Serotypes and Antimicrobial Susceptibility in Clinical Isolates of *Haemophilus influenzae* from Korean Children in Prevaccination Era

Fifty-five strains of *Haemophilus influenzae* recovered at a children's hospital in Korea from 1992 through 1997, were analyzed for serotype and antibiotic resistance. Antimicrobial susceptibility was tested by broth dilution method. Among the 55 strains, 26 were from normally sterile body fluids, of which 17 were from the immunocompetent children. Spectrum in the immunocompetent included meningitis (47%), bacteremic pneumonia (18%), and bacteremia without focus (35%). Three (12%) of 26 invasive infections were caused by non-type b: one type d and two type f. Nine of 29 non-sterile body fluid isolates belonged to one of encapsulted serotypes: four a, two c, one of each of b, d and e. Thirty two (58%) strains were resistant to ampicillin, and all of which produced β-lactamase. All of the strains were highly susceptible to amoxicillin/clavulanate, cefixime, cefuroxime, azithromycin and ciprofloxacin, while 1 (2%), 7 (13%), 4 (7%) and 4 (7%) strains were intermediate to cefprozil, cefaclor, loracarbef, and clarithromycin, respectively. The serotype distribution of *H. influenzae* in Korean children is similar to those in developed countries before the introduction of Hib conjugate vaccine, and ampicillin resistance rate is among the highest published to date.

Key Words : *Haemophilus influenzae; Serotyping; Drug Resistance, Microbial; Korea*

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INTRODUCTION

Haemophilus influenzae is an important pathogen of invasive bacterial infections such as meningitis, septicemia, pneumonia and epiglottitis in infants and children, as well as of focal respiratory tract infections such as sinusitis and otitis media. Among the six encapsulated serotypes (a through f) and unencapsulated, nontypable strains, type b (Hib) has been the leading cause of invasive disease in infants and children until the introduction of protein-conjugate Hib vaccine (1). Other serotypes and/or unencapsulated organisms also have a significant role in invasive infections in the neonates (2), adults (3) and immunocompromised hosts (3) and in the developing countries (4, 5). Recently, they have come to attention with marked decrease of Hib disease after universal immunization of Hib conjugate vaccine in the developed countries. The majority of focal, noninvasive respiratory infections by *H. influenzae* are caused by unencapsulated organisms (6-8).

Antimicrobial resistance of *H. influenzae* is an enlarging problem worldwide. Production of β-lactamase among

H. influenzae strains is steadily increasing since the first documented cases in the early 1970s (9). Prevalence of β-lactamase production has ranged from 1% to 40% in different parts of the world (10, 11) and has exceeded 30% in the U.S.A. in the survey of 1992-3 (12). Recent identification of β-lactamase positive amoxicillin-clavulanate resistant strain (13) as well as β-lactamase negative ampicillin resistant strain (14) raises more concern.

Epidemiology, serotype and antimicrobial susceptibility of *H. influenzae* infection are well-characterized in the developed countries and some variations among the different countries or ethnic groups have been reported. English literature, however, on the aforementioned aspects of *H. influenzae* infection among Asian countries is quite limited and so is in Korea (15). Information on *H. influenzae* infections in terms of clinical spectrum, serotype and antimicrobial susceptibility in a particular community is essential in the establishment of policy for the Hib conjugate vaccine and empirical antimicrobial therapy.

In this report, *H. influenzae* recovered at a university hospital in Korea over six year period from 1992 through

1997, when less than 20% of children received conjugate Hib vaccine, was analyzed with special reference to clinical spectrum of the infections, serotype and resistance to commonly used antimicrobials.

MATERIALS AND METHODS

Isolates

This study included 55 strains of *H. influenzae* collected prospectively at the microbiology laboratory of the Seoul National University Children's Hospital (SNUCH), a tertiary care and referring center, over six-year period from January 1992 to December 1997. In that period a total of 64 strains were identified and kept frozen, and 55 of them were successfully recovered. The organisms were recovered from a variety of clinical specimens, including normally sterile body fluid (blood, cerebrospinal fluid), respiratory tract specimens (specimen from lower respiratory tract, throat swab, aspirate of paranasal sinus), middle ear fluid, conjunctival swab and genitourinary tract discharge. None of the strains that failed to regrow were from normally sterile body fluid. Isolates were identified as *H. influenzae* by using conventional method and all isolated strains were kept frozen at -70℃. Specimens were later thawed for serotyping and antimicrobial susceptibility testing. Only one isolate from each patient was included in this series.

Serotyping

Serotyping was performed by the Quellung reaction, slide agglutination method using commercially available antisera (Difco Laboratories, Detroit, MI, U.S.A.). Each isolate was evaluated with pooled sera, negative control (normal saline) and six monospecific antisera for serotypes "a" through "f" to control cross-reactivity and autoagglutination.

Antimicrobials

The antimicrobials used for susceptibility test were ampicillin, amoxicillin, amoxicillin/clavulanate, cefaclor, cefixime, cefprozil, cefuroxime, loracarbef, azithromycin, clarithromycin and ciprofloxacin.

Determination of minimum inhibitory concentrations (MIC)

MICs were determined by the broth dilution method using microwell plate. Broth microdilution tests were performed according to the National Committee for

Clinical Laboratory Standards (NCCLS) guidelines (1997) (16). All microdilution trays were made with Haemophilus Test Medium (PML Microbials, Tualatin, OR, U.S.A.). Inoculum size was confirmed by performing viable organism counts for each strain of organism. The *H. influenzae* quality-control (QC) strains [ATCC (American Type Culture Collection) 49247 and ATCC 49766], as specified by the NCCLS (16) for *H. influenzae* MIC testing, were included for each testing. In addition, *Escherichia coli* ATCC 35218, the NCCLS recommended TEM-1 β-lactamase-producing strain (16), was used to control the inhibitor component of amoxicillin/clavulanic acid. The results of clinical strain testing were only accepted for analysis if all QC results were within the limits specified by the NCCLS (16).

Interpretive category criteria used for determination of susceptibility were from the NCCLS performance standards for antimicrobial susceptibility testing document (16). Because no interpretive criteria have been established for amoxicillin, only MIC range, MIC_{50} and MIC_{90}, was presented (Table 3).

Production of β-lactamase

β-lactamase activity in whole bacteria was sought in a chromogenic cephalosporin assay (Cefinase; BBL Microbiology Systems, Cockeysville, MD, U.S.A.) (17). β-lactamase-positive and -negative organism controls were included for each testing.

Clinical data

Medical records for all patients were reviewed retrospectively for age, site(s) of infection, clinical diagnosis, pre-existing condition, immunosuppression, and pertinent clinical data. Patients were regarded as immunosuppressed if they were undergoing chemotherapy and/or radiotherapy for malignant disease or if they were taking immunosuppressive agents such as corticosteroids.

RESULTS

Sources of organisms and clinical characteristics of the patients

Among the total of 55 strains, 26 were from normally sterile-body fluids: 20 from blood and 6 from cerebrospinal fluid. Two of the patients with positive blood culture had evidence of meningitis, 6 had pneumonia and one had cellulitis (Table 1). The rest of the strains were from respiratory tract (12 strains from specimen from lower respiratory tract, 1 from throat swab, 1 from para-

Table 1. Disease spectrum of invasive infections caused by *H. influenzae* at the Seoul National University Children's Hospital, by immune status and/or underlying diseases

Diseases	Immunocompetent			Immunosuppressed[+]	Total
	Without underlying diseases[+]	With underlying diseases[+]	Subtotal		
Meningitis	6*	2	8	0	8
Pneumonia (bacteremic)	2	1	3	3	6
Bacteremia (no focus)	2	4	6	5	11
Cellulitis	0	0	0	1	1
Total	10	7	17	9	26

*One patient had arthritis in addition to meningitis.
[+]Underlying diseases are as follows: 2 patients with meningitis - hydrocephalus and ventriculoperitoneal shunt, respectively; the patient with pneumonia - seizure disorder; the 4 patients with bacteremia without focus - cardiomyopathy, convulsive disorder, bronchiolitis obliterans and corrosive esophagitis, respectively.
[+]Immunosuppresive disorders include acute leukemia (4 cases), chronic myelogenous leukemia (1), aplastic anemia (1), primary B-cell deficiency (hyper IgM syndrome; 1) and nephrotic syndrome (2).

Table 2. Distribution of serotypes among 55 strains of *H. influenzae* isolated from children at Seoul National University Children's Hospital, by source of specimen

Serotype	Source of isolates						Total
	Sterile body fluid		Respiratory* specimen	Conjunctival swab	Middle ear fluid	Vaginal swab	
	Blood	CSF					
Nonencapsulated	0	0	11	2	5	2	20
Encapsulated							
Type a	0	0	1	2	0	1	4
Type b	17	6	1	0	0	0	24
Type c	0	0	0	2	0	0	2
Type d	1	0	1	0	0	0	2
Type e	0	0	0	0	1	0	1
Type f	2	0	0	0	0	0	2
Total	20	6	14	6	6	3	55

*Includes specimen from lower respiratory tract, sinus aspirate and throat swab

nasal sinus), middle ear (6 strains), eye (6 strains) and genitourinary tract (3 strains) (Table 2).

Out of the 26 invasive infections, 17 developed in the immunocompetent hosts (7 of them had underlying diseases), and the remaining 9 in the immunosuppressed (Table 1). Spectrum in the immunocompetent children included meningitis (47%), bacteremic pneumonia (18%), and bacteremia without focus (35%), and there was no case of cellulits or epiglottitis. One of the patients with meningitis had pyogenic arthritis also. One patient with cellulitis had nephrotic syndrome receiving corticostroid, and the lesion developed at the thigh.

Age of immunocompetent patients with invasive infections ranged from 1 to 61 months, while that of the immunocompromised ranged from 3 to 115 months and 5 of 9 were 60 months of age or older.

Serotypes

Distribution of serotypes by source of specimen is sum-

marized in Table 2. Three (12%) of 26 invasive infections were caused by non-type b *H. influenzae*. One type d infection occurred in 61 months-old boy associated with bacteremic pneumonia. Both of type f infection occurred in patients with nephrotic syndrome; a 66 months-old girl had cellulits and a 115 months-old boy had bacteremia without focus.

Nine of 29 non-sterile body fluid isolates belonged to one of encapsulted serotypes: four type a strains from conjunctival swab, specimen from lower respiratory tract and vaginal swab; one type b from specimen from lower respiratory tract; both of two type c from conjunctival swab; one type d from aspirate of paranasal sinus; and one type e from middle ear fluid.

Antimicrobial susceptibility

Susceptibility test results and MIC values for *H. influenzae* are summarized in Table 3. Among 55 strains, 32 (58%) strains displayed resistance to ampicillin. All

Table 3. Susceptibility test results and MIC values for isolates of *H. influenzae* isolated from children at the Seoul National University Children's Hospital, 1992-1997

Antimicrobial	MIC range / MIC$_{50}$ / MIC$_{90}$ (μg/mL)	Susceptibility test results*					
		Percentage of strains With indicated result			Breakpoint (μg/mL)[†]		
		S	I	R	S	I	R
Ampicillin	0.12->16 / 16 / >16	42	0	58	$\leq$1	2	$\geq$4
Amoxicillin	0.12->16 / 16 / >16						
Amoxicillin/clavulanate	0.12-4 / 0.5 / 1	100	0	0	$\leq$4/2	-	$\geq$8/4
Cefixime	0.008-16 / 0.03 / 0.06	100	0	0	$\leq$1	-	-
Cefuroxime	0.25-4 / 0.5 / 2	100	0	0	$\leq$4	8	$\geq$16
Cefprozil	0.5-0.12 / 2 / 8	93	7	0	$\leq$8	16	$\geq$32
Cefaclor	0.06-16 / 4 / 16	87	13	0	$\leq$8	16	$\geq$32
Loracarbef	0.5-16 / 1 / 8	98	2	0	$\leq$8	16	$\geq$32
Azithromycin	0.12-2 / 0.5 / 1	100	0	0	$\leq$4	-	-
Clarithromycin	0.5-16 / 4 / 8	93	7	0	$\leq$8	16	$\geq$32
Ciprofloxacin	0.03-0.6 / 0.03 / 0.03	100	0	0	$\leq$1	-	-

*S, susceptibility; I, intermediate resistance; R, resistance
[†]Based on the interpretive guidelines of the National Committee for Clinical Laboratory Standards (1997) (21). Because no interpretive criteria have been established for amoxicillin, only MIC range, MIC50 and MIC90 information was presented.

of the ampicillin resistant strains produced β-lactamase and were susceptible to amoxicillin/clavulanate. There was no ampicillin resistance mediated by mechanism other than β-lactamase production. Ten (50%) of 20 nontypable strains, 16 (67%) of 24 type b and 6 (55%) of 11 non-type b encapsulated strains produced β-lactamase. Annual prevalence of β-lactamase production was 10/17 in 1992, 2/3 in 1993, 2/3 in 1994, 4/10 in 1995, 10/15 in 1996 and 4/8 in 1997.

The frequency of *H. influenzae* resistance to ampicillin varied with the source of the specimens from which the isolate came. Values ranged from 50% (3/6) for ear fluid to 100% (6/6) for CSF, and included 67% (4/6) for conjunctival swab, 72% (8/11) for respiratory specimens and 45% (9/20) for blood. Fifty-seven percent of 26 isolates from invasive disease were resistant to ampicillin.

All of the strains were highly susceptible to cefixime (MIC$_{90}$, 0.06 μg/mL) and cefuroxime (MIC$_{90}$, 2 μg/mL), while 1 (2%), 7 (13%) and 4 (7%) strains were intermediate to cefprozil, cefaclor, and loracarbef, respectively. Azithromycin (MIC$_{90}$, 1 μg/mL) was more active than clarithromycin (MIC$_{90}$, 8 μg/mL). All the strains were susceptible to ciprofloxacin with very low MICs (MIC$_{90}$, 0.03 μg/mL).

DISCUSSION

Major clinical entities of invasive *H. influenzae* infections include meningitis, epiglottitis, pneumonia, arthritis, cellulitis, etc (18, 19). Relative frequency of each of these may be variable in different countries and ethnic

groups depending on background epidemiology of *H. influenzae* infections. For example, among the children of Alaskan natives who are at very high risk of invasive *H. influenzae* infections in the first year of life, epiglottitis is very rare (20), while it is second or third most common manifestation of invasive *H. influenzae* infections in most of other studies in developed countries.

Information on epidemiology including clinical spectrum of invasive *H. influenzae* infections among Asian children has been limited. In recent publications from Far Eastern countries including China and Japan, the annual incidence of invasive *H. influenzae* infections in these areas is reported as <10/100,000 of children under 5 years of age (21-24). Lau et al. observed that among 39 cases of invasive *H. influenzae* infections developed in Chinese residing in Hong Kong, similarly to the SNUCH series, there was no case of epiglottitis, septic arthritis or cellulitis (in immunocompetent children), and 14 of them had pre-existing medical condition (21). In a review on 29 cases of septic arthritis diagnosed during 12 year-period from 1986 to 1997 at the SNUCH, most cases were caused by *Staphylococcus aureus*. The case of septic arthritis associated with meningitis, which was included in the results section, was the only case of arthritis caused by *H. influenzae* (unpublished data). Also, a lot of viral croup has been diagnosed in this hospital, but, suspected bacterial epiglottitis has been extremely rare (25). Although the possibility of underdetection and overuse of antibiotics contributing to the apparent low incidence can not be completely excluded, above publications as well as the results of this paper suggest that the incidence of invasive *H. influenzae* infections in Asian children may

be lower and that the spectrum of diseases may be different compared to those of western countries.

Of the six serotypes and unencapsulated strains of *H. influenzae*, type b has been the most frequent cause of invasive diseases in children before the introduction of the conjugate vaccine. However, there is a considerable variation in the role of *H. influenzae* non-type b (nontypable and other serotypes) organisms in invasive infections in different countries or populations. Precise proportion caused by non-type b in serious disease of children is difficult to determine, because heterogenous groups of population such as neonates, adults or immunocompromised hosts were included in many of the studies. These groups are frequently infected by non-type b.

In most studies performed in industrialized societies, non-type b was isolated in <1% to 5% of invasive *H. influenzae* infections (18, 19, 26). In the developing countries, invasive diseases caused by non-type b are more frequently observed. In New Guinea, type b strains were isolated from only 82% of patients with meningitis, and 13 (18%) of 73 strains causing meningitis were non-type b: 9 (12%) type a, 1 (1%) type f and 3 (4%) nontypable (5). Type a organism is also known to cause meningitis in African (27) and Apache Indian children (28). In addition, pneumonia in the developing countries is caused more frequently by non-type b. Of 32 strains of *H. influenzae* isolated from blood and/or lung aspirates of New Guinean children with pneumonia, only 6 (19%) strains were type b; the rest were non-type b serotypes (8 strains, 25%; 1 type a, 3 type c, 2 type d, 1 of each of type e and f) or nontypable (18 strains, 56%) (4).

Nontypable strain is mainly associated with localized sinopulmonary infection and can cause invasive disease in hosts with underlying immunosuppressive or cardiopulmonary disorders. In contrast, non-b type encapsulated strains are considered less virulent and less commonly identified as cause of clinical disease. Compared with type b disease, invasive disease caused by non-type b serotypes was similar to that caused by nontypable organisms in that a higher proportion of disease occurred in older persons and non-type b organisms were less likely to cause meningitis in younger age group (1).

In the present study, only 1 of 17 strains from immunocompetent children was non-type b serotype (type d), and both of 2 strains of non-type b from the immunocompromised were of type f and from patients with nephrotic syndrome. This result is similar to the serotype distribution of the developed countries in the pre-vaccination era. According to Wenger et al., type f strain was the second common cause of systemic infections by *H. influenzae* in the U.S.A. (1), and is being considered as an emerging cause of invasive disease in children as well as in the immunocompromised and elderly patient

(7). Reports on invasive diseases caused by serotype d are scanty but clinical feature of type d infection is not so different from those caused by other encapsulated serotype organisms (29).

In areas where Hib conjugate vaccine is routinely administered, invasive disease caused by type b has been decreased dramatically (30). As a consequence, nontypable and non-b type encapsulated strains became proportionally more common pathogen of invasive disease (1, 7, 8).

After ampicillin resistance was reported among clinical isolates of *H. influenzae* in 1972 in European country and in 1974 in the United States (9), increasing tendency of ampicillin resistance has been reported in many countries. In the United States, national multi-center surveillance in 1994-1995 and 1996-1997 revealed that overall 33-39% of the isolates were found to be resistant to ampicillin (13, 31, 32). In European countries, the collaborative study in 1986 indicated the overall ampicillin resistance rate of 10% with the highest in Spain (30.6%) and the lowest in Germany (1.6%) (33), and current rates are 30-40% in Spain, 30% in Greece, 20-30% in France, 25% in Italy, 10-20% in the United Kingdom, and 10% in Sweden (34). Egypt (7%) and Central Africa Republic (1.4%) have low resistance rates (10). Information from Asian countries is quite limited; ampicillin resistance rates were 15.8% in Japan (1994-1995) (35), 27% in Taiwan (1989) (36), and 40.5% in Singapore (1996) (11).

In Korea, there have been a few data on the antimicrobial resistance of *H. influenzae*. In 1985, 22.6% of 51 respiratory isolates were found to be ampicillin-resistant by disk diffusion test (37). Among 239 strains from various clinical specimens isolated from 1988 to 1989, ampicillin resistance rate was 21.9% by disk diffusion test (38). In 1991, 31% of 288 strains from respiratory specimens produced β-lactamase (39). The present paper presents 58% of ampicillin resistance by production of β-lactamase, and annual rates were similar throughout the 6 year study period. These observations indicate that the ampicillin resistance of *H. influenzae* in Korea has increased rapidly during the last 10 years and that the current resistance rate is among the highest published to date.

The mechanism of resistance to ampicillin usually involves plasmid-mediated β-lactamase enzyme content. After the first report on ampicillin-resistant strains by production of plasmid-mediated β-lactamase, TEM-1 (9), another β-lactamase enzyme, named rob or ROB-1 was found (40). More recently identified mechanism includes production of altered penicillin-binding-protein (PBP) (14), resulting in β-lactamase negative ampicillin resistant and β-lactamase positive amoxicillin-clavulanate resistant strains (13). In this series, β-lactamase negative and ampicillin resistant strains were not identified despite

the high rate of ampicillin resistance. Because the number of strains included in this series is rather small, however, existence of resistance by such mechanism in Korea cannot be excluded. Continued surveillance for prevalence and mechanism of ampicillin resistance of *H. influenzae* is needed.

In conclusion, result of this study suggests that the clinical spectrum of invasive *H. influenzae* infections in Korean children may be different from those of western countries, though serotypes are not different. Ampicillin resistance of *H. influenzae* resulting from β-lactamase production is as high as almost 60% and treatment of *H. influenzae* infection is becoming a challenging problem in Korea.

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