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Comparative Molecular Analysis of *Haemophilus influenzae* Isolates from Young Children with Acute Lower Respiratory Tract Infections and Meningitis in Hanoi, Vietnam

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Thirty-seven *Haemophilus influenzae* strains from nasopharyngeal swabs (NP) and 44 *H. influenzae* strains from cerebrospinal fluid (CSF) were investigated. Of the 37 *H. influenzae* isolates from NP, the serotypes of 30 isolates were nontypeable, 4 were type b, 2 were type c, and 1 was type a, whereas all of the 44 isolates from CSF were type b. The MICs of 16 antibiotics for the *H. influenzae* isolates from NP and CSF were similar, and no β-lactamase-negative ampicillin-resistant strain was found. Molecular typing by pulsed-field gel electrophoresis (PFGE) showed that the 37 *H. influenzae* strains from NP had 22 PFGE patterns, with none predominating, and the 44 *H. influenzae* strains from CSF had 9 PFGE patterns, with patterns α (22 isolates) and β (12 isolates) predominating. Our results indicate that two predominant types of *H. influenzae* type b strains have the potential to spread among children with meningitis in Hanoi, Vietnam.

Nontypeable *Haemophilus influenzae* (NTHi) can cause a variety of infections, including otitis media, bronchitis, and pneumonia (7), whereas *H. influenzae* type b (Hib) is a common cause of meningitis in children (11). Hib infection rates have been dramatically reduced in countries that have implemented Hib conjugate vaccine programs as part of routine infant immunizations (10). It has also recently been reported that β-lactamase-negative ampicillin (AMP)-resistant (BLNAR) strains have increased in some countries (6, 12), although their global prevalence remains low (4, 5). The aim of our study was to investigate the characteristics of *H. influenzae* among children less than 5 years of age in Vietnam.

Thirty-seven *H. influenzae* strains were isolated from the nasopharyngeal swabs (NP) of 37 children aged 2 to 60 months (mean age, 11 months) who were diagnosed with acute lower respiratory tract infections between 2001 and 2002, and 44 *H. influenzae* strains were isolated from the cerebrospinal fluid (CSF) of 44 children aged 1 to 24 months (mean age, 9 months) who were diagnosed with meningitis between 2002 and 2003, in Hanoi, Vietnam. No patient with an acute lower respiratory tract infection overlapped a patient with meningitis. *H. influenzae* isolates were serotyped by slide agglutination with antisera purchased from Difco Laboratories (Detroit, Mich.), and β-lactamase production was detected by a disk impregnated with nitrocefin (Becton Dickinson, Sparks, Md.). PCR was carried out for *H. influenzae* isolates by using mixed primers (Wakunaga Pharmaceutical Co., Hiroshima, Japan), as described previously (3). MICs were determined by the agar dilution method according to the NCCLS guidelines (8). The susceptibilities of 81 *H. influenzae* isolates to the following 16 antibiotics were tested: penicillin G (Meiji Seika Kaisha, Tokyo, Japan), AMP (Meiji Seika Kaisha), amoxicillin-clavulanic acid (AMC) (GlaxoSmithKline K.K., Tokyo, Japan), cefatrizine (Taiyo Yakuhin Co., Nagoya, Japan), cefuroxime (Sankyo Co., Tokyo, Japan), ceftriaxone (Chugai Pharmaceutical Co., Tokyo, Japan), cefotaxime (Aventis Pharma, Tokyo, Japan), imipenem (Banyu Pharmaceutical Co., Tokyo, Japan), minocycline [Lederle (Japan), Tokyo, Japan], chloramphenicol (Sankyo Co.), clarithromycin (Taisho Pharmaceutical Co., Tokyo, Japan), erythromycin (Dainippon Pharmaceutical Co., Osaka, Japan), gentamicin (Schering-Plough K.K., Osaka, Japan), levofloxacin (Daiichi Pharmaceutical Co., Tokyo, Japan), norfloxacin (Kyorin Pharmaceutical Co., Tokyo, Japan), and sulfamethoxazole-trimethoprim (Shionogi & Co., Osaka, Japan). After digestion with SmaI (Takara Shuzo Co., Shiga, Japan), pulsed-field gel electrophoresis (PFGE) was performed on the 37 *H. influenzae* isolates from the NP and the 44 *H. influenzae* isolates from the CSF, as described previously (16), and the interpretation of PFGE patterns was based on the criteria described by Tenover et al. (13).

Of the 37 *H. influenzae* isolates from NP, the serotypes of 30 isolates were nontypeable, 4 were type b, 2 were type c, and 1 was type a, whereas the 44 isolates from CSF were all type b. Twenty-six strains (70.3%) from NP and 23 strains (52.3%) from CSF were β-lactamase producing, and the remaining strains were β-lactamase negative by the nitrocefin disk assay. PCR analysis to identify the resistance genes indicated that 25 strains from NP and 21 strains from CSF were β-lactamase-producing AMP-resistant isolates which had the TEM-1-type β-lactamase gene; 11 strains from NP and 22 strains from CSF were β-lactamase-negative AMP-susceptible isolates, all of which lacked all resistance genes; and 1 strain each from NP and CSF were β-lactamase-producing AMC-resistant isolates

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TABLE 1. Distribution of MICs against 16 antibiotics for *H. influenzae* strains isolated from nasopharyngeal swabs and cerebrospinal fluid from children in Vietnam

Antibiotic	MIC (μg/ml) for isolates from:					
	NP (n = 37)			CSF (n = 44)		
	Range	50%	90%	Range	50%	90%
Penicillin G	0.5–128	16	32	≤0.004–128	2	32
Ampicillin	0.25–64	8	32	0.125–32	1	8
Amoxicillin-clavulanic acid	0.25–2	0.5	0.5	0.25–1	0.25	0.25
Cefatrizine	2–32	4	8	2–16	4	16
Cefuroxime	0.5–4	1	4	0.016–4	1	2
Ceftriaxone	≤0.004–0.032	0.008	0.016	≤0.004–0.032	0.008	0.008
Cefotaxime	0.008–0.125	0.032	0.032	≤0.004–0.125	0.032	0.063
Imipenem	0.25–4	2	2	0.25–1	0.25	1
Minocycline	0.5–2	1	2	0.5–2	1	1
Chloramphenicol	0.5–16	4	8	0.5–16	8	16
Clarithromycin	0.25–16	8	16	4–16	8	8
Erythromycin	0.25–4	4	4	0.016–8	2	4
Gentamicin	1–2	1	2	0.016–2	0.5	2
Levofloxacin	0.016–0.063	0.032	0.032	≤0.004–0.032	0.032	0.032
Norfloxacin	0.063–0.125	0.125	0.125	0.063–0.125	0.063	0.125
Sulfamethoxazole-trimethoprim	1–≥128	≥128	≥128	0.032–≥128	128	≥128

which had the TEM-1-type β-lactamase gene and the *ftsI* gene with the same substitution as the low-BLNAR strains. Although all isolates from NP which had the TEM-1-type β-lactamase gene were β-lactamase producing by the nitrocefin disk assay, one isolate from CSF which had the TEM-1-type β-lactamase gene was β-lactamase negative and two isolates from CSF which did not have the TEM-1-type β-lactamase gene were β-lactamase producing by the nitrocefin disk assay. No BLNAR strain was found. Table 1 shows the MIC range, the MICs at which 50% of isolates were inhibited (MIC_{50}), and the MIC_{90} of 16 antibiotics for 37 *H. influenzae* isolates from NP and 44 *H. influenzae* isolates from CSF. Although the MICs of the *H. influenzae* isolates from NP against penicillin G and AMP appear to be higher than those from CSF, the antimicrobial susceptibilities of the *H. influenzae* isolates from NP and CSF were similar. Molecular typing by pulsed-field gel electrphoresis (PFGE) showed that the 37 *H. influenzae* strains from NP had 22 PFGE patterns (A to V), without any predominant pattern (Fig. 1). The PFGE patterns of *H. influenzae* types a, b, and c were different from those of NTHi. Four isolates of type b had two PFGE patterns (I and K), and two isolates of type c had two PFGE patterns (H and Q). Forty-four *H. influenzae* strains from CSF had nine PFGE patterns (α to ι), with patterns α (22 isolates) and β (12 isolates) predominating. The PFGE patterns of 4 *H. influenzae* type b strains from NP were quite different from those of the 44 *H. influenzae* type b strains from CSF (Fig. 2).

Infants and young children tend to acquire *H. influenzae* in the upper respiratory tract because of their low immunity (16), and subsequent colonization can become a risk factor for invasive diseases caused by *H. influenzae* (2, 11). Since it has recently been reported that BLNAR NTHi and Hib have increased in some countries (3, 6, 12), the primary objective of this study was to investigate such resistant strains among children in Vietnam. In fact, no BLNAR strains were found in either NP or CSF, although more than half the isolates were β-lactamase producing and had the TEM-1-type β-lactamase gene. Hib remains the major cause of meningitis after the introduction of Hib vaccine in many advanced nations, because that vaccine is not usually available in Vietnam (14). Therefore, a secondary objective of this study was to examine the transmission route of *H. influenzae*. It has recently been reported that children can acquire *H. influenzae* at day care centers (9, 16) or from their parents at home (15). Our PFGE studies showed that NTHi did not have dominant genetic patterns but that Hib had two dominant genetic patterns. The results provide evidence to show that at least two types of Hib strains are spreading horizontally among children with meningitis in Vietnam. The Hib conjugate vaccine appears to be effective, not only for the prevention of invasive diseases, but also for the reduction of nasopharyngeal carriage in young children (1, 10).

In conclusion, our results demonstrate that BLNAR strains are not prevalent and that two predominant types of Hib

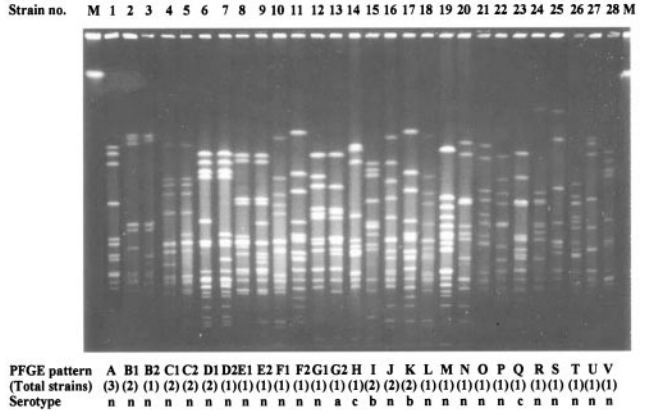

FIG. 1. PFGE patterns of SmaI-digested DNA from 37 *H. influenzae* isolates from NP of 37 children with acute lower respiratory tract infections. Molecular typing by PFGE demonstrated that 37 *H. influenzae* strains from the NP had 22 PFGE patterns (A to V), without any predominant pattern. The PFGE patterns of *H. influenzae* types a, b, and c were different from those of the nontypeable strains.

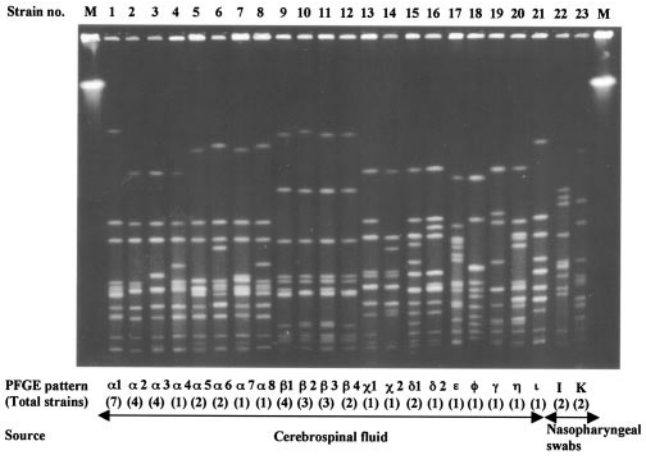

FIG. 2. PFGE patterns of SmaI-digested DNA from 48 Hib isolates from the CSF of 44 children with meningitis and the NP of 4 children with acute lower respiratory tract infections. Molecular typing by PFGE demonstrated that the 44 Hib strains from the CSF had nine PFGE patterns (α to ι), with patterns α (22 isolates) and β (12 isolates) predominating. PFGE patterns of 4 Hib strains from the NP were quite different from those of 44 Hib strains from CSF.

strains have the potential for spreading among children with meningitis in Hanoi, Vietnam. Therefore, the introduction of the Hib conjugate vaccine for young children should be considered in order to prevent invasive diseases caused by Hib.

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REFERENCES

1. **Barbour, M. L., R. T. Mayon-White, C. Coles, D. W. Crook, and E. R. Moxon.** 1995. The impact of conjugate vaccine on carriage of *Haemophilus influenzae* type b. J. Infect. Dis. **171:**93–98.
2. **Faden, H., L. Duffy, R. Wasielewski, J. Wolf, D. Krystofik, and Y. Tung.** 1997. Relationship between nasopharyngeal colonization and the development of otitis media in children. J. Infect. Dis. **175:**1440–1445.
3. **Hasegawa, K., N. Chiba, R. Kobayashi, S. Y. Murayama, S. Iwata, K. Sunakawa, and K. Ubukata.** 2004. Rapidly increasing prevalence of β-lactamase-nonproducing, ampicillin-resistant *Haemophilus influenzae* type b in patients with meningitis. Antimicrob. Agents Chemother. **48:**1509–1514.
4. **Hoban, D., and D. Felmingham.** 2002. The PROTEKT surveillance study: antimicrobial susceptibility of *Haemophilus influenzae* and *Moraxella catarrhalis* from community-acquired respiratory tract infections. J. Antimicrob. Chemother. **50:**49–59.
5. **Karlowsky, J. A., I. A. Critchley, R. S. Blosser-Middleton, E. A. Karginova, M. E. Jones, C. Thornsberry, and D. F. Sahm.** 2002. Antimicrobial surveillance of *Haemophilus influenzae* in the United States during 2000–2001 leads to detection of clonal dissemination of a beta-lactamase-negative and ampicillin-resistant strain. J. Clin. Microbiol. **40:**1063–1066.
6. **Marco, F., J. Garcia-de-Lomas, C. Garcia-Rey, E. Bouza, L. Aguilar, C. Fernandez-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.
7. **Murphy, T. F., and M. A. Apicella.** 1987. Nontypeable *Haemophilus influenzae*: a review of clinical aspects, surface antigens, and the human immune response to infection. Rev. Infect. Dis. **9:**1–15.
8. **National Committee for Clinical Laboratory Standards.** 1998. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7–A4. National Committee for Clinical Laboratory Standards, Wayne, Pa.
9. **Peerbooms, P. G., M. N. Engelen, D. A. Stokman, B. H. van Benthem, M. L. van Weert, S. M. Bruisten, A. van Belkum, and R. A. Coutinho.** 2002. Nasopharyngeal carriage of potential bacterial pathogens related to day care attendance, with special reference to the molecular epidemiology of *Haemophilus influenzae.* J. Clin. Microbiol. **40:**2832–2836.
10. **Peltola, H.** 2000. Worldwide *Haemophilus influenzae* type b disease at the beginning of the 21st century: global analysis of the disease burden 25 years after the use of the polysaccharide vaccine and a decade after the advent of conjugates. Clin. Microbiol. Rev. **13:**302–317.
11. **Saito, M., K. Okada, K. Takemori, and S. Yoshida.** 2000. Clonal spread of an invasive strain of *Haemophilus influenzae* type b among nursery contacts accompanied by a high carriage rate of non-disease-associated strains. J. Med. Microbiol. **49:**845–847.
12. **Suzuki, K., T. Nishimura, and S. Baba.** 2003. Current status of bacterial resistance in the otolaryngology field: results from the Second Nationwide Survey in Japan. J. Infect. Chemother. **9:**46–52.
13. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
14. **Tran, T. T., Q. T. Le, T. N. Tran, N. T. Nguyen, F. K. Pedersen, and M. Schlumberger.** 1998. The etiology of bacterial pneumonia and meningitis in Vietnam. Pediatr. Infect. Dis. J. **17**(Suppl. 9)**:**S192–S194.
15. **Watanabe, H., K. Hoshino, R. Sugita, N. Asoh, K. Watanabe, K. Oishi, and T. Nagatake.** 2004. Possible high rate of transmission of nontypeable *Haemophilus influenzae* including β-lactamase-negative ampicillin-resistant strains between children and their parents. J. Clin. Microbiol. **42:**362–365.
16. **Yano, H., M. Suetake, A. Kuga, K. Irinoda, R. Okamoto, T. Kobayashi, and M. Inoue.** 2000. Pulsed-field gel electrophoresis analysis of nasopharyngeal flora in children attending a day care center. J. Clin. Microbiol. **38:**625–629.