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Frequency and Properties of Naturally Occurring Adherent Piliated Strains of *Haemophilus influenzae* Type b

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We found that 41 of 75 (55%) children with *Haemophilus influenzae* type b disease (70 cases of meningitis, 2 of cellulitis, 2 of septic arthritis, and 1 of epiglottitis) and 2 of 120 (1.7%) children with upper respiratory infection were colonized with *H. influenzae* type b in the nasopharynx (NP). Of these 43 NP strains from children with systemic *H. influenzae* type b disease, 7 (16%) adhered to human buccal epithelial cells. The strains isolated from the systemic site of all children, including children from whose NP adherent bacteria were isolated, did not adhere to buccal epithelial cells in vitro. Each adherent NP strain had biotype (I), serotype (b), and antibiotic susceptibility (sensitive) similar to that of the corresponding nonadherent systemic isolate. With one exception, all NP-systemic pairs had similar major outer membrane proteins. Six of the seven NP strains had a protein band in the whole cell lysate preparation with a molecular weight between 22,000 and 23,000, which could not be seen in the nonadherent cerebrospinal fluid strains. Electron micrographs of all adherent strains showed that more than 95% of the organisms examined were highly piliated, whereas the nonadherent strains were not piliated. All piliated strains agglutinated human erythrocytes. Adherence to buccal epithelial cells and agglutination of erythrocytes could not be blocked by mannose or α-methyl-D-mannoside. We speculate that piliation is not important for NP colonization by *H. influenzae* type b and that the loss of pili may be required for host invasion.

We previously reported that strains of *Haemophilus influenzae* type b (HiTb) isolated from sites of systemic disease did not adhere (<3 HiTb organisms per buccal epithelial cell [BEC]) to human BECs in vitro (10). However, two isolates, one from the nasopharynx (NP) of the sibling of a child with meningitis, and one from the middle ear of a child with otitis media, did adhere (8 and 25 HiTb organisms per BEC, respectively) to human BECs. Pichichero et al. (17) subsequently reported that most strains of HiTb isolated from the NP and all of the strains isolated from systemic sites adhered weakly (0.2 to 0.6 HiTb organism per BEC) to human epithelial cells. They did, however, find two strains from the NP of children that demonstrated much greater adherence to BECs (9.4 and 15.3 bacteria per cell). These investigators demonstrated that strains with enhanced adherence to BECs also agglutinated erythrocytes (RBCs), contained pilus-like structures by electron microscopy, and had an extra protein band in the outer membrane with a molecular weight (MW) of 20,000, as demonstrated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). None of these properties were associated with nonadherent strains.

Studies with an infant rat model of HiTb meningitis produced by intranasal inoculation of adherent piliated HiTb demonstrated that the bacteria subsequently isolated from the blood or cerebrospinal fluid (CSF) are not adherent to human BECs, do not have pili by electron microscopy, and have lost a protein band at MW 25,000 (8). Nonpiliated strains isolated from the systemic site of infection in the same patient are as virulent as piliated NP strains in the infant rat model and remain unchanged and nonadherent on subsequent isolation from blood or CSF (8).

This study describes the frequency of occurrence and selected properties of adherent piliated strains of HiTb in children with systemic HiTb infection to further our understanding of the piliated phenotype for HiTb.

MATERIALS AND METHODS

Population and specimens. Patients less than 12 years of age who were seen in the pediatric clinic of the Ben Taub Hospital with the primary complaint of upper respiratory infection were enrolled in the study. Likewise, patients admitted to Texas Children's Hospital with systemic disease suspected of being caused by HiTb were enrolled in this study as a part of a larger antibiotic treatment study. The history of any prior or current antibiotic usage was noted at the time the culture was obtained.

Pharyngeal cultures were obtained by swabbing the posterior pharynx with a cotton-tipped swab, which was plated to selective agar medium containing Leventhal base, brain heart infusion (BHI) agar, bacitracin, and burro antiserum to the polyribosylphosphate capsule of HiTb (kindly provided by John Robbins) (15). After incubation for 48 h at 35°C, multiple colonies surrounded by a precipitin halo were picked, transfered to chocolate agar, and identified and biotyped by standard methods (9). Confirmed HiTb isolates were stored frozen at −70°C in buffered tryptic soy broth with 10% glycerol. Adherence assays were performed within 1 week of initial isolation.

Quantitation of adherence. Experiments to determine the ability of strains to adhere to human BECs were performed by two independent methods, a fluorescent antibody stain and a radiolabeled adherence assay, both previously described (10). Two independent methods were used as a way to confirm the adherence results, and as reported previously, the results obtained by these two methods correlated well

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TABLE 1. Effect of antibiotic treatment on the ability to isolate HiTb from the NP

Antibiotic therapy before culture	No. of children:		
	Not colonized	Colonized	
		Nonadherent strains	Adherent strains
None	2	10	0
1 Dose	15	19	6
>1 Dose	17	5	1

(10). All the isolates were tested after initial isolation on selective differential agar, and in separate experiments it was found that up to three passages on this medium did not effect the ability of adherent stains to adhere to BECs. With the immunofluorescence method, bacteria were found to be evenly distributed on all buccal cells and that more than 90% of buccal cells had adherent bacteria when a strain was determined to be adherent. Bacteria for adherence studies were grown overnight in supplemented BHI broth (BHI plus 10 μg of each of hemin and NAD per ml). Viability in this medium after overnight growth was consistently 10^9 CFU/ml. BECs were obtained from healthy adult laboratory workers. The effects of mannose and α-methyl-D-mannoside on adherence were studied by using the above two techniques, except that the bacteria were incubated in a 3% solution of the appropriate sugar at 37°C for 1 h before mixing with the epithelial cells. The incubation of the bacteria with the epithelial cells also was performed in the presence of 3% solutions of the sugars or their derivatives in phosphate-buffered saline. The degree of adherence was measured by determining the average number of bacteria adhering to 25 epithelial cells with the fluorescent antibody method or by calculating the percent adherence with the radiolabeled method by the formula: [(counts per minute of BEC + ^{3}H-HiTb) − (counts per minute of ^{3}H-HiTb alone)/(counts per minute for 1 ml of ^{3}H-HiTb)] × 100, as previously described (10). An adherent HiTb strain was defined as having an average of ≥3 adherent bacteria per BEC by the fluorescent antibody method and a percent adherence of ≥0.3% by the radiolabeled method (10).

SDS-PAGE analysis. HiTb cells were grown overnight in 10 ml of supplemented BHI broth. Outer membrane proteins were prepared by the method of Barenkamp et al. (2) with sonic disruption of the cells followed by extraction of the outer membranes with *N*-lauroyl sarcosine. SDS-PAGE was performed in 10% acrylamide gel by the method of Laemmli as modified by Lugtenberg (11). Whole-cell lysates were prepared by centrifuging 10 ml of overnight culture in supplemented BHI broth and resuspending the pellet in 1 ml of distilled water. The cells were lysed by the addition of 1 ml of sample buffer, resulting in a final concentration of 2% SDS and 5% mercaptoethanol. All preparations were heated at 100°C for 5 min before electrophoresis.

Hemagglutination. The ability of the bacteria to hemagglutinate human RBCs was determined by a modification of the assay described by Pichichero et al. (17). Bacteria were suspended in phosphate-buffered saline (pH 7.2) to a concentration of 10^9 CFU/ml, and 0.1 ml of this suspension was added to the well of a microtiter plate. Human RBCs were washed in phosphate-buffered saline and resuspended to a concentration of 10%. The assay was performed by adding 0.01 ml of the RBC suspension to 0.1 ml of the bacteria and incubating at 37°C for 1 h. Hemagglutination was considered positive when the RBCs showed strong immediate agglutination macroscopically.

Electron microscopy. One drop of an overnight broth culture of HiTb washed in phosphate-buffered saline and resuspended to a concentration of 10^9 CFU/ml was placed on agarose for negative staining with 0.5% uranyl acetate by a pseudoreplicating technique (12). BECs incubated with adherent and nonadherent bacteria were prepared as previously described (10), except that after removal of the BECs and adherent bacteria from the filter, they were fixed in 3% phosphate-buffered glutaraldehyde and subjected to critical-point drying with carbon dioxide. The grids were coated with 150 angstroms (15 nm) of gold palladium in a Denton vacuum evaporator. Specimens were viewed with a Jeol 100/CS electron microscope.

Statistical analysis. Statistical analysis of the data was by the chi-square test with the Yates correction.

RESULTS

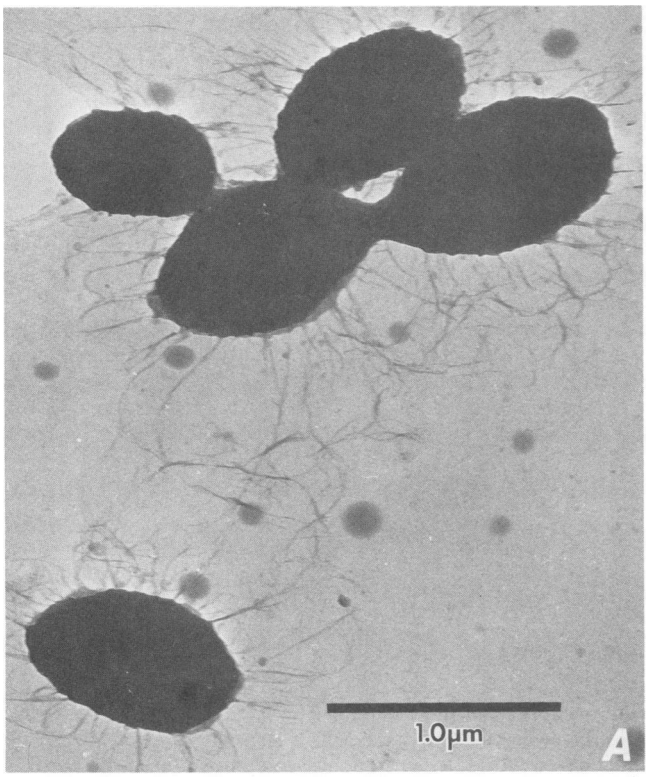
A total of 120 children with upper respiratory infections had NP cultures obtained between November 1981 and October 1982. Two children were found to have HiTb in their throat (1.7%), and these two isolates were not adherent to BECs in vitro. A total of 75 children with systemic HiTb disease (70 cases of meningitis, 2 of cellulitis, 2 of septic arthritis, and 1 of epiglottitis) were cultured between November 1981 and February 1984. Forty-one (54.7%) were colonized in the NP with HiTb. Seven (17.1%) of these NP strains from children with meningitis, but none of the strains isolated from the systemic site in the same patients, adhered to BECs in vitro. In every instance the NP-systemic pair of isolates had concordant biotypes, serotypes (all type b), and antibiotic susceptibility.

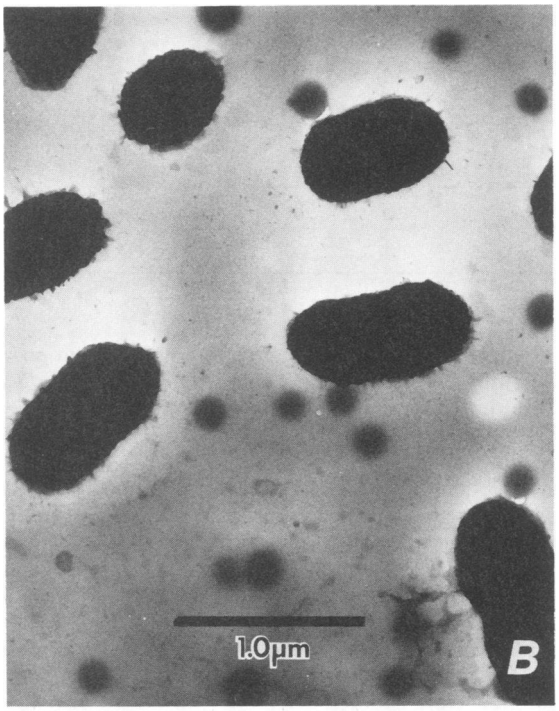
Prior administration of more than one dose of antibiotic significantly affected the ability to isolate HiTb from the NP ($\chi^2 = 9.33$; $P = 0.002$) (Table 1). A total of 10 of 12 (83.3%) children who had received no antibiotic before the NP culture was obtained and 25 of 40 (62.5%) of those children who had received only one dose before the NP culture was obtained were colonized by HiTb. In contrast, only 6 of 23 (26.1%) children were colonized when they had received

TABLE 2. Effect of sugars on the adherence of HiTb to human BECs[a]

Strain	Method	No. of HiTb cells per BEC (FA) or % radiolabeled adherence (RA) with:		
		Control	Mannose	Mannoside
884	FA	16.1 ± 4.9	15.5 ± 6.8	19.1 ± 9.5
	RA	1.8 ± 1.6	1.0 ± 1.0	1.6 ± 1.4
1009	FA	30.2 ± 17.9	35.8 ± 8.3	35.6 ± 16.3
	RA	3.0 ± 0.8	4.1 ± 1.2	3.4 ± 0.2
1264	FA	12.6 ± 3.4	19.3 ± 4.3	21.8 ± 2.4
	RA	1.0 ± 0.2	0.6 ± 0.1	1.0 ± 0.2
1540	FA	11.6 ± 1.4	15.0 ± 1.9	9.7 ± 1.3
	RA	1.4 ± 0.1	1.1 ± 0.4	1.1 ± 0.3
1549	FA	11.3 ± 1.4	12.5 ± 1.5	15.6 ± 1.6
	RA	1.2 ± 0.5	1.0 ± 0.3	1.1 ± 0.3
1565	FA	4.6 ± 0.4	5.2 ± 0.5	6.7 ± 0.7
	RA	1.0 ± 0.3	0.8 ± 0.2	0.7 ± 0.2
1601	FA	17.3 ± 1.7	15.2 ± 1.7	11.1 ± 0.9
	RA	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0

[a] FA, Fluorescent antibody method; RA, radiolabeled method. Values represent the mean ± standard error of the mean. The mean is the average of at least three separate experiments.





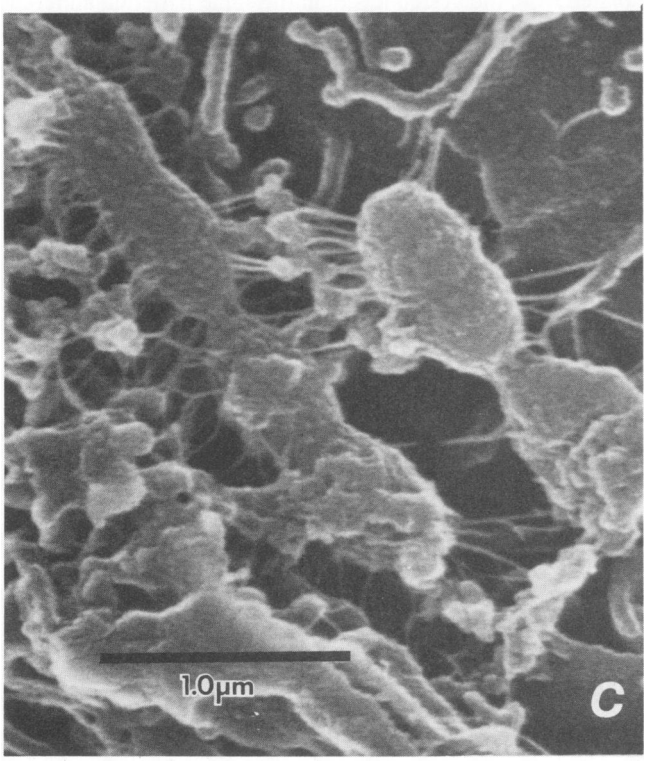


FIG. 1. (A) Transmission electron micrograph of adherent *H. influenzae* 1009 showing highly piliated cells. ×31,922. (B) Transmission electron micrograph of nonadherent, HiTb strain 1007 showing nonpiliated cells. ×26,520. (C) Scanning electron micrograph of BEC with adherent HiTb on the surface. ×33,750.

more than one dose of antibiotic before NP culture. There were no differences between the ages of colonized and noncolonized children (mean ages, 1.3 and 1.2 years, respectively).

Of the 75 systemic isolates, 22 (29.3%) elaborated beta-lactamase, which is a rate of ampicillin resistance similar to that observed in our population over the last several years. There was no association between beta-lactamase production or biotype and colonization and adherence.

All bacterial isolates from the NP and systemic sources from children with invasive disease due to HiTb were screened for adherence to human BECs in vitro by two independent methods. RBC enrichment procedures were not employed. Bacteria used in each primary adherence assay were overnight stationary cultures which had been passed once on artificial medium. None of the isolates from systemic sources were adherent to human BECs in either assay. The seven NP isolates that were found to adhere to BECs in vitro were all isolated from patients with meningitis and did not differ in their rate of isolation after antibiotic therapy, in their biotype, or in their antibiotic susceptibility when compared with those from the larger population of nonadherent strains isolated from colonized individuals. One adherent strain (strain 1009) was isolated from a child who had a twin sibling who was simultaneously ill with meningitis. The NP of the twin was colonized with an HiTb strain which did not adhere to BECs in vitro but had an identical outer membrane protein pattern.

Repeated experiments with two methods to demonstrate adherence revealed that mannose and α-methyl-D-mannoside did not inhibit the attachment of the seven adherent HiTb strains to adult human BECs (Table 2). Incubation of the systemic isolates with either mannose or α-methyl-D-mannoside had no effect on their inability to adhere to BECs. Experiments with the same matched isolates revealed that only the adherent NP isolates, not the systemic isolates, caused agglutination of human type B RBCs, which was not altered by these sugars.

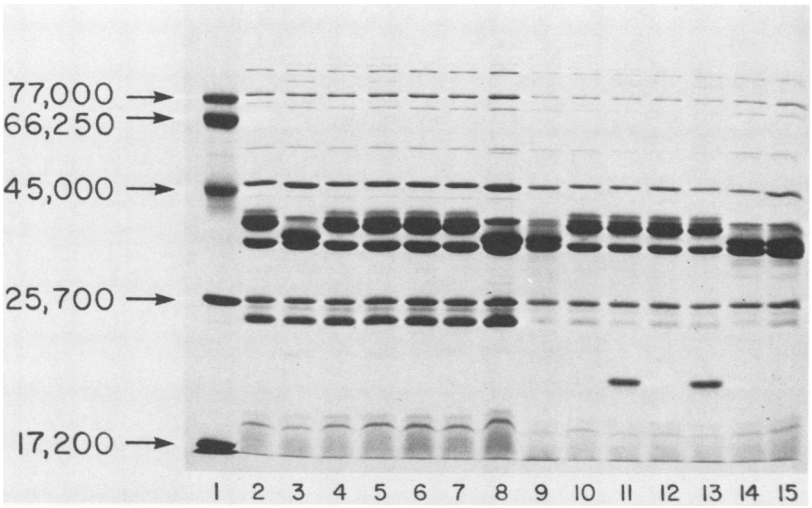


FIG. 2. SDS-PAGE analysis of outer membrane protein preparations of systemic-nonadherent and NP-adherent isolate pairs. Lanes 2 and 3, strains 880 and 884*; lanes 4 and 5, strains 1007 and 1009*; lanes 6 and 7, strains 1228 and 1264*; lanes 8 and 9, strains 1539 and 1540*; lanes 10 and 11, strains 1548 and 1549*; lanes 12 and 13, strains 1558 and 1565*; lanes 14 and 15, strains 1596 and 1601* (asterisks designate NP-adherent isolates). The conditions for PAGE were a 44:0.8 acrylamide-bisacrylamide ratio and 11% acrylamide stained with Coomassie blue R. Lane 1, Molecular weight standards (ovotransferrin, bovine serum albumin, ovalbumins, carbonic anhydrase, and myoglobin). Electrophoresis was at a constant current of 25 mA.

Transmission electron microscopy revealed that >95% of the adherent strains of HiTb were covered with fine pilus-like structures (Fig. 1A). Similar electron micrographs of identical preparations of nonadherent bacteria showed a complete lack of these structures (Fig. 1B). Scanning electron microscopy of BECs incubated with adherent bacteria and then washed free of any nonadherent cells showed the bacteria attached to the surface of the BEC (Fig. 1C).

Analysis of the major outer membrane proteins by PAGE confirms the similarity of the strains isolated from a systemic source and those isolated from the NP. Figure 2 shows that, with the exception of a single pair (strains 880 and 884), each pair of isolates had identical major outer membrane proteins. Strains 880 (systemic) and 884 (NP) had different major outer membrane proteins patterns. Routine screening of the nonadherent NP isolates and the systemic isolates from the same patient also showed concordance in the major outer membrane proteins of the pairs. Adherent NP strains 1549 and 1565, which were isolated from the NP of two different children, differed from their nonadherent systemic isolate counterparts by the presence of an additional protein band at MW 24,000. These bands are not considered to be the major outer membrane proteins used by other authors to differentiate strains of *H. influenzae*. Because these NP isolates adhered to BECs, were piliated, and agglutinated human RBCs, the possibility that these bands represented pili was likely. These bands could not be demonstrated consistently in the outer membrane preparations of the other five adherent strains. Figure 3 shows the presence of proteins which range in MW between 20,050 and 22,850 in six of the seven adherent stains (lanes 3, 5, 7, 9, 11, and 13). Corresponding protein bands in the nonadherent, nonpiliated isolates from the systemic sites in these patients could not be demonstrated. This protein band could not be demonstrated in one of the adherent, piliated strains in either the outer membrane preparation or in the whole-cell lysate (lane 15). These extra protein bands in the whole-cell lysates of the adherent isolates appear to be similar to the bands seen in the outer membrane protein preparations.

DISCUSSION

The ability of pathogenic bacteria to attach to host epithelial surfaces appears important in the pathogenesis of infections of the urogenital, digestive, and respiratory tract (6, 18). In many instances, the ability to cause disease is directly correlated to the presence of bacterial pili (18). Knowledge of the virulence attributed to the presence of pili has come primarily from the study of *Escherichia coli* causing urinary tract infections or gastroenteritis (22), *Neisseria gonorrhoeae* causing urogenital infection (13), and various enteric bacteria causing respiratory infection in patients on mechanical ventilators (6). These infections are not usually associated with bacteremia. The mechanisms by which other invasive encapsulated bacteria (*H. influenzae* type b, *Streptococcus pneumoniae*, and *Neisseria meningitidis*) colonize and initiate disease are less well known (20). Isolates of *S. pneumoniae* from healthy carriers and patients with recurrent otitis media were found to be more adherent to pharyngeal epithelial cells than those strains isolated from sites of systemic infection (1). Similarly, piliated isolates from the throats of healthy carriers of *N. meningitidis* were significantly more adherent to BECs in vitro than were isolates from the CSF or blood of patients with meningococcal disease (3). Adherence of two strains of HiTb from nonsystemic infections was first reported by Lampe et al. (10). Isolates of HiTb from systemic sources were not adherent to buccal or pharyngeal epithelial cells from several individuals, including children and adults.

Guerina et al. (5) reported that clinical isolates of HiTb can express or suppress pili under routine laboratory conditions. They suggested that pili play a role in oropharyngeal adherence and colonization by HiTb and described a nonpiliated HiTb strain isolated from CSF and a highly piliated HiTb strain isolated from the throat of this same patient. A third strain isolated from the blood of another patient was initially nonpiliated but became moderately piliated and adherent to BECs after enrichment procedures were performed for isolation of a subpopulation. Pichichero et al. (17) isolated two

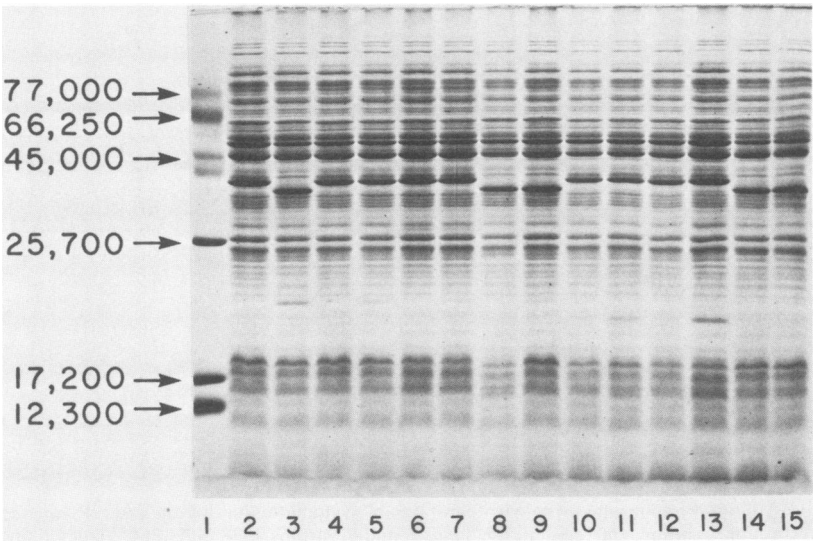


FIG. 3. SDS-PAGE analysis of whole-cell lysate preparations of systemic-nonadherent and NP-adherent isolate pairs. Lanes 2 and 3, strains 880 and 884*; lanes 4 and 5; strains 1007 and 1009*; lanes 6 and 7, strains 1228 and 1264*; lanes 8 and 9, strains 1539 and 1540*; lanes 10 and 11, strains 1548 and 1549*; lanes 12 and 13, strains 1558 and 1565*; lanes 14 and 15, strains 1596 and 1601* (asterisks designate NP-adherent isolates). The conditions for PAGE were 30:0.8 acrylamide-bisacrylamide ratio and 12.5% acrylamide stained with Coomassie blue R. Lane 1, Molecular weight standards (ovotransferrin, bovine serum albumin, ovalbumins, carbonic anhydrase, myoglobin, and cytochrome c). Electrophoresis was at a constant current of 25 mA.

highly piliated strains of HiTb directly from the oropharynx and piliated subpopulations of HiTb from several systemic isolates by using RBC enrichment. These authors felt that pili may be expressed by HiTb colonizing the oropharynx and demonstrated antibody to pilus protein in the serum of a child recovering from HiTb disease.

Mannose-resistant pili with a subunit MW of 24,000 from HiTb have been recently characterized by Stull et al. (21). Although this study was not designed to be a systematic search for adherent piliated HiTb isolates, the authors found that pili were not uniformly present in type b strains isolated from the NP or CSF of patients. Further investigation into the genetics of pili expression, however, revealed that 86% of the strains isolated from the CSF contained the genetic information necessary for the expression of pili (21).

We described here seven strains of highly piliated HiTb which were isolated from the NP but not the CSF of patients with meningitis. Biochemical characteristics and major outer membrane protein patterns of these strains, with one exception, appear to be identical to those of the systemic strains isolated from the same patient, except that the systemic strains did not have characteristics associated with piliation. These seven strains were not selected by RBC enrichment, a procedure which concentrates piliated strains, but were isolated in this highly piliated state directly from the NP of these children. They retained this high degree of piliation in storage and on multiple passage and caused macroagglutination of human RBCs.

Two adherent HiTb NP strains had an additional minor protein band at 24,000 daltons which may be pilin subunits that were not noted in the paired systemic isolates. Although we could not demonstrate this protein in the outer membranes of the other five adherent isolates, we showed similar bands in the 20,000- to 25,000-MW range in whole-cell lysates of these adherent, piliated strains. Similarly Pichichero et al. reported the presence in the outer membrane of a 20,000-MW protein band from the NP isolate but not the CSF HiTb isolate from the same patient (17). Stull et al. (21) purified the pilin protein of strains of *H. influenzae* and estimated the subunit MW as 24,000. The apparent subunit MW of the HiTb pilin is within the range noted for the pilin protein subunit of other bacteria (16). We found that all adherent HiTb strains were mannose resistant, a characteristic which may be more common for pathogenic *E. coli* strains than for commensals (4, 14). Previous investigators have noted mannose-resistant pili for other *Haemophilus* species (7, 19), as well as for *N. meningitidis* (23).

If piliation of HiTb were important in colonizing the NP of children before the development of systemic disease, one would expect to find piliated bacteria colonizing the NP early in the disease. Since only 17% of the children colonized in the NP with HiTb had piliated bacteria isolated from their throats, we conclude that either piliation is not necessary for colonization in children, or that piliated HiTb may lose this property soon after colonization is initiated. We do not feel that piliation is lost during subculture, since our highly piliated strains did not lose pili after multiple passages in vitro and remained pilitated in storage at −70°C. Therefore, the finding that only 17% of the NP isolates are piliated is probably real. Furthermore, CSF and blood isolates from infant rats inoculated with piliated HiTb were no longer piliated (8). Likewise, Guerina et al. (4) showed that only nonpiliated bacteria were isolated from blood cultures when neonatal rats were fed piliated *E. coli*. Perhaps, as is suggested by the loss of pili on invasion, piliation is lost early, on contact with mucosal epithelial cells, and this loss is a prerequisite for invasion.

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