JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2011, p. 1323–1330 Vol. 49, No. 4
0095-1137/11/$12.00 doi:10.1128/JCM.02200-10

Haemophilus influenzae Type b Carriage and Novel Bacterial Population Structure among Children in Urban Kathmandu, Nepal[▽]

E. J. Williams,[1,2]* J. Lewis,[1,2] T. John,[1] J. C. Hoe,[1] L. Yu,[1] S. Dongol,[2] D. F. Kelly,[1] D. T. Griffiths,[3] A. Shah,[2] B. Limbu,[2] R. Pradhan,[2] F. Mawas,[4] S. Shrestha,[2] S. Thorson,[2] A. M. Werno,[5] D. R. Murdoch,[5,6] N. Adhikari,[2] and A. J. Pollard[1]

Oxford Vaccine Group, Oxford University, Oxford, United Kingdom[1]; Patan Hospital, Kathmandu, Nepal[2]; Department of Microbiology, John Radcliffe Hospital, Oxford, United Kingdom[3]; Bacteriology Division, National Institute for Biological Standard and Control, Health Protection Agency, Potters Bar, United Kingdom[4]; Canterbury Health Laboratories, Christchurch, New Zealand[5]; and University of Otago, Christchurch, New Zealand[6]

Received 31 October 2010/Returned for modification 7 December 2010/Accepted 16 January 2011

***Haemophilus influenzae* type b (Hib) is a major cause of invasive bacterial infection in children that can be prevented by a vaccine, but there is still uncertainty about its relative importance in Asia. This study investigated the age-specific prevalence of Hib carriage and its molecular epidemiology in carriage and disease in Nepal. Oropharyngeal swabs were collected from children in Kathmandu, Nepal, from 3 different settings: a hospital outpatient department (OPD), schools, and children's homes. Hib was isolated using Hib antiserum agar plates, and serotyping was performed with latex agglutination. Hib isolates from children with invasive disease were obtained during active microbiological surveillance at Patan Hospital, Kathmandu, Nepal. Genotyping of disease and carriage isolates was undertaken using multilocus sequence typing (MLST). Swabs were taken from 2,195 children, including 1,311 children at an OPD, 647 children attending schools, and 237 children in homes. Overall, Hib was identified in 5.0% (110/2,195; 95% confidence interval [95% CI], 3.9% to 6.4%). MLST was performed on 108 Hib isolates from children carrying Hib isolates and 15 isolates from children with invasive disease. Thirty-one sequence types (STs) were identified, and 20 of these were novel STs. The most common ST isolates were sequence type 6 (ST6) and the novel ST722. There was marked heterogeneity among the STs from children with disease and children carrying Hib. STs identified from invasive infections were those commonly identified in carriage. This study provides evidence of Hib carriage among children in urban Nepal with genetically diverse strains prior to introduction of universal vaccination. The Hib carriage rate in Nepal was similar to the rates observed in other populations with documented high disease rates prior to vaccination, supporting implementation of Hib vaccine in Nepal in 2009.**

Haemophilus influenzae type b (Hib) is a major cause of invasive bacterial infection and pneumonia in childhood. Globally, Hib is estimated to cause over 3 million cases of serious disease and 400,000 deaths, primarily among children in resource-poor countries (36, 37). Protein-polysaccharide conjugate Hib vaccines are highly effective in preventing disease when used in routine infant immunization schedules. There is increasing evidence from large-scale Hib vaccine studies in the Gambia (17), Bangladesh (4), and Lombok, Indonesia (7) of the significant impact of immunization on disease caused by Hib. In 2006, the World Health Organization recommended inclusion of this vaccine in all routine immunization programs, estimating that 20% of all cases of pneumonia were attributable to Hib and variable proportions of childhood meningitis were attributable to Hib, depending on the geographical location (37). The Global Alliance for Vaccines and Immunization (GAVI) has pledged to assist with the introduction of routine infant immunization against Hib in the world's poorest countries. Nepal is one of these countries and introduced Hib vaccine into the infant immunization schedule in 2009 after this study was completed.

Small studies have previously identified Hib as an important cause of meningitis in Nepal (26, 34), and recent studies of children in hospitals in Kathmandu, Nepal, suggest that Hib caused 2 to 8% of the cases of meningitis (12, 25). However, the absolute number of cases in the study was small, and there is still uncertainty about the relative importance of Hib in Asia. In urban Nepal, we have previously identified a significant burden of pneumonia in children less than 5 years old admitted to a hospital (12, 38). While Hib is likely to be the cause of many of these cases of pneumonia, most children with Hib pneumonia have negative blood cultures, and confirmation is therefore not possible (6).

In areas of the world where Hib is a demonstrably important cause of invasive disease in children, carriage rates of 3 to 9% have been documented in children under 5 years of age (20). The presence of significant Hib carriage in a region, such as Nepal, where there is limited population data on the rates of invasive disease, would be suggestive of its importance as a cause of both invasive and mucosal disease in these children.

* Corresponding author. Present address: Neonatal Unit, Royal Victoria Infirmary, Queen Victoria Road, Newcastle-upon-Tyne NE1 4LP, United Kingdom. Phone: 44 191 2821614. E-mail: dreleri.williams@btinternet.com.

▽ Published ahead of print on 26 January 2011.

This study was undertaken to document the age-specific carriage rates of Hib as supporting evidence for the importance of Hib in this population. Furthermore, the presence of Hib carriage data prior to a countrywide immunization program provides an opportunity to assess the impact of the program, using carriage as an accessible surrogate of vaccine efficacy. For health ministries balancing a limited budget, data on Hib carriage among children could support both vaccine implementation and an ongoing program after donor funding has been phased out.

Although genetic profiling of Hib isolates using multilocus sequence typing (MLST) is well described, there are few data describing the molecular epidemiology of Hib isolates from Asia. It is of particular interest to know whether carriage and disease strains are similar before and after the introduction of Hib vaccine.

The primary objective of this study was to determine age-specific rates of oropharyngeal carriage of Hib in children in urban Nepal. The secondary objective was to describe the variation in incidence among 3 cohorts of healthy children in an urban hospital outpatient department (OPD), in schools, and in children's homes. In addition, the study aimed to compare the molecular epidemiology of isolates from children carrying Hib and children with invasive disease.

MATERIALS AND METHODS

Recruitment and study groups. Children were recruited from either (i) the clinic treating patients under 14 years of age in the outpatient department (OPD) at Patan Hospital, Kathmandu, Nepal, (ii) local schools, or (iii) children's homes for socially disadvantaged children. Patan Hospital is an urban hospital with the second largest children's department in Kathmandu, Nepal. The OPD is the primary health clinic for local children in the Patan area in Nepal. There is no known epidemiological connection among these children apart from the fact that they access the same hospital clinic and mostly reside in the catchment area of the clinic. The parents or guardians of children were approached by research officers in the clinic waiting area, and informed written consent was obtained from the parents or guardians. Schools were chosen within a 10-km area surrounding Patan Hospital in Nepal, Kathmandu. Six of seven schools approached agreed to take part in this study. All of the schools participating in the study were within 10 km of Patan Hospital, and 5 were in Patan District; the six schools included free government schools and private schools. All children attending these schools were approached. Written information on the study, a consent form, and a questionnaire were sent to the parents/guardians of the children. Children's homes for socially disadvantaged children in Patan District care for children who were orphaned or whose parents are unable to provide for them. All of the 7 children's homes approached agreed to take part in the study. One large home ($n = 165$) was a government home, and the remaining 6 were funded by charities. All of these orphanages had shared dormitories and living spaces. Written consent was obtained from the 2 governors/staff members of the homes with responsibility for the children. As far as we are know, none of the children in the study had contact with children with invasive Hib disease during the course of this study.

Information was gathered from the parents/guardians of the children on their age, gender, household size, ages of other children living at home, cigarette smoking, Hib vaccination history, and history of antibiotic use in the preceding 28 days. Day care attendance information was also collected in the OPD setting.

The exclusion criteria were children who were admitted to the hospital as inpatients, febrile children with an axillary temperature of ≥38°C, current respiratory symptoms, or the use of antibiotics within the preceding 28 days.

Sample size. Based on estimated Hib carriage rates of 1.9% for children less than 5 years old and 3.2% for children 5 years old and older, we aimed to recruit 1,300 children less than 5 years old and 700 children 5 years old or older so that a 50% reduction in the carriage rate could be identified in future studies with 5% significance and 90% power.

Microbiology. A single oropharyngeal swab was taken from each child using a cotton-tipped wooden swab (Aimes charcoal swab with wooden shaft; Medical Wire Equipment, Wiltshire, United Kingdom). In the OPD, swabs were directly plated onto Hib antiserum agar plates (Colombia base agar, NAD, hemin, bacitracin, and sheep anti-Hib serum [National Institute for Biological Standard and Control, Potters Bar, United Kingdom]). In schools and homes, the swab ends were cut off into a 2-ml bijoux containing *Haemophilus* transport medium (HTM) (tryptone soya broth, NAD, and hemin) and transported to the laboratory and plated onto Hib antiserum agar within 6 h.

The samples were incubated at 37°C in a CO_2-enriched environment (5 to 10%) and checked after 18 to 24 and 48 h for iridescence or precipitation in the agar surrounding a colony (resulting from binding of the Hib antiserum to the type b capsular polysaccharide which is shed from the bacterial surface). After storage for a further 5 days, the samples were discarded if no precipitation was found. Colonies identified by iridescence and/or precipitation were confirmed as Hib using X (haem) & V (NAD) dependence and slide latex agglutination (Remel *Haemophilus* agglutinating sera; Remel, Lenexa, KS). Samples that were X & V dependent were tested using polyvalent and type-specific latex agglutination (Difco). The positive control for Hib strains was the Eagan strain (ATCC 51654). All organisms that were X & V dependent were frozen at −70°C and transported to the United Kingdom for genetic typing using MLST. Information on the samples was removed from the samples to eliminate bias in genetic typing.

Multilocus sequence typing (MLST). The allelic profiles of the Hib isolates were obtained by sequencing internal fragments of the 7 housekeeping genes by the method of Meats et al. (15). Samples were sequenced at the Department of Zoology, University of Oxford, with an Applied Biosystems 3730xl DNA analyzer. The forward and reverse sequences were analyzed using the Sequence Typing Analysis and Retrieval System (STARS) (University of Oxford) (http://sara.molbiol.ox.ac.uk/userweb/mchan/stars/) and submitted to the MLST website (http://www.mlst.net) for assignment of allele numbers and sequence type (ST).

Isolates from children with invasive disease. Since April 2005, as part of a microbiological surveillance study, all children admitted to the children's ward of Patan Hospital with suspected invasive bacterial disease or pneumonia have routinely had blood samples and, where appropriate, cerebrospinal fluid (CSF) samples cultured. All pathogens isolated from blood and/or CSF samples were stored at −70°C (12, 38). Hib disease-causing isolates were obtained from this collection for comparison with isolates from children carrying Hib.

Statistical analysis. The carriage prevalence, for each group of children (i.e., OPD, schools, and homes), was estimated by dividing the number of observed cases by the number of subjects swabbed. The confidence intervals were adjusted to reflect the fact that the children were not sampled independently but within an OPD, schools, and homes (i.e., adjusted for clustering effect). Statistical analyses were carried out using Stata v.10. Comparisons of prevalence between groups were performed by using logistic regression. For calculation of genetic diversity, Simpson's index of diversity (SDI) was used, and SDI was calculated by the following formula: $100 \times [1 - [\text{summ}]n_i(n_i - 1)/N(N - 1)]$, where n_i is the number of strains belonging to the ith type and N is the total number of strains (11, 27).

Ethics. Ethical approval for this study was obtained by the Oxford Tropical Research Ethics Committee (approval no. OXTREC 011-07) and the Nepal Health Research Council (NHRC).

RESULTS

Oropharyngeal swabs were obtained from a total of 2,195 children, including 1,311 children at the outpatient department (OPD), 647 children attending schools, and 237 children in children's homes between June and December 2007. Demographic data on the 3 groups of children studied are summarized in Table 1.

In the OPD, 1,761 children were approached from May to December 2007. A total of 434 children were excluded, as they had fever (94 children), respiratory symptoms (8 children), antibiotic use in the preceding 28 days (324 children), or other reasons (8 children). Sixteen parents or guardians did not consent to having a swab taken from the child. Swabs were collected from 1,311 children.

Of the 2,868 children approached who attended local schools, 738 parents or guardians (26%) agreed to participate, 72 (3%) parents/guardians refused consent, and 2,058 did not return the forms. Ninety-one of the children whose parents/

TABLE 1. Demographic data for the 3 populations of children

Characteristic	No. of children or parameter value (%, unless range is specified)[a]		
	OPD (n = 1,311)	Schools (n = 647)	Homes (n = 237)
Sex			
Female	606 (46.2)	342 (52.9)	118 (49.8)
Male	705 (53.8)	304 (47.1)	119 (50.2)
Unknown		1	
Age, yr [median (range)]	1 (0.3–11.7)	8.6 (3.3–12.9)	4.5 (0.3–11.9)
No. of adults at home			
1 or 2	675 (51.5)	281 (57.6)	N/A
3 to 5	406 (31.0)	152 (31.3)	
6 to 9	205 (15.6)	47 (9.6)	
≥10	25 (1.9)	8 (1.6)	
No data	0	159	
No. of children at home			
1 or 2	643 (87.7)	393 (83.3)	N/A
3 to 5	81 (11.1)	72 (15.2)	
6 to 9	7 (1.0)	7 (1.5)	
≥10	2 (0.3)	0	
No data	578	175	
Smokers in family	493 (37.6)	235 (36.3)	N/A
Attended day care center	180 (13.7)	N/A	N/A
Received Hib vaccine			
1 dose	4	4	
2 doses	2	3	
3 doses	2	12	
Unknown	5	0	
Total no. receiving Hib vaccine	13	19	0

[a] The three populations of children studied are explained in detail in Materials and Methods. The the number of children (n) in each population or study group is shown. OPD, outpatient department. N/A, not available.

guardians gave consent to participate in the study were excluded on the day of swabbing, because they were absent (67 children) or were taking antibiotics (24 children). Swabs were collected from 647 children.

Written consent was provided for 246 of the 490 children present in the children's homes approached. Of these children, 9 were excluded either because of antibiotic usage (8 children) or absence on the day of swabbing (1 child). Swabs were collected from 237 children.

Overall, Hib was identified in 5.0% (110/2,196; 95% confidence interval [95% CI], 3.9% to 6.4%) of samples with population-specific carriage rates of 4.1% (54/1,311; 95% CI, 3.0% to 5.2), 5.4% (35/648; 95% CI, 2.8% to 10.3%), and 8.9% (21/237; 95% CI, 3.8% to 19.1%) for children at the OPD, schools, and homes, respectively. The age-specific carriage rates are shown in Table 2. Overall, there was no significant difference in carriage prevalence in different settings or age groups. There was a trend to the lowest prevalence of Hib carriage being in children ≥5 years of age in all settings, but this was not significant for any individual setting.

The risk factors for Hib carriage are shown in Table 3. There were no significant differences between the median number of adults or children at the children's homes, smokers, or attendance at day care centers. No cases of Hib carriage were identified in children known to have had any dose of Hib vaccine.

Encapsulated *Haemophilus influenzae* bacteria belonging to a type other than type b were isolated from 20 (0.9%) of the children. These bacteria were most commonly types d and f with 6 isolates of each being identified. Three isolates of type a, 3 isolates of type c, and 2 isolates of type e were also confirmed using slide agglutination. In 33 children, a nontypeable *H. influenzae* was identified. These are likely to be underestimates, as the methods used with anti-Hib serum agar are selectively designed to identify Hib isolates.

MLST was performed on 108 isolates from children carrying Hib (2 isolates failed to grow after transport) and 15 isolates from children with invasive disease (Table 4). For the isolates from children carrying Hib, 31 different STs were identified; 64 (59%) isolates had 1 of 11 previously described STs, and 44 isolates (41%) had novel STs (20 novel STs).

Overall, the population structure of the isolates from children carrying Hib was one of high diversity. No single ST accounted for greater than 22% of the population, and the overall Simpson's diversity index (SDI) was 90.7%.

Although there was no difference in the overall Hib carriage prevalence in children in the three settings (homes, schools, and OPD), there were striking differences in the molecular epidemiology of the populations (Table 5). The isolates from homes showed a lower diversity (SDI, 44%) than those from schools (SDI, 92%) or the OPD (SDI, 93%). For schools and OPD combined, ST722, a novel ST, was the most common ST (18% of isolates), followed by ST6 (10% of isolates). ST95 was unusual in being a relatively frequent isolate from the OPD (5/53 [9%]) but was not found among the school population.

The relative prevalence of STs in disease-causing Hib isolates closely mirrored that in isolates from children carrying Hib (Fig. 1). The 15 disease-causing isolates were made up of 7 STs. The most common disease-causing ST was ST722, which

TABLE 2. Hib carriage rates by age group and population

Age group	Hib carriage rate (95% CI) [n][a]			
	OPD	Schools	Homes	Overall
3 to 11 mo	3.1 (1.8–4.5) [640]	N/A	11.1 (0.3–84.2)[b] [27]	3.4 (2.5–4.7)[b] [667]
1 to 4 yr	5.7 (3.6–7.3) [603]	11.1 (0.2–87.8) [9]	11.6 (4.7–26.2)[b] [103]	6.4 (4.7–8.8)[b] [715]
≥5 yrs	1.5 (0.04–7.9) [68]	5.3 (2.7–10.3)[b] [638]	5.6 (2.7–11.2)[b] [107]	5.0 (3.1–8.1)[b] [813]
Overall	4.1 (3.0–5.2)	5.4 (2.8–10.3)[b]	8.9 (3.8–19.1)[b]	5.0 (3.9–6.4) [2,195]

[a] The Hib carriage rate and 95% confidence intervals (95% CIs) are percentages. n is the number of children. N/A, not available.
[b] These confidence intervals were adjusted for clustering effect.

TABLE 3. Demographic data and Hib vaccination details of children carrying Hib and children not carrying Hib

Characteristic	No. of children or parameter value (%, unless SD or range is specified)	
	Not carrying Hib (n = 2,085)	Carrying Hib (n = 110)
Sex		
Female	1,005 (48.2)	61 (55.4)
Male	1,080 (51.8)	48 (43.6)
Unknown		1
Age (yr)		
Mean (SD)	4.2 (3.7)	4.2 (3.4)
Median (range)	2.9 (0.3–12.9)	3.0 (0.3–12.0)
No. of adults at home		
0	0	0
1 or 2	911 (52.9)	44 (57.1)
3 to 5	534 (31.0)	24 (31.2)
6 to 9	243 (14.1)	9 (11.7)
≥10	33 (1.9)	0
Total no.	1,721	77
No. of other children at home		
0	554 (32.6)	23 (28.4)
1 or 2	989 (58.1)	47 (58.0)
3 to 5	142 (8.3)	11 (13.6)
6 to 9	14 (0.8)	0
≥10	2 (0.1)	0
Total no.	1,701	81
Smokers	689/1,868 (36.9)	39/88 (44.3)
Attended day care center	172/2,085 (8.2)	8/110 (7.3)
Received Hib vaccine		
1 dose	8	0
2 doses	5	0
3 doses	14	0

TABLE 4. Multilocus sequence typing profiles of strains from children carrying Hib[a]

Sequence type (ST)[b]	MLST allelic profile for *adk-atpG-frdB-fucK-mdh-pgi-recA* genes	No. of isolates (%) from children:	
		Carrying Hib	With invasive disease
6	10–14–4–5–4–7–8	24 (22.2)	
722*	10–14–4–54–4–7–11	16 (14.8)	4 (26.7)
53	10–14–5–7–4–7–8	11 (10.2)	3 (20)
118	10–14–4–5–4–7–22	8 (7.4)	2 (13.3)
222	6–20–23–1–33–29–7	6 (5.6)	1 (6.7)
721*	31–14–4–9–4–7–8	6 (5.6)	2 (6.7)
44	10–14–4–3–4–3–8	5 (4.6)	
95	31–14–4–5–4–7–8	5 (4.6)	
724*	10–20–21–5–4–7–8	5 (4.6)	2 (6.7)
57	14–7–13–7–17–13–17	1 (0.9)	
82	5–15–10–1–8–5–11	1 (0.9)	
93	6–30–23–1–33–29–7	1 (0.9)	
641	10–14–4–7–4–7–8	1 (0.9)	
719*	8–14–4–9–4–7–8	1 (0.9)	
725*	22–6–11–12–58–18–22	1 (0.9)	
726*	22–6–11–12–58–18–11	1 (0.9)	
728*	6–20–114–1–33–29–7	1 (0.9)	
729*	10–14–115–54–4–7–11	1 (0.9)	
730*	10–14–4–43–4–7–8	1 (0.9)	
731*	6–11–51–1–33–65–7	1 (0.9)	
732*	10–14–5–7–187–7–8	1 (0.9)	
736*	10–14–58–5–4–7–22	1 (0.9)	
738*	6–20–38–1–33–29–7	1 (0.9)	
740*	3–9–8–7–14–8–4	1 (0.9)	
741*	10–14–116–27–4–7–43	1 (0.9)	
744*	10–19–21–5–4–7–8	1 (0.9)	
749*	10–10–5–7–4–7–8	1 (0.9)	
750	10–14–5–11–35–7–8	1 (0.9)	
751	26–14–121–27–78–55–21	1 (0.9)	
752	18–6–3–7–10–28–8	1 (0.9)	
754	10–14–121–5–7–7–8	1 (0.9)	
488	1–1–101–1–149–1–5		1 (6.7)

[a] Multilocus sequence typing (MLST) profiles of Hib strains carried by the children (presented in order of total carriage frequency).

[b] The sequence type (ST) is shown (e.g., ST6, ST722, ST53, etc.). An asterisk indicates that a new ST number has been assigned for isolates identified in this study.

was the most commonly carried isolate in the schools (6 STs identified in 5 schools) and OPD populations (10 STs). The 9 most commonly carried STs accounted for 13/15 (87%) of disease-causing isolates. An exception to the relationship between Hib carriage and disease-causing isolates was ST6. Despite ST6 being the most prevalent ST in isolates from children carrying Hib, there were no disease-causing isolates that were ST6. However, the majority (15/24 isolates) of ST6 carriage isolates were identified as clusters in the home population with all of these isolates identified in 2 of the 7 children's homes studied.

There appeared to be no relation between ST and age groups (<1 year, 1 to 4 years, and >5 years), and the SDI values were similar for children in the different age groups.

The phylogenetic relationships of the Hib carriage and disease-causing isolates (n = 123) are shown in Fig. 1. Linkage analysis of the Hib isolates linking isolates with 6/7 common identical alleles demonstrated that there are 2 clonal complexes around ST6 (103 isolates) with 33% (41/123) sharing 6/7 alleles with ST6 and a further clonal complex around ST222 (10 isolates). ST725 and ST726 (n = 2) were also clonal, sharing 6/7 alleles, and the remaining 8 STs had no similarity between their alleles and hence no clonal relationships.

DISCUSSION

This study provides evidence of Hib carriage in children aged 3 months to 12 years in urban Nepal in 2007 prior to universal vaccination. Allowing for the clustering effect of cases in schools and homes, the carriage rates in all three settings were similar. The rates are comparable to the Hib carriage prevalence of 3 to 7% seen in the United States (16) and United Kingdom (5) in an era prior to routine infant immunization with Hib. There has been uncertainty about the rates of disease caused by Hib in Asia. In some specific populations in Asia with a low incidence of Hib disease, as determined by microbiological surveillance, carriage prevalence has been reported as being as low as 0 to 1% (10, 13, 35). This is similar to the rates reported for other populations after the introduction of routine infant immunization (1, 14). However, the current study has shown a Hib carriage rate of 5.0%,

TABLE 5. Multilocus sequence typing profiles of Hib strains carried by children by population[a]

Sequence type (ST)[b]	No. (%) of children		
	OPD (n = 1,311)	Schools (n = 647)	Homes (n = 237)
6	3 (5.6)	6 (17.1)	15 (75)
722*	10 (18.9)	6 (17.1)	
53	4 (7.5)	5 (14.2)	2 (10)
118	6 (11.3)	2 (5.7)	
222	4 (7.5)	2 (5.7)	
721*	4 (7.5)	2 (5.7)	
44	2 (3.7)	3 (8.5)	
95	5 (9.4)		
724*	3 (5.6)	2 (5.7)	
57	1 (1.9)		
82			1 (5)
93	1 (1.9)		
641			1 (5)
719*	1 (1.9)		
725*	1 (1.9)		
726*	1 (1.9)		
728*	1 (1.9)		
729*	1 (1.9)		
730*	1 (1.9)		
731*	1 (1.9)		
732*	1 (1.9)		
736*	1 (1.9)		
738*		1 (2.9)	
740*			1 (5)
741		1 (2.9)	
744*		1 (2.9)	
749*		1 (2.9)	
750		1 (2.9)	
751		1 (2.9)	
752		1 (2.9)	
754	1 (1.9)		
Total no. of Hib strains	53	35	20

[a] Multilocus sequence typing (MLST) profiles of Hib strains carried by the children (presented in order of total carriage frequency). n is the number of children in the three study groups or populations.

[b] The sequence type (ST) is shown (e.g., ST6, ST722, ST53, etc.). An asterisk indicates that a new ST number has been assigned for isolates identified in this study.

similar to that found in Indonesia where carriage rates were 4.6% (8). In the Indonesian population, a Hib vaccine probe study indicated that the incidence rate of Hib-caused meningitis is >70/100,000 children under 5 years old (7), a rate 20 times the rate identified from hospital-based microbiological studies in this Nepali population (12). The Hib carriage prevalence in these areas contrasts with populations of notably high disease incidence, including Alaska (28) and the Gambia (1) in their respective prevaccine eras, where a Hib carriage prevalence of 7 to 10% was found and the disease rates were correspondingly >70/100,000 children (2, 28). The carriage rate we report here is lower than the rate of 7.7% reported in a study from north India where Hib disease rates are unknown (24). In keeping with other studies, although Hib is the second most common cause of bacterial meningitis in children in Kathmandu, Nepal, the absolute number of cases is relatively small (12). While Hib may cause a more significant burden of disease as pneumonia, it is only a small minority of cases that will be bacteremic. The population in Kathmandu, Nepal, has a high rate of prehospital antibiotic usage, and this is likely to result in a further underestimate of Hib disease burden (38; E. J. Williams, et al., presented at the 26th Annual Meeting of the European Society of Paediatric Infectious Diseases, Graz, Austria, 13 to 17 May 2008). This study of Hib carriage provides useful data to set alongside the previous microbiological surveillance of children with invasive bacterial disease. The prevalence of Hib carriage in this population supports the hypothesis that Hib is likely to be a significant pathogen in the country. It is likely that routine infant immunization with Hib conjugate vaccines will have a significant impact on the burden of pneumonia and meningitis in Nepali children. Such an impact could be documented after implementation by further investigation of the Hib carriage rate as a surrogate of vaccine effectiveness.

In keeping with previous Hib carriage studies, we found a trend to higher carriage rates in preschool children. Children in children's homes had a higher prevalence of Hib carriage, although when the prevalence was adjusted for crowding, it was not significant. This is consistent with other studies from day care centers (3) and "orphanages" (30) which show a higher rate of Hib carriage in these "closed" populations than in the general population with "open" transmission (i.e., OPD and school). This study provides evidence that this vulnerable population has at least as much circulating Hib as the general population and if universal vaccination reduces the population carriage rate of Hib and hence produces herd immunity, as has been found in other settings (9), it would be of benefit to this vulnerable population.

With the knowledge that the data generated in this study showed a high carriage rate in the children's homes, the local pediatricians provided free Hib vaccine to children aged 3 months to 5 years who were living in these orphanages. In order to ensure that all children in the orphanage were immunized, the team visited each month to immunize any new admissions. Six months after this program started (and with full ethical approval), we reswabbed children in the orphanage in the target age group and found that the carriage rate had dropped to 2/109 children (1.8%). Although this population is small and nonstatic, these data suggest that vaccination in this setting may reduce carriage rates, as has been widely described elsewhere.

These data are the first to show the genetic diversity of Hib strains in South Asia and are, to our knowledge, the largest MLST study of Hib isolates responsible for colonization and disease in the same population. The most striking feature of the population structure of Hib isolates in Kathmandu, Nepal, is the high level of diversity of STs in the study population. Previous studies of Hib carriage in the United Kingdom showed an SDI of 25% (19). In the Netherlands, the SDIs of Hib strains before and after immunization were 39% and 48%, respectively (23). In the current study, the overall SDI was 91%. This high level of diversity may be a general feature of open crowded urban settings in the unvaccinated developing world where there may be high rates of long-standing Hib circulation of diverse strains. In comparison, the "closed" populations of the children in children's homes in Kathmandu, Nepal, with relatively limited mixing of children but likely rapid transmission of colonizing strains could explain the lower diversity of strains identified.

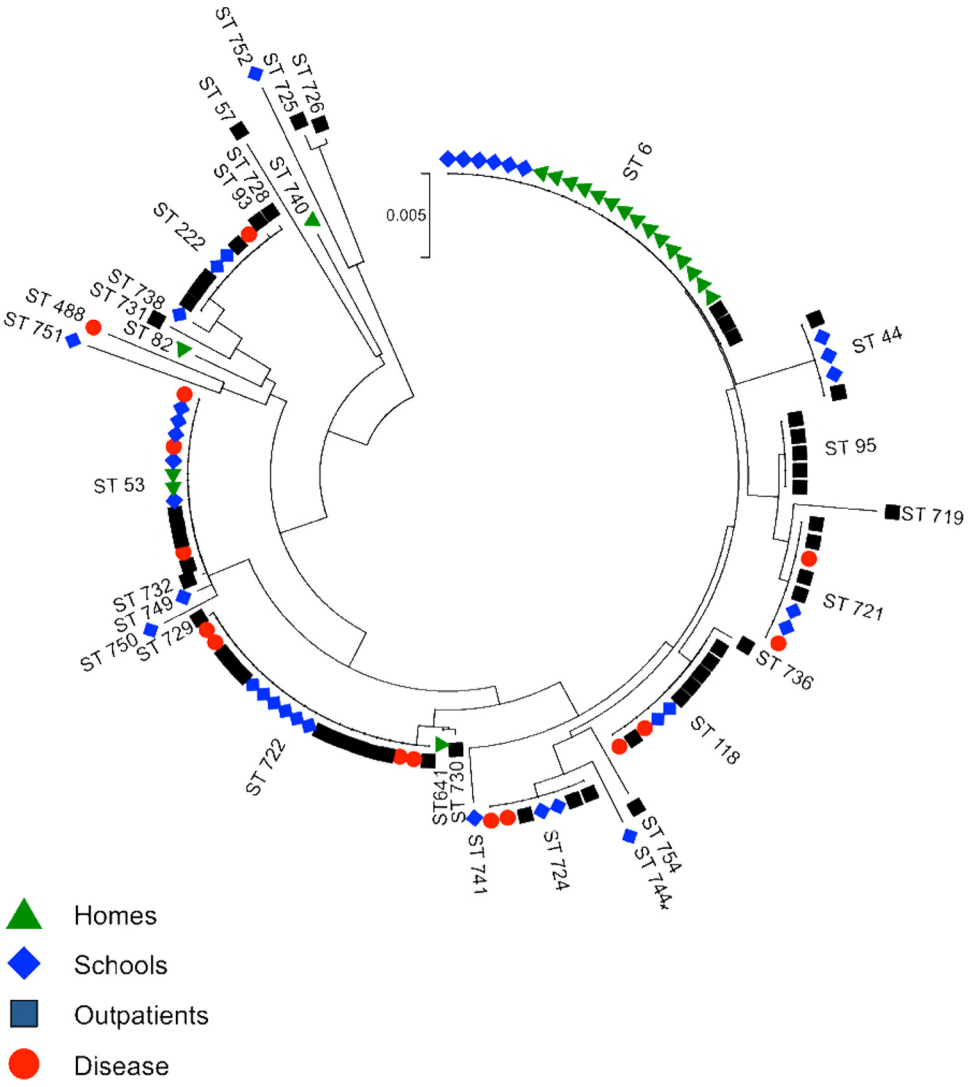

FIG. 1. Phylogenetic tree of Hib isolates from children in urban Nepal, from children carrying Hib and children with Hib-caused disease. The evolutionary history was inferred using the neighbor-joining method (22). The optimal tree with the sum of branch length equal to 0.14325348 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (32) and are in the units of the number of base substitutions per site. There were a total of 3,057 positions in the final data set. Phylogenetic analyses were conducted using MEGA4 software (31).

In keeping with most other studies of Hib carriage, ST6 was one of the most common strains, but unlike other studies, it was found in only one-fifth of cases in our population compared to 39 to 86% of Hib isolates in the United States and Europe (19, 21, 23). A further 40% STs were closely related to ST6 when phylogenetic analysis was undertaken, again in keeping with other studies. Other frequently identified STs (ST722, ST53, and ST118) were not predominant strains in the United States (21), Europe (19, 23), or China (33). This is not unexpected, as a large-scale multiregion study of Hib genetic profiles using electrophoretic typing showed that only 5 to 15% clones were shared between regions (18), and relatively few Asian Hib isolates have been submitted for sequence typing. Overall, almost half of the strains identified by MLST in this population have not previously been described, and the majority of these do not appear to have any clonal phylogenetic relationships to other isolates. This finding of frequent novel STs in Hib is similar to other studies from the Netherlands (23), Poland (29), and China (33) where between 40 and 78% are novel STs. These findings are in contrast to those from a United Kingdom carriage study, more than a decade after vaccine implementation, where there were no new STs identified (19).

Considering that this study was undertaken prior to universal vaccination in the country and that the genetic diversity of Hib increased with vaccination in other settings (23), the effect of universal vaccination on genetic diversity in Kathmandu, Nepal, cannot be readily predicted.

As described by previous investigators (18), most of the clones identified in individuals with invasive infections were

also those that were most frequently associated with carriage. The exception to this was ST6, as despite being the most prevalent ST in carriage, there were no disease-causing isolates that were ST6. However, the majority (15/24 isolates) of ST6 carriage was identified in the home population. As noted previously, the Hib isolates from children in the homes appear to have a population structure different from that of the more general population, and this finding of ST6 clustering could represent the close contact that these children share. The lack of ST6 disease-causing isolates suggests that the relatively high carriage prevalence of ST6 is skewed by the inclusion of the isolates from children living in children's homes and is further evidence that these children are not representative of the general population. An alternative explanation would be that the ST6 organisms have a low potential for invasive disease, but this appears unlikely from previous studies in other regions. It is not clear if all Hib bacteria are equally capable of causing invasive disease. The relationship between carriage and disease isolates suggests that vaccination strategies that reduce Hib carriage in this population should reduce the disease burden by reducing transmission to susceptible individuals (herd immunity).

This study has provided important data on the presence and genetic characterization of Hib carriage among children in urban Nepal. Comparison with studies of Hib carriage and disease in other areas of the world indicates that there is likely to be a significant burden of disease in the children in Kathmandu, Nepal. Following introduction of vaccine, a repeat of this carriage study with genetic phenotyping may be a more straightforward and low-cost means to provide a surrogate for the impact of a Hib vaccine program than a formal effectiveness study. Of particular interest is the very high level of genetic diversity of the Hib isolates compared to previously investigated populations. This may be a general feature of crowded urban populations in the developing world and would be explained by high levels of Hib transmission. Given the importance of herd immunity to vaccine effectiveness, it will be important to monitor the effect of routine immunization on carriage in such populations after implementation of routine immunization.

ACKNOWLEDGMENTS

This project was funded by the Oxford Vaccine Group, University of Oxford, United Kingdom with support from the NIHR Oxford Biomedical Research Centre (BRC). D.F.K. receives salary support from the NIHR BRC. A.J.P. acts as the chief investigator for clinical trials conducted on behalf of Oxford University (the clinical trials are sponsored by vaccine manufacturers, including manufacturers of pneumococcal vaccines) but does not receive any personal payment from them. Industry-sourced honoraria for lecturing or writing and travel expenses and grants for organization of educational activities are paid directly to an educational/administrative fund held by the Department of Paediatrics, University of Oxford. We have no other conflicts of interests to declare.

REFERENCES

1. **Adegbola, R., E. Mulholland, O. Secka, S. Jaffar, and B. Greenwood.** 1998. Vaccination with a Haemophilus influenzae type b conjugate vaccine reduces oropharyngeal carriage of H. influenzae type b among Gambian children. J. Infect. Dis. **177:**1758–1761.
2. **Adegbola, R., et al.** 2005. Elimination of Haemophilus influenzae type b (Hib) disease from The Gambia after the introduction of routine immunisation with a Hib conjugate vaccine: a prospective study. Lancet **366:**144–150.
3. **Akçakaya, N., Y. Camcioğlu, S. Belbek, G. Eçskazan, and H. Cokuğraçs.** 2001. Haemophilus influenzae type b colonization in children in a hospital-based day care center. Eur. J. Epidemiol. **17:**313–316.
4. **Baqui, A., et al.** 2007. Effectiveness of Haemophilus influenzae type B conjugate vaccine on prevention of pneumonia and meningitis in Bangladeshi children: a case-control study. Pediatr. Infect. Dis. J. **26:**565–571.
5. **Barbour, M., R. Mayon-White, C. Coles, D. Crook, and E. Moxon.** 1995. The impact of conjugate vaccine on carriage of Haemophilus influenzae type b. J. Infect. Dis. **171:**93–98.
6. **Feikin, D., et al.** 2004. Rapid assessment tool for Haemophilus influenzae type b disease in developing countries. Emerg. Infect. Dis. **10:**1270–1276.
7. **Gessner, B., et al.** 2005. Incidences of vaccine-preventable Haemophilus influenzae type b pneumonia and meningitis in Indonesian children: hamlet-randomised vaccine-probe trial. Lancet **365:**43–52.
8. **Gessner, B., et al.** 1998. A population-based survey of Haemophilus influenzae type b nasopharyngeal carriage prevalence in Lombok Island, Indonesia. Pediatr. Infect. Dis. J. **17:**S179–S182.
9. **Heath, P. T., and J. McVernon.** 2002. The UK Hib vaccine experience. Arch. Dis. Child. **86:**396–399.
10. **Hu, Y., S. Yu, G. Liu, W. Gao, and Y. Yang.** 2002. Antimicrobial susceptibility of Haemophilus influenzae among children in Beijing, China, 1999–2000. Acta Paediatr. **91:**136–140.
11. **Hunter, P. R., and M. A. Gaston.** 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. **26:**2465–2466.
12. **Kelly, D. F., et al.** 2011. The burden of vaccine-preventable invasive bacterial infections and pneumonia in children admitted to hospital in urban Nepal. Int. J. Infect. Dis. **15:**e17–e23.
13. **Lau, Y., et al.** 1998. Haemophilus influenzae type b infections in Hong Kong. Pediatr. Infect. Dis. J. **17:**S165–S169.
14. **McVernon, J., A. Howard, M. Slack, and M. Ramsay.** 2004. Long-term impact of vaccination on Haemophilus influenzae type b (Hib) carriage in the United Kingdom. Epidemiol. Infect. **132:**765–767.
15. **Meats, E., et al.** 2003. Characterization of encapsulated and noncapsulated Haemophilus influenzae and determination of phylogenetic relationships by multilocus sequence typing. J. Clin. Microbiol. **41:**1623–1636.
16. **Michaels, R., C. Poziviak, F. Stonebraker, and C. Norden.** 1976. Factors affecting pharyngeal Haemophilus influenzae type b colonization rates in children. J. Clin. Microbiol. **4:**413–417.
17. **Mulholland, K., et al.** 1997. Randomised trial of type-b tetanus protein conjugate for prevention of pneumonia and meningitis in Gambian infants. Lancet **349:**1191–1197. (Erratum, **350:**524.)
18. **Musser, J. M., et al.** 1990. Global genetic structure and molecular epidemiology of encapsulated Haemophilus influenzae. Rev. Infect. Dis. **12:**75–111.
19. **Oh, S. Y., et al.** 2008. School-aged children: a reservoir for continued circulation of Haemophilus influenzae type b in the United Kingdom. J. Infect. Dis. **197:**1275–1281.
20. **Olsen, S., S. Dejsirilert, L. Sangsuk, S. Chunsutiwat, and S. Dowell.** 2005. Frequent Haemophilus influenzae type B colonization in rural Thailand. Pediatr. Infect. Dis. J. **24:**739–742.
21. **Sacchi, C. T., et al.** 2005. High level of sequence diversity in the 16S rRNA genes of Haemophilus influenzae isolates is useful for molecular subtyping. J. Clin. Microbiol. **43:**3734–3742.
22. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
23. **Schouls, L. M., et al.** 2005. Increase in genetic diversity of Haemophilus influenzae serotype b (Hib) strains after introduction of Hib vaccination in The Netherlands. J. Clin. Microbiol. **43:**2741–2749.
24. **Sekhar, S., A. Chakraborti, and R. Kumar.** 2009. Haemophilus influenzae colonization and its risk factors in children aged <2 years in northern India. Epidemiol. Infect. **137:**156–160.
25. **Shah, A., et al.** 2009. Invasive pneumococcal disease in Kanti Children's Hospital, Nepal, as observed by the South Asian Pneumococcal Alliance network. Clin. Infect. Dis. **48**(Suppl. 2)**:**S123–S128.
26. **Sharma, P., et al.** 1996. Intravenous chloramphenicol plus penicillin versus intramuscular ceftriaxone for the treatment of pyogenic meningitis in Nepalese children. Trop. Doct. **26:**84–85.
27. **Simpson, E.** 1949. Measurement of diversity. Nature **163:**688.
28. **Singleton, R., et al.** 2000. Experience with the prevention of invasive Haemophilus influenzae type b disease by vaccination in Alaska: the impact of persistent oropharyngeal carriage. J. Pediatr. **137:**313–320.
29. **Skoczynska, A., M. Kadlubowski, J. Empel, and W. Hryniewicz.** 2005. Characteristics of Haemophilus influenzae type b responsible for meningitis in Poland from 1997 to 2004. J. Clin. Microbiol. **43:**5665–5669.
30. **Sulikowska, A., P. Grzesiowski, E. Sadowy, J. Fiett, and W. Hryniewicz.** 2004. Characteristics of Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis isolated from the nasopharynges of asymptomatic children and molecular analysis of S. pneumoniae and H. influenzae strain replacement in the nasopharynx. J. Clin. Microbiol. **42:**3942–3949.
31. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599.

32. **Tamura, K., M. Nei, and S. Kumar.** 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. U. S. A. **101:**11030–11035.
33. **Tian, G., et al.** 2009. Genotypic characteristics of Haemophilus influenzae isolates from pediatric pneumonia patients in Chengdu city, Sichuan, China. J. Microbiol. **47:**494–497.
34. **Tiwari, P. N.** 2003. Meningitis in children - a prospective study with latex agglutination test of the cerebrospinal fluid. J. Nepal Paediatr. Soc. **21:**20–33.
35. **Wang, S., et al.** 2008. Low rate of nasopharyngeal carriage and high rate of ampicillin resistance for Haemophilus influenzae among healthy children younger than 5 years old in northern Taiwan. J. Microbiol. Immunol. Infect. **41:**32–40.
36. **Watt, J. P., et al.** 2009. Burden of disease caused by Haemophilus influenzae type b in children younger than 5 years: global estimates. Lancet **374:**903–911.
37. **WHO.** 2006. WHO position paper on Haemophilus influenzae type b conjugate vaccines. Wkly. Epidemiol. Rec. **81:**445–452.
38. **Williams, E., et al.** 2009. Hospital-based surveillance of invasive pneumococcal disease among young children in urban Nepal. Clin. Infect. Dis. **48**(Suppl. 2)**:**S114–S122.