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Prevalence of the *hifBC*, *hmw1A*, *hmw2A*, *hmwC*, and *hia* Genes in *Haemophilus influenzae* Isolates

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Adherence of *Haemophilus influenzae* to respiratory epithelial cells is the first step in the pathogenesis of *H. influenzae* infection and is facilitated by the action of several adhesins located on the surface of the bacteria. In this study, prevalences of *hifBC*, which represent the pilus gene cluster; *hmw1A*, *hmw2A*, and *hmwC*, which represent high-molecular-weight (HMW) adhesin genes; and *hia*, which represents *H. influenzae* adhesin (Hia) genes were determined among clinical isolates of encapsulated type b (Hib) and nonencapsulated (NTHi) *H. influenzae*. *hifBC* genes were detected in 109 of 170 (64%) Hib strains and in 46 of 162 (28%) NTHi isolates ($P = 0.0001$) and were more prevalent among the invasive type b strains than invasive NTHi strains ($P = 0.00003$). Furthermore, *hifBC* genes were significantly more prevalent ($P = 0.0398$) among NTHi throat isolates than NTHi middle ear isolates. *hmw1A*, *hmw2A*, *hmwC*, and *hia* genes were not detected in Hib strains. Among NTHi isolates, the prevalence of *hmw1A* was 51%, the prevalence of *hmw2A* was 23%, the prevalence of *hmwC* was 48%, and the prevalence of *hia* was 33%. The *hmw* genes were significantly more prevalent among middle ear than throat isolates, while *hia* did not segregate with a respiratory tract site. These results show the variability of the presence of adhesin genes among clinical *H. influenzae* isolates and suggest that hemagglutinating pili may play a larger role in *H. influenzae* nasopharyngeal colonization than in acute otitis media whereas the HMW adhesins may be virulence factors for acute otitis media.

Haemophilus influenzae organisms are gram-negative bacilli characterized by the presence or absence of a polysaccharide capsule; strains that bind antibodies directed against one of six capsular types are designated serotypes a to f and strains that do not bind these antibodies are designated nontypeable. *H. influenzae* possessing the type b capsule (Hib) causes serious invasive infections such as meningitis, epiglottitis, septic arthritis, and facial and periorbital cellulitis accompanied by bacteremia in nonimmune individuals, while nontypeable *H. influenzae* (NTHi) possessing no capsule are important causes of nonbacteremic respiratory infections such as acute otitis media, sinusitis, and bronchitis.

Irrespective of the presence or absence of the capsule, the first step in the pathogenesis of both respiratory and invasive *H. influenzae* infections is asymptomatic colonization of the nasopharynx. *H. influenzae* organisms are inhaled through the upper respiratory tract and, following initial interactions with respiratory mucus (21, 49), utilize a number of adhesins on the bacterial surface to adhere to respiratory epithelial cells. Both Hib and NTHi adhere to respiratory cells by means of hemagglutinating pili (15), P5 fimbriae (2), lipo-oligosaccharide (43), *H. influenzae* adherence and penetration protein (Hap) (39), opacity-associated protein (OapA) (32), and *Haemophilus* surface fibrils (Hsf) (38). In addition to these adhesins, NTHi organisms, which are more genetically diverse than Hib (30), possess additional epithelial cell adhesins, including the high-molecular-weight (HMW) proteins HMW1 and HMW2 (40) and *H. influenzae* adhesin (Hia) (41), which is an allele of the Hsf of Hib (38).

Among the most extensively studied of *H. influenzae* adhesins, hemagglutinating pili are peritrichous, hair-like polymeric structures that protrude from the *H. influenzae* outer membrane (15, 42) and mediate adherence to sialic acid-containing lactosylceramide structures on epithelial cell surfaces (45). Biosynthesis of pili requires the products of five genes, *hifA* through *hifE*, located in the pilus gene *hif* cluster (15, 24, 47, 50). Among NTHi strains, *hifA* (which encodes the pilus structural gene), *hifD* (which encodes a pilus terminal protein), and *hifE* (which encodes the pilus adhesin) show considerable strain-to-strain variation in their nucleotide sequences (9, 24, 34). Among Hib strains, *hifA* shows sequence diversity while *hifD* and *hifE* show sequence homogeneity (15). The nucleotide sequences of *hifB* (which encodes a chaperone-like protein) and *hifC* (which encodes an assembly platform [usher] protein) are highly conserved among all *H. influenzae* strains (15, 24).

Recent studies of many *H. influenzae* strains have documented dramatic genetic variation within the *hif* gene region, which is located between genes homologous to *pepN* and *purE* of *Escherichia coli* and is flanked by dyad repeat sequences (33) that may facilitate recombination. NTHi strains exhibit insertions, deletions, duplications, and rearrangements both within and flanking the *hif* cluster; in some strains the entire cluster is deleted (9, 13, 24, 27, 33, 34). A subset of NTHi strains asso-

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TABLE 1. Oligonucleotides used for constructing DNA probes

Probe	Primer[a]	Nucleotide sequence of primers	Size (bp)	Position	Prototype *H. influenzae* strain
hif BC	hiB-F	5′-CGCCGCCTGTTGCTCGAGTG-3′	1,191	1571–3020	Eagan (GenBank accession no. U13254)
	hifC-R	5′-CACGCCACGCACCACGGGGG-3′			
hia	hia-F	5′-CGCGGCTTGGGCTGGGTCATTTCT-3′	766	2201–2967	Strain 11 (GenBank accession no. U38617)
	hia-R	5′-TCAGCCGTACCGTCAGCATTCAGTTCA-3′			
hmwC	hmwC-F	5′-TTATGGGCAGGGAATCAACAACTTT-3′	703	553–1255	Strain 12 (GenBank accession no. AF 180945)
	hmwC-R	5′-CACTGCCCACATAATCATCTTCTACGA-3′			
hmw1A	hmw1-F1	5′-CCACCGGTGATGATACCAGAGGTG-3′	923	1959–2881	Strain 12 (GenBank accession no. U08876)
	hmw1-R1	5′-CGGCTTTCCTGGAGCCAAAGGTGA-3′			
hmw2A	hmw2-F2	5′-GTCGCCCAGGGCACTGTAACCATT-3′	731	2122–2852	Strain 12 (GenBank accession no. U08875)
	hmw2-R2	5′-CCGCCCAGAATGGATATGTTGTAG-3′			
pepN	pepN-F	5′-GATGGTCGCCATTGGGTGG-3′	918	1219–2137	Rd (GenBank accession no. NC_000907)
	pepN-R	5′-GATCTGCGGTTGGCGGTGTGG-3′			

[a] Primers were named for their respective genes. F and R correspond to forward and reverse direction, respectively.

ciated with conjunctivitis, the so-called *H. influenzae* biogroup aegyptius strains, possess a second copy of the *hif* cluster, located between genes homologous to *pmbA* and *hpt* genes of *E. coli*.

Bacterial expression of hemagglutinating pili is altered through a process called phase variation, which is mediated by slipped-strand mispairing (15), suggesting a means by which *H. influenzae* may rapidly adapt to changing environments. By extension, this also suggests that the presence or absence of the pilus gene cluster is important to *H. influenzae* in its adaptation to the environment (29).

The HMW adhesins HMW1 (125 kDa) and HMW2 (120 kDa), members of the auto-transporter family of proteins, are encoded by genes present in two separate chromosomal loci, *hmw1AC* and *hmw2AC. hmw1A* and *hmw2A*, which encode the adhesive molecule, show 71% identity and 80% similarity (3, 40) among NTHi strains, suggesting that they may be alleles. Recombinant *E. coli* strains expressing either HMW1 or HMW2, however, exhibit different binding characteristics to several human cell lines (18); HMW1 binds to sialylated glycoproteins, whereas the receptor for HMW2 is undefined (37). Recent studies report that both HMW1 and HMW2 are glycosylated (16). *hmwA* genes are located immediately upstream of the accessory genes *hmwB*, which encodes an outer membrane protein responsible for translocation of HMW1 and HMW2 across the outer membrane, and *hmwC*, which encodes a cytoplasmic protein that stabilizes HMW1 and HMW2 (40). HMW1B and HMW2B are 99% identical, while HMW1C and HMW2C are 97% identical (4). Previous studies have shown that 75% of NTHi strains and a few type a, e, and f strains express proteins belonging to the HMW1 and HMW2 family (35, 41).

Hia, found in NTHi strains and in some type a, e, and f strains (35), is an auto-transporter protein encoded by the 3.3-kb *hia* gene (5). Hia shows 72% amino acid identity and 81% similarity to the Hsf adhesins, expressed by Hib (38), suggesting they represent allelic variants. Previous studies revealed that a *hia* homolog is present in approximately 80% of HMW1/HMW2-deficient NTHi strains (5, 35).

To gain better understanding of the roles of *H. influenzae* adhesins in the pathogenesis of *H. influenzae* infections, we investigated the prevalences of the *hifBC*, *hmw1A*, *hmw2A*, *hmwC*, and *hia* genes in a collection of Hib and NTHi invasive and respiratory mucosal isolates. To assess the potential importance of these adhesins in mediating adherence to various body tissues, we stratified the strains by body site of isolation. These studies were predicated on the evolutionary principle that stochastic gain or loss of genetic material through recombination events provides a plastic population of bacteria whose members, because of their variability, are capable of rapidly adapting to environmental changes (17, 22).

MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. influenzae* strains obtained from clinical samples from ill children included 170 Hib and 97 NTHi strains isolated between 1982 and 1995 in Michigan as well as 26 NTHi strains isolated between 1979 and 1982 in Minnesota, 16 NTHi strains isolated between 1996 and 1997 in Missouri, and 3 NTHi strains from Kentucky and 2 NTHi strains from Pittsburgh isolated in 2002. In addition, 18 NTHi throat strains were isolated from healthy children attending day care in Michigan in 1998. Bacterial strains were grown for 6 to 20 h on chocolate agar plates or in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with hemin (10 μg/ml) and NAD (4 μg/ml) at 37°C with 5% CO_2 in a humid atmosphere (1). The strains were identified by standard methods (19) and serotyped by agglutination with antisera specific for *H. influenzae* capsule types a to f (Difco Laboratories).

Isolation of genomic DNA from *H. influenzae*. Genomic DNA was isolated from *H. influenzae* strains using the Wizard genomic DNA purification kit (Promega, Madison, Wis.) according to the manufacturer's instructions.

DNA gene probes. DNA probes were designed to detect conserved regions within *hifB*, *hifC*, *hmwC*, *hmw1A*, *hmw 2A*, *hia*, and *pepN* and are shown in Table 1 and Fig. 1. All primers were synthesized at the University of Michigan Biomedical Research Core Facility and by Invitrogen (Carlsbad, Calif.). The final probes were confirmed by Southern hybridization using positive and negative controls.

The probes were generated by PCRs using a model PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, Mass.). The nucleotide sequence of each probe was confirmed by sequence analysis, performed at the University of Michigan Sequence Core Laboratory.

PCR amplification for *hifBC* regions. In a standard 50-μl reaction mixture, 50 ng of *H. influenzae* strain Eagan genomic DNA was mixed with 20 pmol of *hifB*-F and *hifC*-R primers and 45 μl of PCR Supermix (Gibco BRL, Gaithersburg, Md.). The final PCR mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), and 1 U of recombinant *Taq* DNA polymerase along with the *H. influenzae* genomic DNA and primers. The amplification cycle consisted of an initial 1 min hold at 95°C followed by 35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, followed by a final elongation step for 1 min at 72°C. The amplified PCR product of the *hifBC* intergenic region was digested with DraI, and the 1,191-bp fragment was used as the *hifBC* probe.

PCR amplification for *hia*, *hmw1A*, *hmw2A*, *hmwC*, and *pepN* region probes. Genomic DNA from NTHi strain 12 was used as a template for *hmw1A*, *hmw2A*, and *hmwC*; that from NTHi strain 11 was used as a template for *hia*; and that

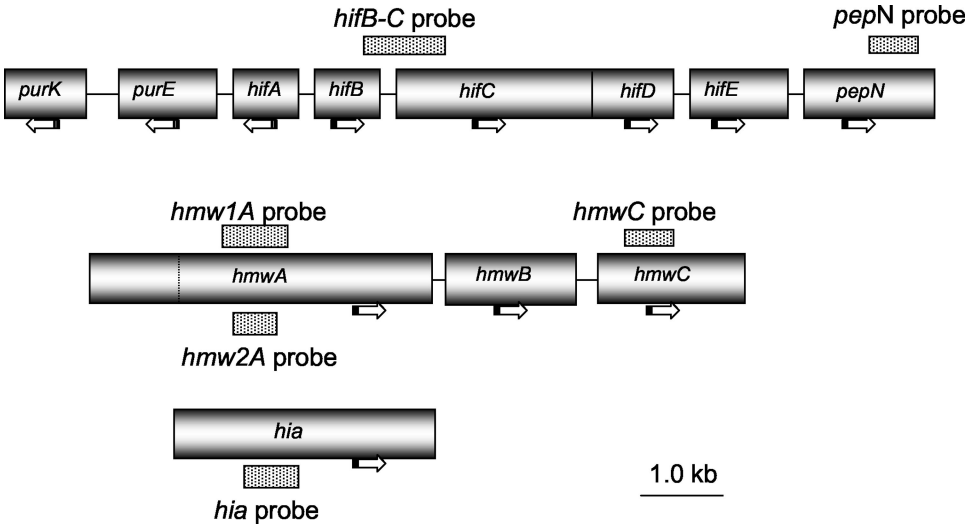

FIG. 1. Localization of probes used in this study. Horizontal arrows indicate the directions of transcription. The dotted line in the *hmwA* gene represents bp 1259; *hmw1A* and *hms2A* genes are highly conserved between bp 1 and 1259.

from strain Rd was used as a template for *pepN*. Using the PCR strategy described above, samples were incubated 5 min at 95°C for an initial denaturation step and were subjected to 30 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min in a final 50-μl reaction mixture. All amplifications were performed with 1 μl of genomic DNA, a 10 mM concentration of each deoxynucleoside triphosphate, 5 mM $MgCl_2$, 4 U of platinum *Taq* polymerase, and 25 pmol of each forward and reverse primer.

PCR amplified DNA, generated using *hmwC*-F and *hmwC*-R primers, was purified using QIAquick Spin PCR purification kit (QIAGEN, Valencia, Calif.) and cloned into the plasmid TOPO vector 2.1 from Invitrogen. The recombinant plasmid DNA was prepared using a plasmid kit (QIAGEN) according to the manufacturer's instruction and digested with EcoRI. The appropriate DNA fragment was used as a *hmwC* probe after gel purification.

Gel purification and labeling. Twenty microliters of the PCR products of *hmw1A*, *hmw2A*, *hia*, *pepN*, the DraI-digested region of *hifBC*, and the plasmid DNAs of *hmwC* were gel purified on 1% agarose gel with modified 1× TEA buffer (40 mM Tris-acetate, pH 8.0; 0.1 mM Na_2-EDTA). Specific bands were excised and purified by using an Ultrafree-DA centrifugal filter device (Millipore, Bedford, Mass.), labeled with fluorescein, and used as DNA probes (ECF Random Prime Labeling Kit; Amersham Pharmacia Biotech, Piscataway, N.J.).

Total DNA isolation and dot blot hybridization. Crude DNA was isolated from *H. influenzae* lysates and used for dot blot analysis as follows. One microliter of defrosted skim milk stock of each *H. influenzae* isolate was grown in microtiter plate wells in 800 μl of brain heart infusion broth supplemented with NAD and hemin by overnight incubation at 37°C (17). The microtiter plates were then centrifuged at 1,000 × *g* for 20 min (IEC HN-SII; International Equipment, Needham Height, Mass.). The supernatant was discarded and the pellets were suspended in 800 μl of lysis buffer (0.4 M NaOH, 10 mM EDTA). The plates were incubated at 70°C for 0.5 h and centrifuged again for 5 min. The final DNA concentrations were determined by spectrophotometry to confirm similar concentrations among DNA preparations. Eighty microliters of DNA lysate from each well was blotted onto Hybond N^+ membranes (Amersham Pharmacia Biotech) with a Bio-Dot Microfiltration Apparatus (Bio-Rad, Hercules, Calif.) and washed with 80 μl of 0.4 M NaOH. After air drying, DNA was cross-linked to the membranes by exposure to UV light for 3 min.

The dot blots were hybridized to fluorescein-labeled DNA fragments under stringent conditions (68°C). Following hybridization, membranes were washed 15 min at 68°C (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate) and 15 min at 68°C (0.1× SSC, 0.1% sodium dodecyl sulfate), reacted with a 1:7,000 dilution of antifluorescein antibody coupled to alkaline phosphatase to amplify the signal, and developed with ECF substrate (Amersham Pharmacia Biotech) to detect chemiluminescence. Duplicate membranes were tested with each probe.

The DNA from appropriate positive and negative control *H. influenzae* strains were placed on each membrane. *Hib* strains Eagan, M43, and *H. influenzae* biogroup aegyptius (F3031) were used as positive controls for *hifBC* probes and as negative controls for *hmw1A*, *hmw2A*, *hmwC*, and *hia* probes, while NTHi strains AAr73 and AAr176 were used as negative controls for *hifBC* probes. NTHi strain 12, originally isolated from the middle ear fluid of a child with otitis media, is the prototype for the *hmw1* and *hmw2* clusters (3) and was used as a positive control for *hmw1A*, *hmw2A*, and *hmwC* and as a negative control for *hia*. NTHi strain 11, from which *hia* was cloned (3), was used as a positive control for *hia* and as negative control for *hmw1A*, *hmw2A*, and *hmwC*. *H. influenzae* strain Rd was used as a negative control for all genes examined in this study.

The signal intensity of each dot on the membranes was detected by using a STORM 860 Phosphor Imager (Storm System; Molecular Dynamics, Sunnyvale, Calif.) and recorded in the form of intensity volume, expressed as a percentage of the positive controls after correcting for the background signal (51). Duplicate measurements were obtained on two different membranes for each probe. A strain with signal intensity above 50% of the positive control for at least one of the replicate measurements was classified as positive. Since all *H. influenzae* strains tested in our laboratory to date contain the *pepN* homolog, a *pepN* probe was used to normalize the quantity of DNA on the membranes. Strain samples giving intermediate or discrepant hybridization intensity results with each probe were confirmed by Southern blot hybridization or by PCR using the appropriate primers for the genes of interest (36).

Data analysis. The data presented in Table 2 were treated as counts corresponding to a three-way representation (isolate site versus strains versus presence or absence of *hifBC* genes). We thus performed a stratified analysis that examined the association between two variables while adjusting for the effects of others. To determine the difference in prevalence rates of *hifBC* genes between the Hib and NTHi strains, adjusting (stratifying) for the isolate site effect, we used Mantel-Haenszel tests for the overall prevalence both at the stratum level as well as for the combined table. Further, odds ratios were calculated at each isolate level and tested for significance. Finally, homogeneity of odds ratios across the isolates was tested. Exact *P* values were computed wherever appropriate.

The prevalence and distribution of the nonpilus adhesin genes in NTHi invasive and respiratory isolates (Table 3) were compared by Fisher's exact test for binomial proportions. Apart from an overall comparison, differences between throat and middle ear strains among the respiratory isolates were of primary interest. Due to the presence of multiple genes in a single isolate (Table 4), the categories presented in Table 3 are overlapping. Consequently, a "marginal" analysis involving each individual gene type was used.

RESULTS

Detection of *hifBC* genes among Hib and NTHi isolates. A total of 170 Hib and 162 NTHi strains isolated from a variety of body sites were screened for hybridization to the *hifBC*

TABLE 2. Prevalence and distribution of *hifBC* genes in various isolates of *H. influenzae*

H. influenzae isolate	No. of strains/total no. of strains isolated (%)[a]	
	Hib	NTHi
Invasive		
Blood	65/100 (65)	3/15 (20)
Cerebrospinal fluid	38/62 (61)	0/4
Joint fluid	2/2	0/2
Pleural fluid	1/1	0
Pus	0/1	0/1
Lung biopsy		1/1
Total invasive	106/166 (64)§	4/23 (17)§
Respiratory		
Throat		23/60 (38)◇
Middle ear		10/50 (20)◇
Sputum		3/13 (23)
Trachea		4/8
Epiglottis	2/3	2/3
Conjunctiva	1/1	0/3
Nose		0/2
Total respiratory	3/4 (75)*	42/139 (30)*
Total	109/170 (64)□	46/162 (28)□

[a] Symbols: §, $P = 0.00003$; ◇, $P = 0.0398$; *, $P = 0.0922$ (Fisher's exact test) or $P < 0.0001$ (Mantel-Haenszel test); □, $P = 0.0001$.

probe, which represents the highly conserved *hifB* and *hifC* genes of the pilus *hif* cluster (Table 2). Of the 170 Hib isolates, 109 (64%) hybridized to the *hifBC* probe compared to 46 of the 162 (28%) NTHi isolates ($P = 0.0001$), consistent with the findings of a previous study examining the presence of *hifA* using PCR (8).

TABLE 4. Distribution of *hifBC*, *hia*, *hmw1A*, *hmw2A*, and *hmwC* genes in combination with each other among *H. influenzae* isolates

Presence of gene					No. with genes (%)	
hifBC	*hmw1A*	*hmw2A*	*hmwC*	*hia*	Hib ($n = 170$)	NTHi ($n = 162$)
+	−	−	−	−	109 (64)	3 (2)
−	+	−	−	−	0	7 (4)
−	+	+	−	−	0	1 (1)
−	+	+	+	−	0	28 (17)
−	+	−	+	−	0	30 (19)
−	−	+	+	−	0	2 (1)
−	+	−	+	+	0	1 (1)
−	+	−	−	+	0	2 (2)
−	−	−	+	−	0	9 (6)
−	−	−	−	+	0	27 (17)
+	−	−	+	+	0	1 (1)
+	−	+	−	−	0	5 (3)
+	+	−	+	−	0	6 (4)
+	+	+	+	−	0	1 (1)
+	+	+	−	−	0	1 (1)
+	+	−	−	+	0	1 (1)
+	+	−	−	−	0	5 (3)
+	−	−	−	+	0	22 (14)
−	−	−	−	−	61 (36)	10 (6)

To examine the relative roles of *H. influenzae* genetic background (as determined by presence or absence of type b capsule) and environmental selection in fostering the presence of the pilus genes, we compared the hybridization of the *hifBC* probe to Hib and NTHi strains stratified by isolation from an otherwise sterile site (thus indicating bacterial invasion) or from the respiratory tract. Among the invasive *H. influenzae* isolates, the *hifBC* probe hybridized to 106 of the 166 (64%) type b strains and 4 of the 23 (17%) nontypeable strains. Fisher's exact test (equivalent to Mantel-Haenszel test in this

TABLE 3. Prevalence and distribution of *hmw1A*, *hmw2A*, *hmwC*, and *hia* genes in NTHi isolates

NTHi isolates	No. of strains possessing gene/total (%)			
	hmw1A	*hmw2A*	*hmwC*	*hia*
Invasive				
Blood ($n = 15$)	4/15 (27)	2/15 (13)	4/15 (27)	8/15 (53)
Cerebrospinal fluid ($n = 4$)	3/4	0/4	2/4	1/4
Joint fluid ($n = 2$)	2/2	0/2	2/2	0/2
Pus ($n = 1$)	0/1	0/1	0/1	1/1
Lung biopsy ($n = 1$)	1/1	1/1	1/1	0/1
Total ($n = 23$)	10/23 (43)	3/23 (13)	9/23 (39)	10/23 (43)
Respiratory				
Throat ($n = 60$)	24/60 (40)◇	9/60 (15)*	22/60 (37)□	23/60 (38)¶
Middle ear ($n = 50$)	38/50 (76)◇	18/50 (36)*	33/50 (66)□	12/50 (24)¶
Sputum ($n = 13$)	5/13 (38)	4/13 (31)	6/13 (46)	5/13 (38)
Trachea ($n = 8$)	3/8 (38)	2/8 (25)	4/8 (50)	2/8 (25)
Epiglottis ($n = 3$)	0/3	0/3	0/3	2/3
Conjunctiva ($n = 3$)	3/3	2/3	3/3	0/3
Nose ($n = 2$)	0/2	0/2	1/2	0/2
Total ($n = 139$)	73/139 (53)	35/139 (25)	69/139 (50)	44/139 (32)
Total ($n = 162$)	83/162 (51)	38/162 (23)	78/162 (48)	54/162 (33)

[a] Symbols: ◇, $P < 0.0001$; *, $P = 0.0099$; □, $P = 0.0019$; ¶, $P = 0.15$.

case) showed $P = 0.00003$, indicating a highly significant difference between Hib and NTHi. Among the respiratory isolates, on the other hand, the difference between *hifBC* rates of hybridization to type b and nontypeable strains failed to reach significance at the 5% level (*P* value based on Fisher's exact test equaled 0.0922). Thus, the differences in prevalence of the *hif* cluster among Hib and NTHi strains appear to be related to the nature of type b organisms (which are known to be highly clonal) rather than to environmental selection. A combined Mantel-Haenszel test for the difference in prevalence of *hifBC* between the two strains adjusting for the isolate effect yielded a *P* value less than 0.0001. This overall significance is presumably attributed to the invasive isolate data which are more evenly balanced in the number of strains.

A slightly different approach to the comparative analysis is provided by the odds ratio, which essentially estimates the likelihood (odds) of finding a *hifBC* gene in a given strain in comparison to the others. In the invasive isolates, the odds ratio is estimated to be 8.4, indicating that the *hifBC* gene is about eight times more likely to be present in Hib than in NTHi strains. The associated exact 95% confidence limit of the odds ratio is [2.6, 35.1]. The corresponding estimate of the odds ratio of the *hifBC* gene in the respiratory isolates is 6.9, with an associated exact 95% confidence interval of [0.53, 367.4]. The extreme width of the interval in the latter case is a reflection of low numbers of Hib respiratory strains. A homogeneity test of odds ratios across the isolates did not find any significant difference ($P = 0.8828$).

To assess the roles of pili in colonization and in otitis media, we compared the presence of the *hif* cluster among NTHi strains isolated from throats of children with its presence among NTHi isolates from the middle ears of children with otitis media. The *hifBC* probe hybridized significantly more frequently to throat (23 of 60 [38%]) than to middle ear (10 of 50 [20%]; exact $P = 0.0398$) isolates, suggesting that *H. influenzae* carrying the *hif* cluster has a selective advantage in the throat compared to the middle ear space.

Prevalence of *hmw1A*, *hmw2A*, *hmwC*, and *hia* genes among *H. influenzae* isolates. The *hmw1A*, *hmw2A*, and *hmwC* probes hybridized to none of the type b strains, consistent with results from previous studies (41). Furthermore, the *hia* probe did not hybridize with type b strains, even though the Hia adhesin of NTHi is a homologue to the Hsf adhesin found on Hib (38). Sequence analysis of our *hia* probe revealed 60% homology with *hsf*, thus explaining its failure to hybridize with type b strains. Overall, among the 162 nontypeable isolates, 83 (51%) hybridized with *hmw1A* and 38 (23%) hybridized with *hmw2A* (Table 3). Furthermore, 52 of 162 (32%) hybridized with *hmw1A* and not *hmw2A*, whereas 7 of 162 (4%) hybridized with *hmw2A* and not *hmw1A*. A total of 31 of 162 (19%) strains hybridized with both, and 72 of 162 (44%) hybridized with neither. In addition, 78 of 162 (48%) hybridized with *hmwC*, and 54 of 162 (33%) hybridized to the *hia* probe, a somewhat higher prevalence than described previously (41). Thus, considerable variability in the presence of these genes was seen among the nontypeable strains tested.

There was no difference between hybridization of *hmw1A*, *hmw2A*, *hmwC*, or *hia* probes to invasive NTHi isolates compared to that of NTHi respiratory isolates (exact *P* values ranging between 0.29 and 0.5), suggesting that *H. influenzae* expressing the HMW or Hia adhesins is not selected either for or against during systemic invasion.

To assess the roles of HMW and Hia adhesins in throat colonization and in otitis media, we compared the prevalences of these genes among *H. influenzae* throat isolates to middle ear isolates. *hmw1A*, *hmw2A*, and *hmwC* were significantly more prevalent in middle ear isolates (one-sided exact *P* values based on Fisher's test are 0.0001, 0.0099, and 0.0019, respectively). There is, however, no significant difference in distribution of *hia* genes between throat and middle ear isolates ($P = 0.15$). This suggests that the HMW adhesin provides a survival advantage in the middle ear space while Hia does not offer a survival advantage in either location.

Table 4 shows the associations of the adhesin genes with each other. Ten of 162 (6%) NTHi isolates and 61 of 170 (38%) Hib isolates did not hybridize with any of the probes. The most common patterns seen with the *hmw* genes was *hmw 1A* and *C* positive (37 strains) and *hmw1A*, *hmw2A*, and *hmwC* positive (29 strains); only 7 strains had *hmw2A* without *hmw1A*, five of which did not carry *hmwC* and, thus, would be incapable of expressing functional HMW (4). Because the *hmwC* probe would be expected to hybridize to all *hmw* genes, our dot blot technique didn't allow us to assess the presence of complete *hmw* gene clusters or to localize these genes in the *H. influenzae* chromosome. More than a third (68 of 162, 42%) of NTHi gave evidence of *hmwA* and *hmwC* genes, suggesting the presence of a complete *hmw* cluster, whereas 62 of 162 (38%) had neither *hmwA* nor *hmwC* genes, suggesting the lack of a *hmw* cluster. Of the 62 NTHi isolates without evidence of an *hmw* cluster, 49 (79%) hybridized with the *hia* probe. A total of 13 of the 162 (8%) NTHi isolates hybridized with neither *hia* nor one of the *hmw* genes, whereas 5 (3%) hybridized with *hia* and at least one of the *hmw* genes, which contradicts previous studies that suggest *hia* and *hmw* are mutually exclusive (35, 41).

DISCUSSION

Recent advances in bacterial genomics have revealed wide genetic variability between organisms of the same species; for example, sequence comparisons have shown up to 25% differences in the gene content of strains of *Neisseria meningitidis*, *Helicobacter pylori*, and *Escherichia coli* (6). While a complete genomic sequence is currently available for only one *H. influenzae* strain (12), studies of individual *H. influenzae* virulence genes have demonstrated considerable variability among strains in both their presence and sequences. The variability of lipo-oligosaccharide and metabolic function genes has been ascribed to frequent recombination events (10, 26), which most likely also facilitate the variability among the adhesin genes in this study.

Previous studies have demonstrated the genetic variability of the *H. influenzae hif* gene region, which contains the *hif* cluster that encodes *H. influenzae* hemagglutinating pili. Geluk et al. (13) used Southern blotting to identify *hifA*, *hifB*, *hifD*, and *hifE* and PCR amplification to identify *hifC* in 83 NTHi respiratory isolates. Only 18% of the strains contained homologues of the entire *hif* region; the remaining genomes contained none of the *hif* genes. This all-or-none dichotomy was not substantiated in the study of Mhlanga-Mutangadura et al. (27), who

analyzed the nucleotide sequences of the *hif* region PCR products from 14 *H. influenzae* strains. All four of the type b strains had intact *hif* regions, while 8 of the 10 nontypeable strains contained no *hif* genes; one nontypeable strain and one type f strain contained pseudo-*hifA* genes; a deletion of the entire *hifB*; and deletions in *hifC*, *hifD*, and *hifE*, as well as additional individual mutations. Furthermore, Read et al. (34) demonstrated the presence of *hifA* and *hifE* homologues by PCR amplification in two of five nontypeable respiratory strains. One of the five also showed a second copy of *hifA*, which was similar in size to the PCR product of the *hifA1* that is found in a second copy of the *hif* cluster described in *H. influenzae* biogroup aegyptius strains associated with conjunctivitis and Brazilian purpuric fever. Finally, Rodriguez et al. (35) showed that 37% of type a *H. influenzae* strains and 8% of type e *H. influenzae* strains hybridized to *hifA*, *hifB*, *hifC*, *hifD*, and *hifE* probes and 82% of type f *H. influenzae* strains hybridized with *hifA*, *hifC*, *hifD*, and *hifE* probes, but none hybridized with the *hifB* probe, demonstrating the genetic conservation described previously with type f strains (7, 9, 26, 31). Thus, the *hif* gene region is highly variable, particularly among nontypeable strains and the *hif* gene cluster, when present, is not intact in some *H. influenzae* strains.

To determine the presence of the *hif* cluster among a large number of Hib and NTHi isolates, we performed dot blot hybridization studies utilizing a probe that spanned the highly conserved *hifB* and *hifC* genes of this cluster. Our results show that the *hifBC* region is significantly more prevalent among type b strains than nontypeable strains, irrespective of whether these strains were isolated from respiratory samples or from invasive samples, suggesting that the high prevalence of pilus genes in type b strains is related to their clonal population structure (30) rather than to environmentally induced selection (28) during invasion. In addition to these studies of the *hif* genes, other studies have shown that pili are expressed more commonly in colonizing Hib isolates than invasive isolates (23, 42), demonstrating the known phase variation of pilus expression (14). In the systemic circulation, Hib expressing pili may be selected against by virtue of their increased susceptibility to phagocytosis (44).

Our results also show increased prevalence of *hifBC* among NTHi isolated from throat samples compared to those from the middle ears of children with otitis media, consistent with a model of hemagglutinating pili playing a larger role in *H. influenzae* nasopharyngeal colonization than in the establishment of infection in the middle ear and corroborate the findings of Krasan et al. (20). These findings differ from the results of Geluk et al. (13), who showed no difference in presence of the pilus gene cluster among *H. influenzae* isolates from patients with otitis media and from healthy carriers. The study by Geluk et al. (13), however, was compromised by small numbers of *H. influenzae* isolates, and the carrier strains were isolated from healthy adults. Our study tested a much larger number of isolates, and the carrier strains were isolated from throat samples from both healthy children and individuals with a respiratory infection.

It is possible that some of our strains contained *hifA*, *hifD*, or *hifE* genes, or their fragments, in the absence of *hifB* and *hifC*; if such were the case, our estimates of the prevalence of any *hif* gene would be artificially low. Such organisms, however, would not be capable of expressing functional pili (25, 46, 50) and, thus, would be similar to strains lacking the *hif* cluster in their susceptibility to natural selection. Likewise, the dot blot hybridization we employed could not distinguish between the presence of complete or partial *hifB* and *hifC*. Based on the findings of other investigators (13, 27), the probability that *hifBC* positive strains represent incomplete *hif* clusters is relatively small.

Prevalence studies of *hmw* and *hia* genes have shown their presence in 80 and 20%, respectively, of nontypeable strains (41). Neither of these genes have been reported in type b strains (3), but *hmw* was seen in 26% of type a strains, 8% of type e strains, and 5% of type f strains, while *hia* was seen in 74% of type a, 92% of type e, and 95% of type f strains (35).

In testing *hmw* prevalence among NTHi strains, St. Geme et al. (41) used a probe from the 5′ region of *hmw1A* which is highly conserved with, and cross-hybridizes with, *hmw2A*. In contrast, our study used *hmw1A* and *hmw2A* probes from the highly diverse 3′ regions and did not demonstrate cross-hybridization (data not shown). In addition, we used a probe from *hmwC*, which is highly conserved (96%) between *hmw1* and *hmw2*. Our results describe variability in the presence of these genes, with NTHi possessing either *hmw1* or *hmw2* or both or neither.

The prevalence of *hmw1* and *hmw2* among nontypeable strains in our study (56%) was somewhat lower than that described in the study by St. Geme (79.7%) (41). This difference may reflect the source of the strains, as our study used primarily NTHi strains from middle ear and throat cultures, while the study of St. Geme used primarily invasive nontypeable strains. We were, however, unable to detect a difference in the prevalences of *hmw1* and/or *hmw2* among NTHi invasive (48%) and noninvasive (55%) strains.

An advantage of the probes used in our study is their ability to distinguish between the prevalence of *hmw1A* and *hmw2A* genes, which appear to be alleles; about half of the nontypeable strains hybridized with the *hmw1A* probe, and a quarter hybridized with the *hmw2A* probe; about a fifth of strains hybridized with both; and almost half hybridized with neither. In addition, a third of the NTHi strains hybridized with *hmw1* but not *hmw2*, and very few hybridized with *hmw2* but not *hmw1*. These results suggest duplication and heterogeneity of *hmw* genes that is reminiscent of the situation with the pilus gene cluster in *H. influenzae* biogroup aegyptius strains (as well as a few other NTHi strains) in which two copies of the *hif* gene clusters may be present, although neither copy is necessarily complete (34). Duplication of *hmwA* may provide a survival advantage to the organisms, although the low number of strains carrying *hmw2A* without *hmw1A* raises the question of the function of HMW2A. HMW1 mediates binding to a sialic acid containing glycoprotein (37), and the receptor characteristics of HMW2 are unknown, although RGD-mediated adherence to the integrin CR3 has been suggested (48). *H. influenzae* strains carrying a duplication of *hif* clusters (which we were unable to detect with the methods used in this study), each with a phase variable *hifA*, assures a higher probability of pilus expression, since nonexpression would require both *hifA* genes to be in the "off" configuration. The *hmw1A* and *hmw2A* genes, on the other hand, appear to differ in function (18). While *hmw1A* and *hmw2A* contain a series of seven base pair repeats

(11) that allows phase variation of their expression from weak to strong, the effect of this variation on adherence is unclear, as *H. influenzae* organisms that carry the *hmw* genes but do not exhibit *hmw*-mediated adherence have not been widely described (35).

Of the 52 strains that hybridized with neither *hmw1A* nor *hmw2A*, 10 hybridized with *hifC*. This suggests that these strains possess *hmw1A* or *hmw2A* with deletions in the variable regions, do not possess these genes at all, or may possess *hmwA* genes that are variable enough from either *hmw1A* or *hmw2A* that they do not hybridize with the probes. Ongoing studies in our laboratory will address this question. van Schilfgaarde (48) et al. describe an *H. influenzae* strain that carries an allele of *hmw* whose genetic sequences and gene product antigenicity differ from those of *hmw1A* and *hmw2A*. The full scope of genetic differences in *hmwA* genes in *H. influenzae* awaits sequence analyses of these genes from a variety of strains.

Previous studies have found that the presence of *hia* and the *hmw* genes are mutually exclusive in both NTHi (41) and type a, e, and f strains (35), although an otitis media strain has been reported to carry both *hmw* and *hia* genes (20). In our study, we identified a small number of NTHi strains (5 of 162 [3%]) that hybridized to the *hia* probe and to at least one of the *hmw* region probes. These findings, along with the variability in presence of *hmw1A*, *hmw2A*, and *hmwC* and the genetic variability of the *hif* cluster (13, 27, 34), underscore the high genetic variability of Hia genes.

In summary, the results of this study repeat the growing theme of significant genetic variability in *H. influenzae* virulence genes, particularly among NTHi strains, which are less clonal in their population structure than type b strains. This variability, facilitated by genetic recombination, appears to allow *H. influenzae* to survive in various environmental niches. In addition, these results suggest that hemagglutinating pili play a more important role in *H. influenzae* nasopharyngeal colonization than in acute otitis media, whereas the HMW adhesins may be virulence factors for acute otitis media.

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