Jpn. J. Infect. Dis., 59, 111-116, 2006

Original Article

Genetic and Phenotypic Characterization of *Haemophilus influenzae* Type b Isolated from Children with Meningitis and Their Family Members in Vietnam

Phan L. T. Huong*, Ngo T. Thi[1], Dang D. Anh, Vu T. T. Huong, Le N. Minh[2], Tran Q. Canh[3], Mayumi Matsuoka[4], Kazunari Kamachi[4], Tsutomu Yamazaki[5], Yoshichika Arakawa[4] and Tsuguo Sasaki[4]

Department of Bacteriology and [2]Department of Immunology & Biotechnology, National Institute of Hygiene and Epidemiology; [1]Department of Microbiology, National Hospital for Pediatric, Hanoi; [3]Hai Duong Medical College, Haiduong Province, Vietnam; [4]Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo 208-0011; and [5]Department of Pediatrics, Saitama Medical School, Saitama 350-0495, Japan

(Received December 26, 2005. Accepted February 21, 2006)

SUMMARY: To investigate *Haemophilus influenzae* type b (Hib) infection in Vietnamese children under the age of 5 years, cerebrospinal fluid (CSF) samples from patients with meningitis were screened for Hib, and isolates were subjected to evaluation of susceptibility to 12 antibiotics, biotyping, and genotyping with pulsed-field gel electrophoresis (PFGE). The major biotype was type II (68.3%), followed by type I (22.8%). Among 79 Hib isolates, 45 (57%) were β-lactamase-producing and ampicillin-resistant (44 and 1 isolates produced TEM-1- and ROB-1-type β-lactamases, respectively), and 34 isolates (43%) were β-lactamase-nonproducing and ampicillin-sensitive. No β-lactamase-nonproducing and ampicillin-resistant isolates were found. The PFGE patterns of Hib isolates were highly divergent, but most could be classified into three clusters. We also investigated Hib colonization in household contacts of patients, and found that Hib isolates from the CSF of patients and from nasopharyngeal cavities of household contacts showed the same PFGE patterns. This observation suggested that household contacts of patients are a possible reservoir of Hib.

INTRODUCTION

Haemophilus influenzae type b (Hib) is a predominant cause of invasive bacterial infections, including meningitis and sepsis, and is the most common cause of bacterial pneumonia in young children under 5 years of age, especially infants under 12 months. Prior to the introduction of routine childhood immunization with conjugate Hib vaccines, it was estimated that there were 600,000 deaths annually due to Hib infection worldwide (1). After the introduction of Hib conjugate vaccines in the 1980s, the incidence of invasive Hib infections has declined dramatically in many countries (1). However, Hib conjugate vaccines have not been introduced into routine national immunization programs in many Asian countries. According to recent reports, the incidence of Hib meningitis in young children under 5 years of age per year is 3.8/100,000 in Thailand (2), 6.0/100,000 in South Korea (3), 16/100,000 in Indonesia (4), and 8.6-8.9/100,000 in Japan (5). These incidence rates are lower than those seen in countries outside Asia before the introduction of Hib conjugate vaccines.

In Vietnam, bacterial meningitis due to Hib received relatively little attention before 2000. In our previous epidemiological study of Hib-associated invasive meningitis in Hanoi during 2000-2002, the incidence of Hib meningitis among children 1-23 months of age was 26/100,000 (1-6 months of age, 30/100,000; 7-11 months of age, 44/100,000), while that among children under 5 years of age was 10/100,000 (6). Meningitis due to Hib infection in children less than 23 months of age accounted for 88-94% of total bacterial meningitis in children under 5 years of age.

In the present study, Hib strains isolated from meningitis patients in Hanoi were characterized by evaluation of susceptibility to antibiotics, biotyping, and pulsed-field gel electrophoresis (PFGE). We also investigated the PFGE profiles of Hib strains isolated from family members in close contact with meningitis patients.

MATERIALS AND METHODS

Clinical isolates: This study was carried out with the approval of the National Institute of Hygiene and Epidemiology (NIHE) Research Ethics Committee. Screening for causative bacteria in 489 samples of cerebrospinal fluid (CSF) from patients with suspected bacterial meningitis was carried out at the National Hospital of Pediatrics in Hanoi from March 2002 to March 2004. CSF specimens were inoculated directly onto chocolate agar plates, and into soybean casein digest (SCD) broth supplemented with 5% Fildes enrichment. Agar plates were incubated at 37°C under 5% CO_2–95% air for 18 h. Colonies that appeared to be *H. influenzae* were subcultured onto new chocolate agar plates and preliminarily identified using X, V, and XV factor disks (Becton-Dickinson, Sparks, Md., USA) on tryptocasein soy agar (TSA). When colonies did not appear on chocolate

*Corresponding author: Mailing address: Department of Bacteriology, National Institute of Hygiene and Epidemiology, 1 Yersin Street, Hanoi, Vietnam. Tel: +84-4-9714408, Fax: +84-4-8210853, E-mail: thanhhuong@hn.vnn.vn

agar plates, small portions of cultures from the SCD were subcultured onto new chocolate agar plates and incubated under the same culture conditions for up to 7 days. Colonies considered to be *H. influenzae* were sent to the NIHE and were further identified using Api-10S for *H. influenzae* (BioMérieux, Marcy l'Etoile, France) and Cefinase (BBL, Cockeysville, Md., USA) for β-lactamase production. The identities of all Hib isolates characterized in this study were reconfirmed at the National Institute of Infectious Diseases in Tokyo by using ID Test NH-20 Rapid (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), the Phadebact Haemophilus Test (Boule Diagnostics AB, Huddinge, Sweden), and *Haemophilus influenzae* Antisera a to f (Denka Seiken Co., Ltd., Tokyo, Japan). Of 98 isolates considered to be Hib, several isolates were found not to be Hib in the confirmatory studies. Finally, we analyzed 79 Hib strains for which satisfactory clinical associations were available concerning the patients.

To investigate the possible existence of a reservoir of Hib in family members in close contact with meningitis patients, nasopharyngeal specimens collected from these family members were screened for Hib on chocolate agar plates. Isolates were characterized in the same way as described above.

Antibiotic susceptibility test: MICs of 12 antimicrobial agents (ampicillin, azithromycin, cefaclor, cefotaxime, cefpodoxime, ceftriaxone, chloramphenicol, clarithromycin, levofloxacin, meropenem, rifampicin, sulbactum/ampicillin), commonly used in the treatment of meningitis and pneumonia were determined for the 79 Hib isolates with the Etest (AB Biodisk, Solona, Sweden). After overnight culture of Hib isolates on chocolate agar plates, the colonies were suspended in 3 ml of saline to give a turbidity equal to that of a 0.5 McFarland turbidity standard. Mueller-Hinton agar (Becton Dickinson) with 0.5% yeast extract, hematin (15 μg/ml), and β-NAD (15 μg/ml) was used. Each Hib suspension was spread onto Mueller-Hinton agar plates with a cotton swab. After drying for 15 min, the Etest strips were placed on the plate, and the plates were incubated at 37°C under 5% CO_2–95% air for 18 h. The MIC was interpreted in accordance with the manufacturer's instructions for the Etest.

PCR methods: Hib colonies grown on chocolate agar plates were suspended in 200 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer containing 1.0 v/v % Triton X-100 and boiled for 5 min. PCR was carried out for β-lactamase-producing Hib isolates to identify the genes encoding for TEM-1 and ROB-1 penicillinases (7). For identification of each gene, the following sets of PCR primers were used: for TEM-1 gene, TEM-F (5′-TGGGTGCACGA GTGGGTTAC-3′) and TEM-R (5′-TTATCCGCCTCCAT CCAGTC-3′), and for ROB-1 gene, ROB-F (5′-ATCAGCCA CACAAGCCACCT-3′) and ROB-R (5′-GTTTGCGATTTG GTATGCGA-3′). PCR products for TEM-1 and ROB-1 were 526 bp and 692 bp, respectively. The composition of the PCR mixture was as follows: 2 μl of template, 30 pmol of forward and reverse primers, and 25 μl of premix *Taq* (TaKaRa EX Taq™ Version; Takara Syuzo Co., Kyoto, Japan), with distilled water to give a final reaction volume of 50 μl. The PCR conditions were 2 min at 94°C first, 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for elongation for 30 cycles, and finally 5 min at 72°C. PCR products were electrophoretically resolved on 2% agarose gels, then visualized under UV illumination after ethidium bromide staining.

PFGE: PFGE was performed according to the reported suggestions for typing of *H. influenzae* strains (8), with minor modifications. In brief, chromosomal DNA was digested overnight at 30°C with 20 U of restriction enzyme *Sma*I (Takara Shuzo) in a 200 μl reaction volume, and the digested fragments were separated on a 1% agarose gel with a CHEF Mapper system (Bio-Rad Laboratories, Hercules, Calif., USA). The run conditions were selected by the autoalgorithm mode of the system with a size range of 20 to 300 kb. The PFGE patterns were analyzed by the unweighted pair-group method with arithmetic averages (UPGMA) using Diversity Database version 1.1 software (PDI, Inc., Suddle River, N. J., USA).

RESULTS

Sources of the 79 clinical isolates of Hib by patient sex, age, and specimen: A total of 79 Hib isolates derived from CSF were used in this study. The sources of Hib isolates by patient sex, age, and specimen, and their β-lactamase production and biotype are summarized in Table 1. Forty-five (57%) isolates were β-lactamase-producing and ampicillin-resistant (BLPAR) and 34 (43%) were β-lactamase-nonproducing and ampicillin-sensitive (BLNAS). Although there are TEM- and ROB-types of β-lactamases, ROB-1 was found in only 1 (2%) of 45 BLPAR isolates. Nearly all cases (99%) of Hib meningitis occurred in young children less than 24 months of age, and 86% occurred in infants less than 12 months. Only 1 case (1.3%) of Hib meningitis was found later than 2 years (at 5 years of age). There was no significant difference in the age distribution in males and females (data not shown). The predominant biotype of Hib isolates was type II (68.3%), followed by type I (22.8%). Although we attempted to isolate pathogens from 489 CSFs of patients with suspected bacterial meningitis, we could not isolate any pathogen from over 50% of the CSFs. Beside Hib, other bacterial pathogens isolated from the 100 CSFs were

Table 1. Source of 79 clinical isolates of Hib by patient sex, age, and specimen

Characteristics of Hib isolates	Total No. (%)	No. (%) of isolates	
		BLPAR	BLNAS
Total	79 (100.0)	45 (57.0)	34 (43.0)
Gender			
Male	44 (55.7)	28 (35.4)	16 (20.3)
Female	35 (44.3)	17 (21.5)	18 (22.8)
Age (M)			
1-6	33 (41.8)	17 (21.5)	16 (20.3)
7-12	35 (44.3)	22 (27.8)	13 (16.4)
13-24	10 (12.6)	5 (6.3)	5 (6.3)
>24	1 (1.3)	1 (1.3)	
Specimen type			
CSF	79 (100.0)		
Biotype			
Type I	18 (22.8)		
Type II	54 (68.3)		
Type III	2 (2.5)		
Type IV	3 (3.8)		
Type V	1 (1.3)		
Type VI	0		
Type VII	1 (1.3)		
Type VIII	0		

BLPAR, β-lactamase-producing and ampicillin-resistant; BLNAS, β-lactamase-nonproducing and ampicillin-sensitive; CSF, cerebrospinal fluid.

Table 2. MICs of 12 antimicrobials for 79 Hib isolates

Antibiotics	MIC (μg/ml)			NCCLS breakpoints	
	Range	50%	90%	Susceptible MIC	Resistant MIC
Azithromycin	1-8	4	4	8	
Clarithromycin	4-32	16	32	16	64
Rifampicin	0.064-256	0.25	0.5		
Chloramphenicol	0.5-64	16	64	2	8
β-LA(+)	8-64	16	64		
β-LA(–)	0.5-64	1	32		
Ampicillin	0.125->256	8	256	1	4
β-LA(+)	2->256	>256	>256		
β-LA(–)	0.125-0.5	0.25	0.5		
Sulbactum/Ampicillin	0.125-4	1	2		
β-LA(+)	0.125-4	1	2		
β-LA(–)	0.125-0.5	0.25	0.5		
Ceftriaxone	<0.016-0.032	<0.016	<0.016	2	
Cefaclor	1-4	2	2	8	32
Cefotaxime	<0.016-0.064	0.016	0.032		
Cefpodoxime	0.016-0.064	0.064	0.064		
Levofloxacin	0.008-0.032	0.016	0.032	2	
Meropenem	0.032-0.25	0.125	0.125	0.5	

β-LA(+), β-lactamase producer; β-LA(–), β-lactamase non-producer.

Streptococcus pneumoniae (15%), *Klebsiella pneumoniae* (8%), *Escherichia coli* (6%), β-Streptococcus spp. (5%) such as *S. agalactiae* and *S. pyogenes*, *Neisseria meningitidis* (4%), *Staphylococcus aureus* (3 %), *Pseudomonas aeruginosa* (2%), and other species (5 %).

MICs of 12 antimicrobials for the 79 Hib isolates: Table 2 summarizes the MICs of 12 different antimicrobial agents that have been widely used in the treatment of bacterial meningitis or pneumonia. Some of these agents are not always used for young children in Vietnam. The most effective antibiotics against Hib isolates on the basis of the MICs were ceftriaxone, cefotaxime, cefpodoxime, levofloxacin, meropenem, and rifampicin. The MICs of chloramphenicol, ampicillin, and sulbactum/ampicillin for Hib isolates depended on the existence of β-lactamase. In the case of BLPAR-Hib, both chloramphenicol and ampicillin resistance genes might be located on transferable plasmids that confer multiple-drug resistance (9,10), since cumulative curves of the MICs of these antimicrobial agents for β-lactamase-positive and negative Hib isolates showed different profiles (Fig. 1).

PEGE analysis of Hib isolated from patients with meningitis: As a first step, 50 randomly selected Hib isolates (22 β-lactamase-producing and 28 β-lactamase-nonproducing) were subjected to PFGE with *Sma*I digestion. Visually distinct PFGE patterns of 24 Hib isolates were subjected to a computer-assisted analysis. The PFGE patterns of these Hib isolates and the computed dendrogram are shown in Fig. 2. Vietnamese Hib isolates showed highly divergent PFGE patterns (22 to 100% similarity), as observed in Alaskan and Australian Hib isolates (11,12). The various PFGE patterns could primarily be classified into three clusters. All Hib isolates belonging to cluster A were β-lactamase-nonproducers. However, the PFGE profiles of Hib isolates belonging to clusters B and C were highly divergent, and no significant tendency of β-lactamase production was apparent. Generally speaking, β-lactamase-nonproducing Hib isolates showed somewhat similar PFGE patterns, but β-lactamase-producing isolates showed a variety of PFGE patterns. The similarity between clusters B and C was about 56%, but the similarity between clusters A and B/C was only 22%. There was no relationship between the clusters and biotypes (data not shown).

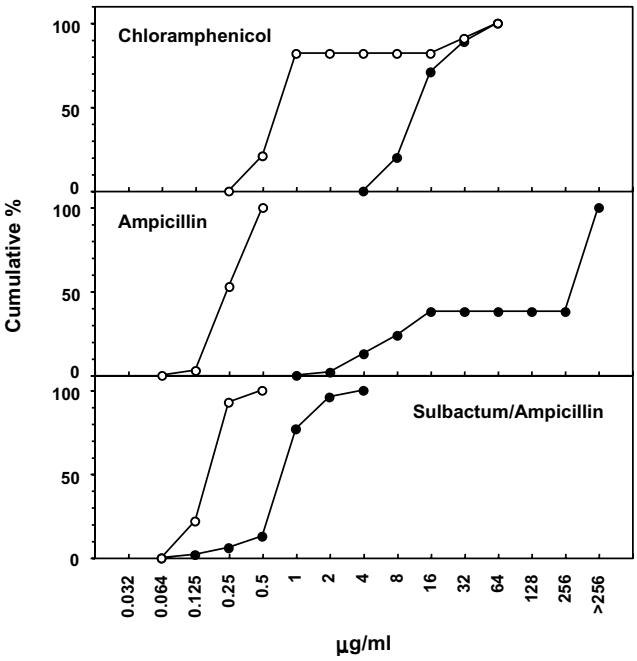

Fig. 1. The cumulative curves of MICs of chloramphenicol, ampicillin and sulbactum/ampicillin for 45 β-lactamase-producing (●) and 34 β-lactamase-nonproducing (○) Hib isolates.

PFGE analysis of Hib isolated from household contacts: To investigate the possible transmission of Hib organisms between meningitis patients and family members in close contact with them, which has been speculated to be one of the major causes of Hib infection in the community, we tried to isolate Hib from nasopharyngeal swabs from members of 11 families by using chocolate agar medium. Nontypable *H. influenzae* or *H. haemolyticum* was isolated from the mem-

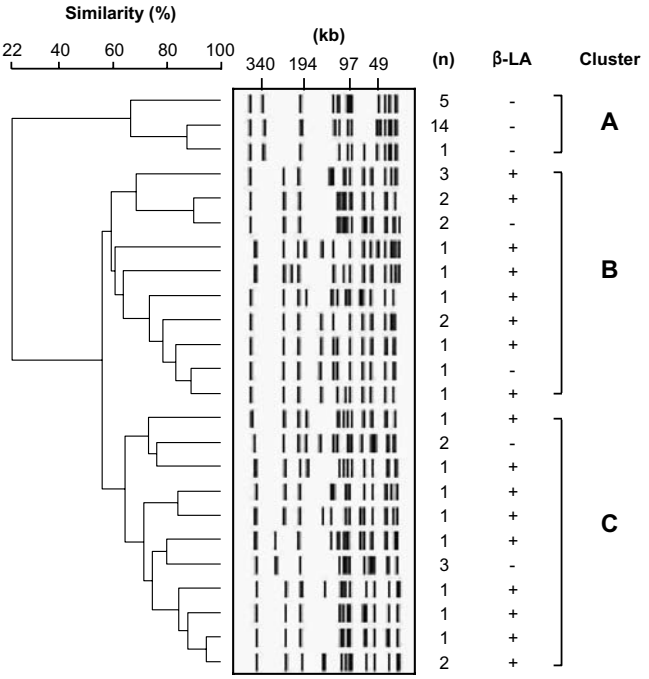

Fig. 2. PFGE patterns of 50 Hib isolates digested with *Sma*I (right) and dendrogram of PFGE results (left). The dendrogram was calculated by UPGMA method.

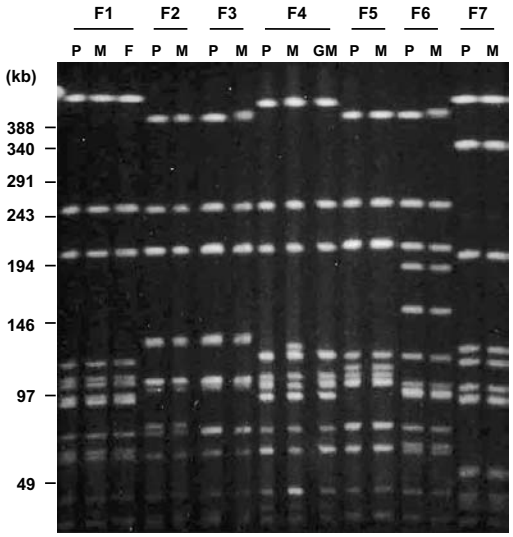

Fig. 3. PFGE patterns of Hib isolates derived from meningitis patients (P) and their family members; mother (M), father (F), and grandmother (GM) in family (F) 1 to 7. Hib isolates from F1, F3, F6, and F7 were β-lactamase-nonproducers, Hibs from F2 were β-lactamase-producers (ROB) and Hibs from F4 and F5 were TEM-1 type β-lactamase-producers.

bers of 4 families. After exclusion of these samples, 16 Hib isolates from the members of 7 families were subjected to PFGE analysis (Fig. 3). All Hib isolates, except for only "M" in F4, derived from any single family showed exactly the same PFGE pattern. Although a minor extra band was found in "M", this isolate showed high similarity (95%) with those from "P" and "GM." Therefore "M" might have originated from the same clones (13). These findings clearly suggest that Hib organisms might be transmitted from a patient and colonize the nasopharynx of family members.

DISCUSSION

Of the 79 Hib strains isolated from the CSFs of meningitis patients in Hanoi, 57% were BLPAR. In Japan, 15.4% of 395 Hib strains isolated from patients with meningitis from 1999 to 2002 were BLPAR, 30.6% were low β-lactamase-nonproducing and ampicillin-resistant (low BLNAR; MIC: 2 μg/ml), and 13.9% were BLNAR (MIC: 8 μg/ml) (5). However, in Vietnam, neither low BLNAR- nor BLNAR-Hib isolates were found in the present study. BLPAR isolates were prevalent in Vietnam, as reported from the United States (14) or Canada (15). Plasmid-mediated β-lactamases, TEM- and ROB-types, have so far been identified in *H. influenzae*, and the former is predominant in general. The global prevalence of *H. influenzae* producing ROB-1 has been reported to be 7 to 11% among β-lactamase-producing isolates (16). However, in this study, ROB-1 was found in only 1 (2%) of 45 β-lactamase-producing Hib isolates. This discrepancy may reflect the kinds of antibacterial agents that have been preferentially used in Vietnam, although the overall isolation frequency of β-lactamase producers is considerably higher than those reported in other countries or districts.

Among the MICs for the 79 Hib isolates, the MICs of chloramphenicol, ampicillin, and sulbactum/ampicillin depended on the production of β-lactamase. β-Lactamases vary considerably from one organism to another. Some are chromosomally encoded, while others are plasmid-mediated; some are constitutive, while others require induction (17). In the case of Hib, both chloramphenicol and ampicillin resistance genes might be located on the transferable plasmids that confer multiple-drug resistance in BLPAR-Hib (9,10). One BLNAS-Hib strain showed a very high MIC to rifampicin (Table 2). We have not yet elucidated the reason for this.

Biotypes of Hib isolates are related to locality and the year of isolation. In the Netherlands, 77 of 80 Hib strains (97%) collected between 1975 and 1982 belonged to biotype I (18). It is said that the vast majority of serotype a, b, and f strains belong to biotype I; serotype c strains are usually biotype II; and strains with serotype d or e capsules are biotype IV (19). However, in Vietnam, the major biotype of Hib isolates was biotype II (68.3%), followed by I (22.8%). This difference may reflect the presence and circulation of specific endemic strains in separate districts, and this shift in the biotypes of Hib isolates is worthy of monitoring hereafter in Vietnam.

PFGE analysis of genomic DNA is one of the most widely accepted standard molecular techniques for epidemiological studies of bacterial isolates. In particular, restriction fragment length polymorphism (RFLP) typing by PFGE is the most practical aid for investigating outbreaks when it is applied to small sets of isolates that are epidemiologically related (13). In our study, the PFGE for Hib strains showed a variety of RFLP patterns from isolate to isolate, but they could primarily be classified into three clusters. All the Hib isolates belonging to cluster A were β-lactamase-nonproducers. However, the productivity of β-lactamases among isolates belonging to clusters B and C was highly divergent. In general, β-lactamase-nonproducing Hib isolates showed somewhat similar PFGE patterns, but β-lactamase-producing isolates showed very divergent PFGE patterns. Hasegawa et al. (20) reported the PFGE profiles of 51 Hib strains treated with the endonuclease *Sma*I. They classified their PFGE profiles into 4 patterns (I to IV). Patterns I and II were BLNAR and showed higher similarity. Although it is difficult to compare our data with those reported by Hasegawa et al., it seems that

Hib isolates belonging to our cluster A (BLNAS) are similar to those belonging to patterns I and II. This finding may suggest the presence of a clonal expansion of the BLNAR lineage of Hib organisms that have been proliferating in separate countries or geographical districts.

Hib isolates derived from family members in close contact with meningitis patients showed exactly the same PFGE patterns as those of the patients. This finding clearly suggests that Hib organisms might be transmitted from meningitis patients to family members and colonize the nasopharynx. These family members may well act as possible spreaders of Hib organisms in the community. It is well known that household contacts of colonized day-care children are a reservoir of Hib (21-23).

As noted in the Introduction, the reported incidences of Hib-associated bacterial meningitis in Asian countries are lower than those in European countries and the US prior to the introduction of Hib conjugate vaccines (2,4,6,15). The apparently low incidences in Asia could be due to inadequate microbiological or epidemiological methods, the effects of antibiotic use, genetic differences in patients and/or Hib strains, or low lumbar-puncture rates (4). Although we attempted to isolate relevant pathogens from 489 CSF samples from patients with suspected bacterial meningitis, we could not isolate any pathogen from over 60% of the samples. Low recovery of pathogens from CSF may be one of the reasons for the apparently low incidence of Hib meningitis in Vietnam. Therefore, we are now developing a multiplex PCR method that should enable us to simultaneously detect multiple pathogens in CSF. In Vietnam, 10 vaccines (diphtheria-tetanus-pertussis, polio, measles, BCG, hepatitis B, Japanese encephalitis, cholera, typhoid) are included in the National Immunization Program (NIP) to prevent vaccine-preventable diseases, but the Hib vaccine has not yet been included. NIP vaccines are free of charge and in principle are obligatory for the target infants and children. In this study, it became clear that nearly all cases (99%) of Hib meningitis occurred in children less than 24 months of age, and 87% of the cases occurred in infants under 12 months. The introduction of Hib conjugated vaccination for infants less than 12 months of age in Vietnam is therefore worthy of urgent consideration (24).

ACKNOWLEDGMENTS

This study was conducted with the support of a grant for Research Promotion on Emerging and Re-emerging Infectious Diseases (H15-Shinko-24) from Ministry of Health, Labour and Welfare of Japan.

REFERENCES

1. 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.
2. Rerks-Ngarm, S., Treleaven, S. C., Chunsuttiwat, S., Muangchana, C., Jolley, D., Brooks, A., Dejsirilert, S., Warintrawat, S., Guiver, M., Kunasol, P., Maynard, J. E., Biggs, B.-A. and Steinhoff, M. (2004): Prospective population-based incidence of *Haemophilus influenzae* type b meningitis in Thailand. Vaccine, 22, 975-983.
3. Kim, J. S., Jang, Y. T., Kim, J. D., Park, T. H., Park, J. M., Kilgore, P. E., Kennedy, W. A., Park, E., Nyambat, B., Kim, D. R., Hwang, P. H., Kim, S. J., Eun, S. H., Lee, H. S., Cho, J. H., Kim, Y. S., Chang, S. J., Huang, H. F., Clemens, J. D. and Ward, J. I. (2004): Incidence of *Haemophilus influenzae* type b and other invasive diseases in South Korean children. Vaccine, 22, 3952-3962.
4. Gessner, B. D., Sutanto, A., Linehan, M., Djelantik, I. G., Fletcher, T., Gerudug, I. K., Ingerani, Mercer, D., Moniaga, V., Moulton, L. H., Mulholland, K., Nelson, C., Soemohardjo, S., Steinhoff, M., Widjaya, A., Stoeckel, P., Maynard, J. and Arjoso, S. (2005): Incidences of vaccine-preventable *Haemophilus influenzae* type b pneumonia and meningitis in Indonesian children: hamlet-randomised vaccine-probe trial. Lancet, 365, 43-52.
5. Kamiya, H., Uehara, S., Kato, T., Shiraki, K., Togashi, T., Morishima, T., Leboulleux, D., Goto, Y. and Sato, O. (2000): Nationwide comprehensive epidemiologic study of childhood bacterial meningitis in Japan. p. 238. Abstracts. The 40th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto.
6. Dang, D. A., Phan, L. T. H., Nguyen, L. H., Nguyen, C. K., Nguyen, H. A., Long, T. H., Kennedy, W., Kilgore, P. E. and Clemens, J. D. (2001): Bacterial meningitis in Hanoi, 2000-2002. J. Prevent. Med., 47, 18-22.
7. Tenover, F. C., Huang, M. B., Rasheed, J. K. and Persing, D. H. (1994): Development of PCR assays to detect ampicillin resistance genes in cerebrospinal fluid samples containing *Haemophilus influenzae*. J. Clin. Microbiol., 32, 2729-2737.
8. Saito, M., Umeda, A. and Yoshida, S. (1999): Subtyping of *Haemophilus influenzae* strains by pulsed-field gel electrophoresis. J. Clin. Microbiol., 37, 2142-2147.
9. Anderson, J. R., Smith, M. D., Kibbler, C. C., Holton, J. and Scott, G. M. (1994): A nosocomial outbreak due to non-encapsulated *Haemophilus influenzae*: analysis of plasmids coding for antibiotic resistance. J. Hosp. Infect., 27, 17-27.
10. Leaves, N. I., Dimopoulou, I., Hayes, I., Kerridge, S., Falla, T., Secka, O., Adegbola, R. A., Slack, M. P. E., Peto, T. E. A. and Crook, D. W. M. (2000): Epidemiological studies of large resistance plasmids in *Haemophilus*. J. Antimicrob. Chemother., 45, 599-604.
11. Lucher, L. A., Reeves, M., Hennessy, T., Popovic, T., Rosenstein, N. and Parkinson, A. J. (2002): Reemergence in southwestern Alaska of invasive *Haemophilus influenzae* type b disease due to strains indistinguishable from those isolated from vaccinated children. J. Infect. Dis., 186, 958-965.
12. Moor, P. E., Collignon, P. C. and Gilbert, G. L. (1999): Pulsed-field gel electrophoresis used to investigate genetic diversity of *Haemophilus influenzae* type b isolates in Australia shows differences between Aboriginal and non-Aboriginal isolates. J. Clin. Microbiol., 37, 1524-1531.
13. Tenover, F. C., Arbeit, D. R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. and Swaminathan, B. (1995): Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing. J. Clin. Microbiol., 33, 2233-2239.
14. Doern, G. V., Brueggemann, A. B., Pierce, G., Holley, H. P., Jr. and Rauch, A. (1997): Antibiotic resistance

among clinical isolates of *Haemophilus influenzae* in the United States in 1994 and 1995 and detection of beta-lactamase-positive strains resistant to amoxicillin-clavulanate: results of a national multicenter surveillance study. Antimicrob. Agents Chemother., 41, 292-297.
15. Karlowsky, J. A., Verma, G., Zhanel, G. G. and Hoban, D. J. (2000): Presence of ROB-1 β-lactamase correlates with cefaclor resistance among recent isolates of *Haemophilus influenzae*. J. Antimicrob. Chemother., 45, 871-875.
16. Galán, J-C., Morosini, M-I., Baquero, M-R., Reig, M. and Baquero, F. (2003): *Haemophilus influenzae* bla_{ROB-1} mutations in hypermutagenic Δ*amp*C *Escherichia coli* conferring resistance to cefotaxime and β-lactamase inhibitors and increased susceptibility to cefaclor. Antimicrob. Agents Chemother., 47, 2551-2557.
17. Handal, T. and Olsen, I. (2000): Antimicrobial resistance with focus on oral β-lactamases. Eur. J. Oral Sci., 108, 163-174.
18. van Alphen, L., Riemens, T., Poolman, J., Hopman, C. and Zanen, H. C. (1983): Homogeneity of cell envelope protein subtypes, lipopolysaccharide serotypes, and biotypes among *Haemophilus influenzae* type b from patients with meningitis in the Netherlands. J. Infect. Dis., 148, 75-81.
19. Kilian, M. (2003): *Haemophilus*. p. 623-635. *In* Murray, P. R., Baron, E. J., Pfaller, M. A., Tenover, F. C. and Yolken, R. H. (ed.), Manual of Clinical Microbiology. 8th ed. American Society for Microbiology, Washington, D.C.
20. Hasegawa, K., Chiba, N., Kobayashi, R., Murayama, S. Y., Iwata, S., Sunakawa, K. and Ubukata, K. (2004): Rapidly increasing prevalence of β-lactamase-nonproducing, ampicillin-resistant *Haemophilus influenzae* type b in patients with meningitis. Antimicrob. Agents Chemother., 48, 1509-1514.
21. Li, K. I., Dashefsky, B. and Wald, E. R. (1986): *Haemophilus influenzae* type b colonization in household contacts of infected and colonized children enrolled in day care. Pediatrics, 78, 15-20.
22. Makintubee, S., Istre, G. R. and Ward, J. I. (1987): Transmission of invasive *Haemophilus influenzae* type b disease in day care settings. J. Pediatr., 111, 180-186.
23. Redmond, S. R. and Pichichero, M. E. (1984): *Haemophilus influenzae* type b disease. An epidemiologic study with special reference to day-care centers. JAMA, 252, 2581-2584.
24. Watanabe, H., Kaji, C., Dang, D. A., Phan, L. T. H., Nguyen, T. H. A., Vu, T. H., Hoang, V. M. P., Ngo, T. T., Pham, T. S., Nguyen, T. T. N., Rusizoka, O. S., Watanabe, K., Nagatake, T. and Oishi, K. (2005): Comparative molecular analysis of *Haemophilus influenzae* isolates from young children with acute lower respiratory tract infections and meningitis in Hanoi, Vietnam. J. Clin. Microbiol., 43, 2474-2476.