible	% Intermediate	% Resistant
Ampicillin	All	≤0.03–>16	0.25	>16	0.25	71.1	0.5	28.5
	β-Lactamase positive	0.5–>16	>16	>16	>16	0.3	1.5	98.3
	β-Lactamase negative	≤0.03–4	0.25	0.5	0.25	99.0	0.1	0.9
Amoxicillin-clavulanate	All	≤0.015/0.008–16/8	0.5	1	0.5	99.9		0.1
	β-Lactamase positive	0.25/0.12–16/8	1	2	1	99.5		0.5
	β-Lactamase negative	≤0.015/0.008–4/2	0.25	1	0.25	100		0
Cefuroxime	All	≤0.03–4	0.5	2	0.5	100	0	0
	β-Lactamase positive	0.06–4	0.5	2	0.5	100	0	0
	β-Lactamase negative	≤0.03–4	0.5	2	0.5	100	0	0
Cefprozil	All	≤0.12–>64	2	8	1	91.4	3.6	5.0
	β-Lactamase positive	0.5–>64	4	64	4	72.2	10.8	17.0
	β-Lactamase negative	≤0.12–64	2	4	1	99.0	0.8	0.2
Azithromycin	All	≤0.015–>64	1	2	1	99.7		
	β-Lactamase positive	0.12–>64	1	2	1	99.8		
	β-Lactamase negative	≤0.015–32	1	2	1	99.6		
Clarithromycin	All	≤0.03–>128	8	16	8	73.9	23.1	3.1
	β-Lactamase positive	0.12–>128	8	16	8	69.0	27.1	3.9
	β-Lactamase negative	≤0.03–>128	8	16	8	75.8	21.5	2.7
Levofloxacin	All	≤0.004–0.12	0.015	0.015	0.015	100		
	β-Lactamase positive	≤0.004–0.12	0.015	0.015	0.015	100		
	β-Lactamase negative	≤0.004–0.12	0.015	0.015	0.015	100		
Gatifloxacin	All	≤0.002–0.06	0.008	0.015	0.008	100		
	β-Lactamase positive	0.004–0.03	0.008	0.015	0.008	100		
	β-Lactamase negative	≤0.002–0.06	0.008	0.015	0.008	100		

[a] Among the isolates tested, 406 were β-lactamase positive and 1,028 were β-lactamase negative.

(Lambda Ladder PFG Marker; New England Biolabs, Inc., Beverly, Mass.) was included on the gel.

Of the 1,434 *H. influenzae* isolates tested, 71.7% ($n = 1,028$) were β-lactamase negative and 28.3% ($n = 406$) were β-lactamase positive. Among the β-lactamase-negative isolates, nine isolates were ampicillin resistant (BLNAR) (Table 1) and one isolate was ampicillin intermediate (the susceptibility of each isolate was confirmed by repeat β-lactamase and MIC testing) (Table 1). All nine BLNAR isolates were collected from adult respiratory specimens; the ampicillin MICs for all nine isolates were 4 μg/ml and were reproducible; and all nine isolates were susceptible to amoxicillin-clavulanate (MICs, 4/2 μg/ml), cefuroxime (MICs, 2 μg/ml), cefprozil (MICs, 2 to 4 μg/ml), macrolides (azithromycin, clarithromycin), and fluoroquinolones (levofloxacin, gatifloxacin) (Table 1). Seven of the nine BLNAR isolates were from outpatients, including six of eight isolates from a hospital in the west-north-central region of the United States (hospital A) and a single isolate from a hospital in the mid-Atlantic region (hospital B). All nine BLNAR isolates were identical by PFGE and were different from each of the three *H. influenzae* control isolates tested (Fig. 1).

Among the β-lactamase-positive isolates, 399 were ampicillin resistant, 6 were ampicillin intermediate, and 1 was ampicillin susceptible (MIC, 0.5 μg/ml; the result was confirmed by repeat β-lactamase and MIC testing) (Table 1). Two BLPACR isolates were also identified; the result for each isolate was confirmed by repeat testing (Table 1), and both isolates were collected from the same hospital in the east-south-central region of the United States (hospital C). For the BLPACR isolates, amoxicillin-clavulanate MICs were 16/8 and 8/4 μg/ml, respectively, and both isolates were resistant to ampicillin (MICs, >16 μg/ml) and cefprozil (MICs, 16 and 8 μg/ml, respectively). The BLPACR isolates were susceptible to cefuroxime, macrolides (azithromycin, clarithromycin), and fluoroquinolones (gatifloxacin, levofloxacin). PFGE analysis showed that the two BLPACR isolates were unrelated (Fig. 1).

Excluding ampicillin, *H. influenzae* demonstrated the highest levels of resistance to cefprozil (5.0%) and clarithromycin (3.1%) (Table 1). All isolates were susceptible to levofloxacin and gatifloxacin, and 99.7% of the isolates were susceptible to azithromycin. The presence of β-lactamase decreased the percentage of isolates that were susceptible to cefprozil and clarithromycin by >5%; the MIC at which 90% of isolates are inhibited (MIC_{90}s) for ampicillin, amoxicillin-clavulanate, and cefprozil were lower for β-lactamase-negative isolates than for β-lactamase-positive isolates. All isolates not susceptible to azithromycin ($n = 5$) were clarithromycin resistant, representing 11.4% of all clarithromycin-resistant isolates ($n = 44$); all clarithromycin-intermediate isolates ($n = 331$; 23.1% of all isolates) were azithromycin susceptible.

The clinical significance of BLNAR and BLPACR isolates

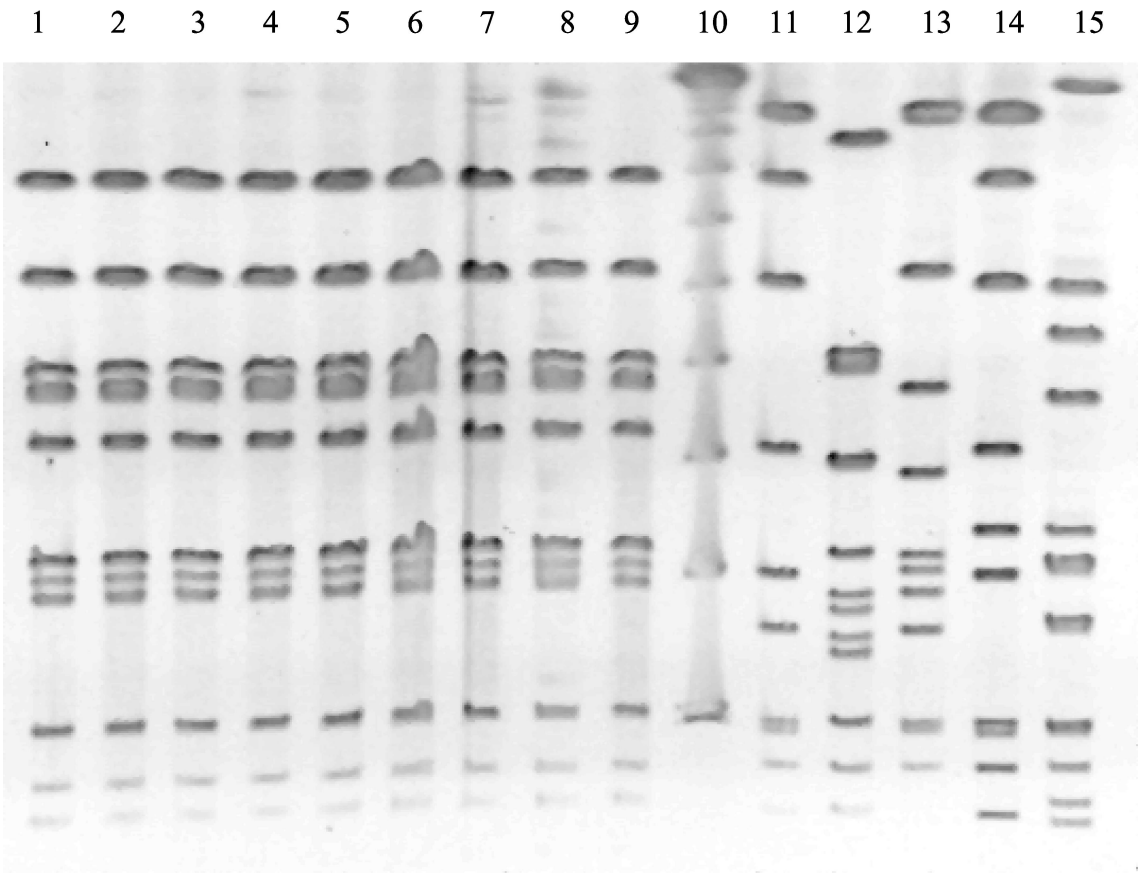

FIG. 1. PFGE results for *H. influenzae* digested with *Sma*I. Lanes 1 to 7 and 9, BLNAR isolates collected at hospital A; lane 8, BLNAR isolate collected at hospital B; lane 10, 50- to 1,000-kb bacteriophage lambda ladder; lanes 11 to 13, β-lactamase-negative and ampicillin-susceptible controls, one from hospital A and two from other hospitals participating in the surveillance study; lanes 14 and 15, BLPACR isolates collected at hospital C.

of *H. influenzae* is unknown. However, BLNAR isolates have been shown to be less susceptible to ampicillin, amoxicillin-clavulanate, and commonly active oral cephalosporins than other β-lactamase-negative isolates (3, 9, 13, 14), suggesting that resistance is likely arising due to mutations within penicillin-binding proteins (24). NCCLS recommends that BLNAR strains be considered resistant to amoxicillin-clavulanate, ampicillin-sulbactam, cefaclor, cefamandole, cefetamet, cefonicid, cefprozil, cefuroxime, loracarbef, and piperacillin-tazobactam, despite the apparent in vitro susceptibilities of some BLNAR strains to these agents (19). BLPACR isolates are generally ampicillin resistant and less susceptible to cefaclor, cefuroxime, cefprozil, loracarbef, or cefpodoxime than comparative β-lactamase-positive and amoxicillin-clavulanate-susceptible isolates (3, 4, 14). The amoxicillin-clavulanate MICs for the two BLPACR isolates in the present study, as has been reported previously, clustered at the resistance breakpoint (8/4 μg/ml) (3, 4, 12, 14).

This study detected nine isolates of *H. influenzae* with a BLNAR phenotype and confirmed the results by repeat MIC and β-lactamase testing. This results in a prevalence of the BLNAR phenotype of 0.9% among the β-lactamase-negative isolates and 0.6% among all *H. influenzae* isolates tested. PFGE analysis revealed that all nine isolates were identical (Fig. 1). A previous U.S. study found that two BLNAR isolates collected from a single institution were clonal, and their genomic DNAs possessed *Sma*I restriction PFGE profiles remarkably similar to that of the currently described clone (14). A second study of 29 BLNAR isolates collected in France between 1987 and 1994 showed 20 unique *Sma*I PFGE banding patterns that suggested limited clonality of the isolates (10). The present study, however, is the first to report a BLNAR clone in two geographically distant institutions and suggests that BLNAR isolates reported in other studies, conducted at different study sites, may also have been clonal. The nine BLNAR isolates identified here represent a prevalence (<1%) similar to that reported in the United States in the 1994–1995 and 1997–1998 respiratory seasons (4, 14, 22) but less than that reported by Doern et al. (3) for the 1994–1995 respiratory season (4% among β-lactamase-negative isolates). Overall, the data suggest no trend toward an increasing prevalence but, rather, suggest the sporadic isolation of BLNAR isolates. The contribution of clonality to the sporadic isolation of BLNAR isolates were not mentioned in the majority of previous studies.

In summary, ≥99.7% of all *H. influenzae* isolates were susceptible to amoxicillin-clavulanate, cefuroxime, azithromycin, and fluoroquinolones, clearly indicating that resistance to these compounds has not emerged. The data reported here

and elsewhere suggest that the proportion of *H. influenzae* isolates that produce β-lactamase may have reached a plateau, at least temporarily. BLNAR isolates remain uncommon (usually <1%) and were shown in the present study to be attributable to clonal spread between patients attending the same hospital and between patients attending geographically distant hospitals.

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REFERENCES

1. **Campos, J. M.** 1999. Haemophilus, p. 539–560. *In* P. R. Murray, E. J. Baron. M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 7th ed. ASM Press, Washington, D.C.
2. **Daum, R. S., M. Murphey-Corb, E. Shapira, and S. Dipp.** 1988. Epidemiology of ROB β-lactamase among ampicillin-resistant *Haemophilus influenzae* isolates in the United States. J. Infect. Dis. **157:**450–455.
3. **Doern, G. V., A. B. Brueggemann, G. Pierce, H. P. Holley, and A. Rauch.** 1997. Antibiotic resistance among clinical isolates of *Haemophilus influenzae* in the United States in 1994 and 1995 and detection of β-lactamase-positive strains resistant to amoxicillin-clavulanate: results of a national multicenter surveillance study. Antimicrob. Agents Chemother. **41:**292–297.
4. **Doern, G. V., R. N. Jones, M. A. Pfaller, K. Kugler, and the SENTRY Participants Group.** 1999. *Haemophilus influenzae* and *Moraxella catarrhalis* from patients with community-acquired respiratory tract infections: antimicrobial susceptibility patterns from the SENTRY Antimicrobial Surveillance Program (United States and Canada, 1997). Antimicrob. Agents Chemother. **43:**385–389.
5. **Doern, G. V., J. H. Jorgensen, C. Thornsberry, D. A. Preston, and the Haemophilus Surveillance Group.** 1986. Prevalence of the antimicrobial resistance among clinical isolates of *Haemophilus influenzae*: a collaborative study. Diagn. Microbiol. Infect. Dis. **4:**95–107.
6. **Doern, G. V., J. H. Jorgensen, C. Thornsberry, D. A. Preston, T. Tubert, J. S. Redding, and L. A. Maher.** 1998. National collaborative study of the prevalence of antimicrobial resistance among clinical isolates of *Haemophilus influenzae*. Antimicrob. Agents Chemother. **32:**180–185.
7. **Doern, G. V., and the Alexander Project Collaborative Group.** 1996. Antimicrobial resistance among lower respiratory tract isolates of *Haemophilus influenzae*: results of a 1992–1993 Western Europe and USA collaborative surveillance study. J. Antimicrob. Chemother. **38**(Suppl. A)**:**59–69.
8. **Felmingham, D., J. Washington, and the Alexander Project Group.** 1999. Trends in the antimicrobial susceptibility of bacterial respiratory tract pathogens–-findings of the Alexander Project 1992–1996. J. Chemother. **11:**5–21.
9. **Fuchs, P. C., A. L. Barry, and S. D. Brown.** 2000. Susceptibility of *Streptococcus pneumoniae* and *Haemophilus influenzae* to cefditoren, and provisional interpretive criteria. Diagn. Microbiol. Infect. Dis. **37:**265–269.
10. **Gazagne, L., C. Delmas, E. Bingen, and H. Dabernat.** 1998. Molecular epidemiology of ampicillin-resistant non-beta-lactamase-producing *Haemophilus influenzae*. J. Clin. Microbiol. **36:**3629–3635.
11. **Hoban, D. J., G. V. Doern, A. C. Fluit, M. Roussel-Delvallez, and R. N. Jones.** 2001. Worldwide prevalence of antimicrobial resistance in *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* in the SENTRY Antimicrobial Resistance Surveillance Program, 1997–1999. Clin. Infect. Dis. **32**(Suppl. 2)**:**S81-S93.
12. **Jacobs, M. R., and S. Bajaksouzian.** 1997. Evaluation of *Haemophilus influenzae* isolates with elevated MICs to amoxicillin/clavulanic acid. Diagn. Microbiol. Infect. Dis. **28:**105–112.
13. **Johnson, D. M., D. J. Biedenbach, M. L. Beach, M. A. Pfaller, and R. N. Jones.** 2000. Antimicrobial activity and in vitro susceptibility test development for cefditoren against *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus* species. Diagn. Microbiol. Infect. Dis. **37:**99–105.
14. **Jones, R. N., M. R. Jacobs, J. A. Washington, and M. A. Pfaller.** 1997. A 1994–95 survey of *Haemophilus influenzae* susceptibility to ten orally administered agents. Diagn. Microbiol. Infect. Dis. **27:**75–83.
15. **Livermore, D. M.** 1995. β-Lactamases in laboratory and clinical practice. Clin. Microbiol. Rev. **8:**557–584.
16. **Marco, F., J. García-de-Lomas, C. García-Rey, E. Bouza, L. Aguilar, C. Fernández-Mazarrasa, and the Spanish Surveillance Group for Respiratory Pathogens.** 2001. Antimicrobial susceptibilities of 1,730 *Haemophilus influenzae* respiratory tract isolates in Spain in 1998–1999. Antimicrob. Agents Chemother. **45:**3226–3228.
17. **Markowitz, S. M.** 1980. Isolation of an ampicillin-resistant, non-β-lactamase-producing strain of *Haemophilus influenzae*. Antimicrob. Agents Chemother. **17:**80–83.
18. **National Committee for Clinical Laboratory Standards.** 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A5. National Committee for Clinical Laboratory Standards, Wayne, Pa.
19. **National Committee for Clinical Laboratory Standards.** 2001. Performance standards for antimicrobial susceptibility testing. Approved standard M100-S11. National Committee for Clinical Laboratory Standards, Wayne, Pa.
20. **Ohkusu, K., A. Nakamura, and K. Sawada.** 2000. Antibiotic resistance among clinical isolates of *Haemophilus influenzae* in Japanese children. Diagn. Microbiol. Infect. Dis. **36**:249–254.
21. **Rittenhouse, S. P., L. Miller, R. L. Kaplan, G. H. Mosely, and J. A. Poupard.** 1995. A survey of β-lactamase-producing *Haemophilus influenzae*: an evaluation of 5750 isolates. Diagn. Microbiol. Infect. Dis. **21:**223–225.
22. **Thornsberry, C., M. E. Jones, M. L. Hickey, Y. Mauriz, J. Kahn, and D. F. Sahm.** 1999. Resistance surveillance of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* isolated in the United States, 1997–1998. J. Antimicrob. Chemother. **44:**749–759.
23. **Tomeh, M. O., S. E. Starr, J. E. McGowan, Jr., P. M. Terry, and A. G. Nahmias.** 1974. Ampicillin-resistant *Haemophilus influenzae* type b infection. JAMA **229:**295–297.
24. **Ubukata, K., Y. Shibasaki, K. Yamamoto, N. Chiba, K. Hasegawa, Y. Takeuchi, K. Sunakawa, M. Inoue, and M. Konno.** 2001. Association of amino acid substitutions in penicillin-binding protein 3 with β-lactam resistance in β-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. Antimicrob. Agents Chemother. **45:**1693–1699.