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# High prevalence of nasal carriage of β-lactamase-negative ampicillin-resistant *Haemophilus influenzae* in healthy children in Korea

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## SUMMARY

This study investigated the carriage of antimicrobial resistant *Haemophilus influenzae* in 582 healthy children attending kindergarten or elementary school at four intervals over a 9-month period in Seoul, Korea. Diverse colonization patterns and a lower level of long-term persistent carriage by *H. influenzae* status were evident in this study. Colonizing *H. influenzae* isolates showed a high rate of resistance to β-lactams including ampicillin (51·9%), cefaclor (52·1%), and amoxicillin/clavulanate (16·3%). Based on the ampicillin resistance mechanism, *H. influenzae* isolates were categorized as β-lactamase-negative, ampicillin-susceptible (BLNAS) (48·1%), β-lactamase-positive, ampicillin-resistant (BLPAR) (22·6%), β-lactamase-negative, ampicillin-resistant (BLNAR) (22·8%), and β-lactamase-positive, amoxicillin/clavulanate-resistant (BLPACR) strains (6·5%). This study provides the first evidence of a high prevalence (22·8%) of BLNAR strains of *H. influenzae* nasal carriage in healthy children attending kindergarten or the first 2 years of elementary school in Korea. The high carriage of these resistant strains in overcrowded urban settings may create reservoirs for development of *H. influenzae*-resistant strains.

**Key words**: BLNAR strains, *Haemophilus influenzae*, nasal carriage.

## INTRODUCTION

Upper respiratory tract infections (URTIs) are a frequent cause of antibiotic prescriptions to outpatients and pose an appreciable public health burden in the community. Potential respiratory pathogens such as *Streptococcus pneumoniae* and *Haemophilus influenzae* often colonize the human URT without producing clinical symptoms [1].

Colonization is regarded as a prerequisite to RTIs or a source for transmission between individuals [2]. For acute otitis media, colonization by potential respiratory pathogens increases the risk of complications and recurrence [3]. Thus, a reduction of carriage of *H. influenzae*, which is frequently associated with acute otitis media, could reduce the incidence of infection [2]. With the widespread use of the conjugate *H. influenzae* type b (Hib) vaccine in many countries, colonization rates have decreased markedly, as has the incidence of *H. influenzae* type b invasive diseases [4].

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Until now, Hib vaccine was not included in the National Immunization Programme and has been administered in the private sector for Korean infants. Thus, there are many limitations of interpretation of Hib disease incidence and the influence of Hib vaccination in our communities.

Given the high prevalence of $\beta$-lactamase production in *H. influenza* strains, the widespread usage of oral cephalosporins and amoxicillin/clavulanate has raised concern about increased resistance to $\beta$-lactam antibiotics in Korea. Adding weight to this concern, the latest Korean Nationwide Acute Respiratory Infections Surveillance (ARIS) study reported that 47·2% of *H. influenzae* clinical isolates were $\beta$-lactamase-positive, ampicillin-resistant (BLPAR), with the emergence of $\beta$-lactamase-negative, ampicillin-resistant (BLNAR) (6·1%) and $\beta$-lactamase-positive, amoxicillin-clavulanate-resistant (BLPACR) (5·2%) *H. influenzae* [5]. These BLNAR and BLPACR strains originated mainly from young children who were examined as outpatients of primary clinics throughout Korea.

In Korea, many young children attend day-care facilities, which are a recognized source of respiratory pathogens. This may be a trigger for the increased incidence of RTIs and the emergence and subsequent spread of resistant *H. influenzae* in the community setting [6, 7]. Little is known about the nasal carriage rate of antibiotic resistant *H. influenzae* in young children attending day-care centres or schools in Korea. The present study investigated the nasal carriage rate of antibiotic-resistant *H. influenzae* in young children attending three kindergartens and one elementary school in a 1-year longitudinal carriage study. We identified BLNAR and BLPACR strains and analysed the genetic characteristics in ampicillin-resistant *H. influenzae* isolates. In addition, we determined the colonization patterns in each individual and characterized the genotypes of *H. infuenzae* repeatedly isolated from the same children during four sampling periods by molecular typing methods.

## METHODS

### Bacteria isolates

All 440 *H. infleunzae* strains were derived from the 2328 nasal aspirates of 582 healthy children attending kindergarten (mean age $5{\cdot}6 \pm 1{\cdot}2$ years) or the first 2 years of elementary school (mean age $8{\cdot}4 \pm 0{\cdot}6$ years) located in Seoul, Korea during the four sampling times (June, September, December 2006, February 2007) in our previous longitudinal nasal carriage study [8]. Of the 440 isolates, 214 *H. influenzae* were isolated from 660 samples of the younger kindergarten children (carriage rate 32·4%) and 226 *H. influenzae* from 1668 samples of the pupils in grades 1 and 2 (carriage rate 13·6%). All *H. influenzae* were confirmed by conventional laboratory methods including Gram staining, growth on chocolate agar (but not blood agar), catalase test, $\beta$-$NAD^+$ (V factor)/hemin (X factor) requirements and an API NH kit. All strains were cultured on chocolate agar at 37 °C in an atmosphere of 5% $CO_2$ and stored at −70 °C as skim milk stocks for subsequent testing.

### Laboratory bacteriological procedures

$\beta$-lactamase production was detected using the chromogenic nitrocefin disk test (BD Biosciences, USA).

### Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of ampicillin, amoxacillin/clavulanate, cefaclor, cefotaxime, azithromycin, tetracycline, levofloxacin, trimethoprim/sulfamethoxazole, and chloramphenicol were determined by the broth microdilution method. Dehydrated microbroth 96-well panels were prepared by Dade-MicroScan (Sacramento, USA) and contained doubling antibiotic dilutions encompassing Clinical and Laboratory Standards Institute (CLSI) recommended interpretative breakpoints [9]. Panels were inoculated with bacteria to achieve a final concentration of $5 \times 10^5$ colony-forming units in 100 $\mu$l and incubated at 35 °C in ambient air for 24 h before reading. The MIC was defined as the lowest concentration of antibiotic inhibiting visible growth. MICs were interpreted using CLSI recommended breakpoints [7]. *H. influenzae* ATCC49247 and ATCC49766 were used as control strains for MIC testing.

### Polymerase chain reaction (PCR)-based detection of $\beta$-lactamase genes

Chromosomal DNA was extracted from each isolate grown on chocolate agar using the Exgene GeneAll Cell SV (Geneall Biotechnology, Korea), according to the manufacturer's protocol. Ampicillin-resistant *H. influenzae* were examined for the presence of

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TEM-1 type or ROB-1 type $\beta$-lactamase gene using previously described primer sets [10]. PCR products were resolved by electrophoresis on a 1% agarose gel for 1 h at 100 V. The gels were stained with ethidium bromide and photographed under ultraviolet (UV) light.

### Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)

PFGE and MLST were performed on continuously repeated isolates of *H. influenzae* from the same children during this survey. PFGE was performed as described previously [5]. After restriction with *Sma*I, DNA fragments were separated by a CHEF III electrophoresis system (Bio-Rad Laboratories, USA). The initial pulse time of 1 s was increased linearly to 25 s at 6 V/cm and 14 °C. The gels were visualized with ethidium bromide and analysed by the Fingerprinting II informatix software (Bio-Rad). PFGE patterns were visually compared and evaluated according to previous criteria [11]. Isolates that showed indistinguishable PFGE patterns were considered to be the same strain. MLST analysis was performed by the procedures on the *H. influenzae* MLST website (http://haemophilus.mlst.net). Briefly, the internal fragments of seven housekeeping genes [*adk* (adenylate kinase), *atpG* (ATP synthase F1 subunit gamma), *frdB* (fumarate reductase iron-sulfur protein), *fucK* (fuculokinase), *mdh* (malate dehydrogenase), *pgi* (glucose-6-phosphate isomerase), and *recA* (RecA protein)] were amplified by PCR from chromosomal DNA using the primer sets. The sequences at each locus were compared to alleles on the *H. influenzae* MLST database, and assigned corresponding allele numbers and sequence types.

### Statistical analysis

Statistical analysis was performed by $\chi^2$ test using SAS software version 9.2 (SAS Institute, USA) as appropriate.

## RESULTS

A total of 440 *H. influenzae* isolates were identified from 2328 samples of the 582 healthy children attending three kindergartens or one elementary school. Antimicrobial susceptibility testing was performed against 430 *H. influenzae* isolates (10 isolates were excluded owing to viability loss during storage at −70 °C). The *in vitro* activities of nine antimicrobial agents among the 430 *H. influenzae* isolates are summarized in Table 1. Slightly over half (51·9%, 223/430 isolates) showed non-susceptibility to ampicillin and 16·3% (70/430) were not susceptible to amoxicillin/clavulanate. The non-susceptibility rates were also high for cefaclor (52·1%) and trimethoprim/sulfamethoxazole (47·7%). However, non-susceptibilities to tetracycline, azithromycin, chloramphenicol, and cefotaxime were rare (2·8%, 2·3%, 2·1%, and 0·9%, respectively) in the *H. influenzae* isolates. All isolates were fully susceptible to levofloxacin. Compared to the isolates from kindergarten children, the isolates from elementary-school pupils were more frequently susceptible to amoxicillin/clavulanate (23·3% *vs.* 9·5%, $P=0{\cdot}0001$) and cefaclor (60·5% *vs.* 44·1%, $P=0{\cdot}0007$).

The prevalences of $\beta$-lactamase-producing strains, BLNAR strains, and BLPACR strains are shown in Table 2. The prevalence of carriage of $\beta$-lactamase-producing strains was 29·5%, 27·1%, 24·8%, and 34·5% at June, September, December 2006, and February 2007, respectively. Strikingly, $\beta$-lactamase production was only detected in 29·1% (125/430 *H. influenzae*) and the prevalence of BLNAR was found to be high (22·8%, 98/430 isolates) in our carriage study. It is noteworthy that BLNAR strains (98 isolates) constituted 44·0% of the 223 ampicillin-resistant strains. Of the 125 $\beta$-lactamase-producing isolates, 113 (90·4%) were positive for the *TEM-1* gene, 10 (8·0%) for the *ROB-1* gene, and two (1·6%) did not have either gene.

Based on their ampicillin-resistance mechanisms, all *H. influenzae* isolates were categorized as follows: $\beta$-lactamase-negative, ampicillin-susceptible (BLNAS) strains ($n=207$, 48·1%); BLPAR strains ($n=97$, 22·6%); BLNAR strains ($n=98$, 22·8%); and BLPACR strains ($n=28$, 6·5%) (Table 3). The $MIC_{50}$s and the $MIC_{90}$s of ampicillin, amoxicillin/clavulante, and cefaclor in the BLNAR strains were 2- to 16-fold higher than those for the BLNAS strains. BLNAR strains were more significantly non-susceptible to amoxicillin/clavulante ($P<0{\cdot}0001$) and cefaclor ($P = 0{\cdot}0007$) than BLNAS strains. In the BLPACR strains, the $MIC_{50}$s of ampicillin, amoxicillin/clavulanate and cefotaxime were 8- to 32-fold higher than those for the BLNAS strains. The BLPACR strains showed more resistance to amoxicillin/clavulante ($P<0{\cdot}0001$) and cefaclor ($P = 0{\cdot}0003$) than the BLNAS strains. BLPACR strains showed much higher MICs to three $\beta$-lactam antibiotics

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Table 1. In vitro *non-susceptibility of 430* Haemophilus influenzae *isolates to nine antimicrobial agents overall, divided according to kindergarten or elementary school*

| | | No. of non-susceptible isolates/no. tested (%) | | | | |
|---|---|---|---|---|---|---|
| Antimicrobial agents | Group | Total | June 2006 | Sept. 2006 | Dec. 2006 | Feb. 2007 |
| Ampicillin | All | 223/430 (51·9) | 53/112 (47·3) | 51/85 (60·0) | 52/117 (44·4) | 67/116 (57·8) |
| | Kindergarten | 105/210 (50·0) | 21/60 (35·0) | 23/46 (50·0) | 19/35 (54·3) | 42/69 (60·9) |
| | Elementary school | 118/220 (53·6) | 32/52 (61·5) | 28/39 (71·8) | 33/82 (40·2) | 25/47 (53·2) |
| Amoxicillin-clavulanate* | All | 70/430 (16·3) | 28/112 (25·0) | 17/85 (20·0) | 12/117 (10·3) | 13/116 (11·2) |
| | Kindergarten | 49/210 (23·3) | 20/60 (33·3) | 12/46 (26·1) | 7/35 (20·0) | 10/69 (14·5) |
| | Elementary school | 21/220 (9·5) | 8/52 (15·4) | 5/39 (12·8) | 5/82 (6·1) | 3/47 (6·4) |
| Cefaclor* | All | 224/430 (52·1) | 65/112 (58·0) | 50/85 (58·8) | 40/117 (34·2) | 69/116 (59·5) |
| | Kindergarten | 127/210 (60·5) | 37/60 (61·7) | 28/46 (60·9) | 17/35 (48·6) | 45/69 (65·2) |
| | Elementary school | 97/220 (44·1) | 28/52 (53·8) | 22/39 (56·4) | 23/82 (28·0) | 24/47 (51·1) |
| Cefotaxime | All | 4/430 (0·9) | 1/112 (0·9) | 3/85 (3·5) | 0/117 (0·0) | 0/116 (0·0) |
| | Kindergarten | 4/210 (1·9) | 1/60 (1·7) | 3/46 (6·5) | 0/35 (0·0) | 0/69 (0·0) |
| | Elementary school | 0/220 (0·0) | 0/52 (0·0) | 0/39 (0·0) | 0/82 (0·0) | 0/47 (0·0) |
| Trimethoprim-sulfamethoxazole | All | 205/430 (47·7) | 57/112 (50·9) | 53/85 (62·4) | 42/117 (35·9) | 53/116 (45·7) |
| | Kindergarten | 93/210 (44·3) | 23/60 (38·3) | 25/46 (54·3) | 14/35 (40·0) | 31/69 (44·9) |
| | Elementary school | 112/220 (50·9) | 34/52 (65·4) | 28/39 (71·8) | 28/82 (34·1) | 22/47 (46·8) |
| Azithromycin | All | 10/430 (2·3) | 1/112 (0·9) | 0/85 (0·0) | 3/117 (2·6) | 6/116 (5·2) |
| | Kindergarten | 4/210 (1·9) | 1/60 (1·7) | 0/46 (0·0) | 0/35 (0·0) | 3/69 (4·3) |
| | Elementary school | 6/220 (2·7) | 0/52 (0·0) | 0/39 (0·0) | 3/82 (3·7) | 3/47 (6·4) |
| Chloramphenicol | All | 9/430 (2·1) | 1/112 (0·9) | 0/85 (0·0) | 5/117 (4·3) | 3/116 (2·6) |
| | Kindergarten | 2/210 (1·0) | 1/60 (1·7) | 0/46 (0·0) | 0/35 (0·0) | 1/69 (1·4) |
| | Elementary school | 7/220 (3·2) | 0/52 (0·0) | 0/39 (0·0) | 5/82 (6·1) | 2/47 (4·3) |
| Tetracycline | All | 12/430 (2·8) | 2/112 (1·8) | 1/85 (1·2) | 1/117 (0·9) | 5/116 (4·3) |
| | Kindergarten | 6/210 (2·9) | 2/60 (3·3) | 0/46 (0·0) | 1/35 (2·9) | 3/69 (4·3) |
| | Elementary school | 6/220 (2·7) | 0/52 (0·0) | 0/39 (2·6) | 3/82 (3·7) | 2/47 (4·3) |
| Levofloxacin | All | 0/430 (0·0) | 0/112 (0·0) | 0/85 (0·0) | 0/117 (0·0) | 0/116 (0·0) |
| | Kindergarten | 0/210 (0·0) | 0/60 (0·0) | 0/46 (0·0) | 0/35 (0·0) | 0/69 (0·0) |
| | Elementary school | 0/220 (0·0) | 0/52 (0·0) | 0/39 (0·0) | 0/82 (0·0) | 0/47 (0·0) |

* There were significant differences in non-susceptibility rates of amoxicillin/clavulanate ($P=0·0001$) and cefaclor ($P=0·0007$) in *H. influenzae* isolates from the children attending kindergarten or elementary school.

(ampcillin, amoxicillin/clavulanate, cefaclor) than the BLNAR strains.

Longitudinal investigations are important for understanding the dynamic status of *H. influenzae* carriage in individuals. Presently, when samples were obtained from 582 volunteers at about 3-month intervals, 48·1% (280 children) were colonized at least once by *H. influenzae*. The number of positive samples per child ranged from one to four over the sampling period (Table 4). Carriers were grouped as persistent (positive at three or all four time points, 6·2%) or occasional (positive at only one or two time points, 41·9%) carriers. Interestingly, there was a significant difference between two age groups of children. We observed higher frequencies of long-term *H. influenzae* carriage in the kindergarten children ($P<0·0001$) than in the elementary-school children ($P=0·0133$). The remaining 51·9% of children displayed no *H. influenzae* during any sampling over the course of the study. For elementary-school children, the proportion of non-carriers was significantly higher (60·9%) than for kindergarten children (29·1%) ($P<0·0001$).

Six children were colonized by *H. influenzae* throughout the study. These *H. influenzae* isolates were subjected to MLST and PFGE genotyping to understand the dynamics of the colonization of *H. influenzae* isolates within an individual. Genotyping using MLST and PFGE of 24 isolates revealed 16 unique sequence types and 18 PFGE patterns (Table 5). Colonization by *H. influenzae* was dynamic: for any pair of two consecutive samples from the same children, the majority contained two different clones [MLST: 61·1% (11/18 pairs);

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Table 2. *Distribution of β-lactam-resistance mechanisms in the* Haemophilus influenzae *isolates from healthy children at kindergarten or elementary school in this study*

| | No. (%) of isolates | | | | |
|---|---|---|---|---|---|
| Resistance class | June 2006 ($n=112$) | Sept. 2006 ($n=85$) | Dec. 2006 ($n=117$) | Feb. 2007 ($n=116$) | All ($n=430$) |
| Ampicillin resistance | 53 (47·3) | 51 (60·0) | 52 (44·4) | 67 (57·8) | 223 (51·9) |
| β-lactamase | 33 (29·5) | 23 (27·1) | 29 (24·8) | 40 (34·5) | 125 (29·1) |
| TEM | 29 (25·9) | 19 (22·4) | 26 (22·2) | 39 (33·6) | 113 (26·3) |
| ROB | 4 (3·6) | 3 (3·5) | 2 (1·7) | 1 (0·9) | 10 (2·3) |
| BLNAR | 20 (17·9) | 28 (32·9) | 23 (19·7) | 27 (23·3) | 98 (22·8) |
| BLPACR | 10 (8·9) | 6 (7·1) | 7 (6·0) | 5 (4·3) | 28 (6·5) |

TEM, TEM-1 β-lactamase; ROB, ROB-1 β-lactamase; BLNAR, β-lactamase-negative, ampicillin-resistant *H. influenzae*; BLPACR, β-lactamase-positive, amoxicillin/clavulanate-resistant *H. influenzae*.

Table 3. *Distribution between β-lactam-resistance mechanisms and non-susceptibility to ampicillin, amoxicillin/clavulanate, and cefaclor in* Haemophilus influenzae *carriage strains*

| β-lactam resistance type (no. of isolates) | Ampicillin | | | | Amoxicillin/clavulanate | | | | Cefaclor | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $MIC_{50}$ | $MIC_{90}$ | Range | % NS | $MIC_{50}$ | $MIC_{90}$ | Range | % NS | $MIC_{50}$ | $MIC_{90}$ | Range | % NS |
| BLNAS (207) | ⩽0·5 | 1 | ⩽0·5–1 | 0·0 | ⩽0·5 | 4 | ⩽0·5–32 | 5·3 | 8 | 32 | ⩽0·5–64 | 31·4 |
| BLPAR (97) | <16 | <16 | <2–16 | 100·0 | 2 | 4 | ⩽0·5–4 | 0·0 | 32 | 64 | 1–64 | 70·1 |
| BLNAR (98) | 8 | 16 | <2–16 | 100·0 | 4 | 16 | ⩽0·5–32 | 32·7 | 16 | 64 | ⩽0·5–64 | 65·3 |
| BLPACR (28) | <16 | <16 | 16< | 100·0 | 8 | 32 | 8–32 | 100·0 | 64 | 64 | 8–64 | 96·4 |

BLNAS, β-lactamase-negative, ampicillin-susceptible; BLPAR, β-lactamase-positive, ampicillin-resistant; BLNAR, β-lactamase-negative, ampicillin-resistant; BLPACR, β-lactamase-positive, amoxicillin/clavulanate-resistant; MIC, minimum inhibitory concentration; NS, non-susceptible.

PFGE: 66·7% (12/18 pairs)]. The longest duration of carriage of a unique MLST and PFGE genotype were 6 months and 3 months, respectively. On average, the colonization period detected for the same genotype clone of *H. influenzae* was about 3 months. In particular, one child (participant no. 81) showed the highest turnover rate of strains, where all four isolates exhibited different MLST and PFGE patterns.

## DISCUSSION

We longitudinally investigated the changes and antibiotic resistance of *H. influenzae* carriage in 582 healthy children at four intervals over a 9-month period in Seoul, Korea. This study provides the first evidence of a high prevalence (22·8%) of BLNAR strains of *H. influenzae* nasal carriage in healthy children attending kindergarten or the first 2 years of elementary school in Korea.

Previous studies have reported a wide range of *H. influenzae* carriage rates (11·9–88·0%) in various age groups [7]. The prevalence of *H. influenzae* carriage was 54·8% from preschool children aged 3–6 years in Spain [12], 37·4% from children aged 3–36 months attending day-care centres in The Netherlands [7], 13% from pre-school children (<7 years) in Sweden [13], 11·9% from children aged 1–7 years in Italy [14], and 15·6% in 683 healthy children aged 5–6 years in Turkey [15]. To the best of our knowledge, these differences may be affected by multiple factors, such as age group, family size, overcrowded living conditions, day-care contact and methodological factors [7]. Our study shows that the overall frequency of *H. influenzae* carriage was 18·9% from healthy children attending kindergarten or elementary school residing in a metropolitan area of Korea. The frequency was lower than expected. However when the age groups were analysed separately, the carriage rates of 32·4% in kindergarten children (3–7 years) and 13·6% in the elementary-school children (7–10 years) were compatible with previous reports from similar age groups [7]. We also confirmed the findings of

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Table 4. *Status of* Haemophilus influenzae *carriage in 582 children during the longitudinal study*

| | Presence or absence of *H. influenzae* carriage in | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | No. (%) of children | | | |
| Group* | June 2006 | Sept. 2006 | Dec. 2006 | Feb. 2007 | No. of children | Kindergarten ($n=165$) | Elementary school ($n=417$) | Total ($n=582$) | *P* value |
| Persistent carriers | + | + | + | + | 6 | 25 (15·1) | 11 (2·6) | 36 (6·2) | $P<0·0001$ |
| | + | + | + | − | 6 | | | | |
| | − | + | + | + | 6 | | | | |
| | + | + | − | + | 10 | | | | |
| | + | − | + | + | 8 | | | | |
| Occasional carriers | + | + | − | − | 8 | 91 (55·2) | 153 (36·7) | 244 (41·9) | $P=0·0133$ |
| | − | − | + | + | 23 | | | | |
| | − | + | + | − | 8 | | | | |
| | + | − | − | + | 16 | | | | |
| | + | − | + | − | 14 | | | | |
| | − | + | − | + | 13 | | | | |
| | + | − | − | − | 48 | | | | |
| | − | + | − | − | 28 | | | | |
| | − | − | + | − | 52 | | | | |
| | − | − | − | + | 34 | | | | |
| Non-carriers | − | − | − | − | 302 | 49 (29·1) | 253 (60·9) | 302 (51·9) | $P<0·0001$ |

+, *H. influenzae* isolated; −, not isolated.

* Persistent carriers, occasional carriers and non-carriers were grouped according to the results of carriage of *H. influenzae* at four time points.

other investigators that the carriage rate of *H. influenzae* was higher in younger children than in older children ($P<0·0001$).

Despite the fact that Hib vaccine is not currently included in the National Immunization Programme and is administered to infants through the private sector in Korea, Hib immunization rates have risen from 16% in 2002 to 50% [16]. However, one of the limitations of this study is that we did not make an accurate investigation of *H. influenzae* type b (Hib) vaccine immunization in the recruited children. Thus, we could not perceive how far the immunization of Hib vaccine directly influenced nasal carriage status in healthy children. In this study, in 440 *H. influenzae* isolates, only 11 (2·5%) isolates were encapsulated (no type b) and the others (97·5%) were non-encapuslated (non-typable) (data not shown). Given that 97·5% of strains were non-typable *H. influenzae*, the relevance of vaccination status to the study's findings is minimal – only Hib carriage would be anticipated to be influenced by Hib conjugate vaccine.

Considering the results of the longitudinal study, we categorized the carriage pattern in each individual into three groups: persistent, occasional, and non-carrier. Many children were occasional carriers (one or two positives at the four sampling times) over the study periods. Persistent carriers were rare (6·2%). We observed that the duration of carriage was inversely correlated with age (15·1% for kindergarten children *vs.* 2·6% for elementary-school children, $P<0·0001$). Of particular interest, 51·9% were non-carriers with no carriage of *H. influenzae* at all four sampling times among all children. We observed higher prevalence of non-carriers in the older children attending elementary school than in the younger children (60·9% *vs.* 29·1%, $P<0·0001$). The above-described discrepancy between pre-school and elementary-school children is probably due to the difference of carriage rates, immunity maturation, and close contacts in the closed community.

We genotyped 32 *H. influenzae* isolates from six children who were colonized by *H. influenzae* for >9 months. Overall, each individual had a mean of three different *H. influenzae* clones in four repeated nasal samples. Of the six children, four carried the same *H. influenzae* strain for 3 months, but two children were never colonized by the same genotype of *H. influenzae*. Although we did not genotype a large number of *H. influenzae*, we observed that nearly half of the children changed strains between the two

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Table 5. *Genetic analysis and the profiles of antibiotic resistance of* Haemophilus influenzae *isolates from the six persistent carriers at the four sampling points*

| Participants | Age (yr) | Institution | MLST sequence type (ST) | | | | PFGE pattern | | | | Antibiotic resistance profiles* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | June 2006 | Sept. 2006 | Dec. 2006 | Feb. 2007 | June 2006 | Sept. 2006 | Dec. 2006 | Feb. 2007 | June 2006 | Sept. 2006 | Dec. 2006 | Feb. 2007 |
| 36 | 7 | Kindergarten A | ST113 | ST113 | ST532 | ST532 | G1 | G1 | G2 | G2 | A/C/S | A/M/C/S | — | A |
| 38 | 7 | Kindergarten A | ST530 | ST531 | ST532 | ST532 | J1 | J2 | J3 | J3 | M | A/C/S | — | — |
| 81 | 7 | Kindergarten B | ST533 | ST543 | ST534 | ST535 | U1 | U2 | U3 | U4 | A/M/C | A/C/S | A/C | A/C |
| 164 | 3 | Kindergarten C | ST536 | ST537 | ST538 | ST539 | S1 | S1 | S2 | S3 | — | A/C | — | A/M/C |
| 399 | 8 | Elementary school (grade 2, class 1) | ST262 | ST262 | ST262 | ST540 | K1 | K1 | K2 | K3 | — | — | — | — |
| 421 | 9 | Elementary school (grade 2, class 2) | ST541 | ST541 | ST542 | ST542 | L1 | L1 | L2 | L3 | A/M/C/S | A/M/C/S | C | A/M/C |

MLST, Multi-locus sequence typing; PFGE, pulsed-field gel electrophoresis.
* A, Ampicillin; M, amoxicillin/clavulanate; C, cefaclor; S, Trimethoprim-sulfamethoxazole.

periods 3 months apart and rarely found infants carrying the same genotype strain among the six children over time. This highlights the dynamic process of nasal colonization in the same children during the study period. It may be that *H. influenzae* carriage in the children attending kindergarten or elementary school could be a very dynamic process. Such a dynamic colonization of *H. influenzae* was shown in the Swedish study of Trottier *et al.* [17] and the high turnover of *H. influenzae* was reported by Sá-Leão *et al.* [18] in Portugal and by Hashida *et al.* [19] in Japan.

Korea is one of the countries exhibiting the highest prevalence of antibiotic resistance by respiratory pathogens. Thus, amoxicillin/clavulanate and oral cephalosporins have been widely used for oral antibiotic treatments for outpatients with acute respiratory infections as a result of high level of $\beta$-lactamase-producing *H. influenzae* and multidrug-resistant *S. pneumoniae*. These frequent prescriptions of empirical antibiotics for patients with RTIs raise concerns about new changes of resistance patterns in our community. In a previous study of the Korean ARIS, we identified $\beta$-lactamase production (52·4%) as the major mechanism of ampicillin-resistant *H. influenzae* and the emergence of BLNAR (6·1%) strains of *H. influenzae* [5].

BLNAR strains are rare globally but their prevalence has increased in some countries, e.g. Japan, Spain, and France [19–21]. Masuda *et al.* [22] reported that 5·5% of *H. influenzae* isolates were BLNAR strains in Japanese children attending day-care centres. Another more, recent survey from Japan revealed that BLNAR strains accounted for 25·0% of the nasopharyngeal *H. influenzae* isolates from healthy children attending day-care centres as a result of increased exposure to cephem antibiotics throughout Japan [19]. The present result showed a high prevalence rate (22·8%) of BLNAR isolates in the *H. influenzae* isolates from healthy Korean children, resembling the increased incidence of BLNAR strains in the aforementioned Japanese studies. A probable explanation for this increase is the marked widespread use of oral cephaosporins for the treatment of children with RTIs in Korea. This will become a major problem for the blind empirical therapy of respiratory infections in our community.

Continued carriage of antibiotic-resistant *H. influenzae* appears to be an important factor in the dissemination of resistant clones throughout the community [6, 19]. In our study, the colonizing

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*H. influenzae* isolates showed a high rate of resistance to $\beta$-lactams including ampicillin (51·9%), cefaclor (52·1%), and amoxicillin/clavulanate (16·3%). These results are consistent with those of the Korean nationwide ARIS study, with the exception of an increase of the resistance against cefaclor [5]. Characteristically, the proportion of carriage of amoxicillin/clavulanate and cefaclor-resistant *H. influenzae* was higher for younger children compared to older children, which was similar to data reported by Hashida *et al*. [19]. This may suggest that younger children attending kindergarten frequently experience acute RTIs and have a greater chance of receiving antibiotic treatment, which would potentially induce resistance.

In conclusion, this study, the largest longitudinal study to date, comprising 582 healthy children attending kindergarten or elementary school in Seoul, Korea, aimed at monitoring levels of carriage rate and antimicrobial resistance of *H. influenzae* isolates. The nasal carriage rate of *H. influenzae* was different depending on the age group: it was significantly more prevalent in younger children attending kindergarten than pupils in elementary school (grades 1 and 2). The healthy children displayed diverse colonization patterns and a lower level of long-term persistent carriage status. We also identified the high rate of resistance to $\beta$-lactam antibiotics and a high proportion (22·8%) of BLNAR strains in *H. influenzae* carriage of healthy children. The high carriage rate of these resistant strains in urban overcrowded facilities such as kindergarten or school may be creating a new situation of *H. influenzae* resistance in our community.

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## DECLARATION OF INTEREST

None.

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