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Haemophilus influenzae: Comparison of Respiratory Tract Isolates with Genitourinary Tract Isolates

WILLIAM L. ALBRITTON,[1]†* JAMES L. BRUNTON,[2,3] MICHAEL MEIER,[3] M. NANCY BOWMAN,[1] AND LESLIE A. SLANEY[1]

Department of Pediatrics and Medical Microbiology, University of Manitoba, Winnipeg, Manitoba,[1] Department of Medicine, Mt. Sinai Hospital, Toronto, Ontario,[2] and Department of Medical Bacteriology and Department of Medicine, University of Alberta, Edmonton, Alberta,[3] Canada

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Haemophilus influenzae isolates recovered from the genitourinary (GU) tract were shown to have a significantly different biotype distribution compared with respiratory tract isolates. Biotype IV strains were recovered more commonly from the GU tract, and most strains were non-serotypable. Antibiotic-susceptible strains isolated from the GU tract more frequently harbored plasmids of <10 megadaltons than did antibiotic-susceptible respiratory tract strains. One 2.8-megadalton plasmid resident in a GU tract isolate and one 1.8-megadalton plasmid resident in a respiratory tract isolate were shown to be related to the small ampicillin resistance plasmids previously described in *H. influenzae*, *Haemophilus parainfluenzae*, *Haemophilus ducreyi*, and *Neisseria gonorrhoeae*. This supports the suggestion that these ampicillin resistance plasmids originated by transposition or recombination of the ampicillin transposon (Tn*A*) with cryptic endogenous *Haemophilus* plasmids.

Genitourinary (GU) tract colonization with *Haemophilus influenzae* appears to be infrequent, and serious infections from this source are uncommon (17, 24, 33). Previous studies have suggested bacteriological differences between *H. influenzae* GU tract infections and their associated neonatal infections and the more frequently reported respiratory infections in infants and adults due to this organism (3, 8). In general, the organisms isolated from the GU tract are non-encapsulated, and the biotype distribution appears to be different.

Since 1976, we have isolated *H. influenzae* from the GU tract of 82 patients; these isolates include strains from two surveys of GU tract colonization due to this organism in asymptomatic pregnant females and symptomatic males and females in an ambulatory population. This study reports the results of biotyping and plasmid analysis of these GU tract isolates compared with a similar analysis of respiratory tract isolates.

MATERIALS AND METHODS

Bacterial strains and plasmids. GU tract isolates of *H. influenzae* from 82 patients were analyzed and compared with 122 consecutively isolated respiratory strains. Sixty-four of the GU tract strains were recovered by routine isolation in the Clinical Microbiology Laboratory of the Health Sciences Centre, Winnipeg, Manitoba, Canada. Five isolates were recovered during a 12-month prospective study of genital tract colonization in 1,724 asymptomatic pregnant women. Twelve isolates were recovered during a 2-month prospective study of 722 symptomatic patients presenting to the Primary Health Care Clinic of the Health Sciences Centre, a clinic responsible for the treatment of sexually transmitted diseases. A single strain was recovered from a genital ulcer lesion in an ongoing prospective study in Nairobi, Kenya. Clinical and demographic data were obtained from a review of hospital charts. The bacterial plasmids used in this study are described in Table 1.

Culture methods. Routine isolations were made on split plates of 1% hemoglobin agar supplemented with CVA enrichment (GIBCO) and modified Thayer-Martin medium. Isolations from the two prospective studies were made on hemoglobin agar or selective media, which included hemoglobin agar with 5 U of bacitracin per ml or supplemented Mueller-Hinton broth containing bacitracin (5 U/ml). Cultures were incubated at 37°C in a 5% CO_2 atmosphere. Organisms were identified as *H. influenzae* if they had typical colony morphology and Gram stain and required both X and V factors for growth on tryptic soy agar. Biotyping was done by the method of Kilian, as previously described (3). Preliminary antibiotic resistance to ampicillin, tetracycline, chloramphenicol, and kanamycin was determined by disk sensitivity testing, as previously described (3). Ampicillin-resistant isolates were screened for the production of β-lactamase with a chromogenic cephalosporin, and the minimum inhibitory concentrations of tetracycline for strains resistant

† Present address: Sexually Transmitted Diseases Research Laboratory, Center for Infectious Diseases, Centers for Disease Control, Atlanta, GA 30333.

TABLE 1. Bacterial plasmids

Plasmid	Molecular mass ($\times 10^6$)	Phenotype[a]	TnA sequences carried	Source	Reference
pJB603	5.4	Ap^r	At least 2.2 Mdal	*H. influenzae*	This study
pHI4564	5.4	Ap^r	At least 2.2 Mdal	*H. influenzae*	This study
pHD131	7.0	Ap^r	100%	*H. ducreyi*	3
pFA3	4.7	Ap^r	40%	*N. gonorrhoeae*	29, 35
RSF1050	5.0	Ap^r, Ie1	100%	pMB8::Tn*3*	18

[a] Ap^r, Ampicillin resistant; Ie1, immunity to ColE1.

to tetracycline were shown to be ≥4 μg/ml by agar dilution susceptibility testing with hemoglobin agar.

Plasmid analysis. The presence or absence of plasmids was determined by agarose gel electrophoresis of cleared lysates according to the method of Meyers et al. (25). Homology was demonstrated non-quantitatively by filter blot hybridization. Plasmid DNA within the agarose gel was depurinated with 0.25 HCl before being denatured and transferred to nitrocellulose filter paper, as described by Southern (34). Probe DNA was rebanded twice in cesium chloride-ethidium bromide density gradients before being nick translated by the method of Maniatis et al. (22). [^{32}P]deoxycytidine triphosphate (New England Nuclear Corp.; 300 Ci/mmol) was used as the labeled nucleotide. Hybridization was carried out in 2× SSC (1× SSC, 0.15 M NaCl and 0.015 M sodium citrate [pH 7.0])–50% formamide–0.1% sodium dodecyl sulfate–1× Denhardt mix (11) with 4×10^6 cpm of probe DNA at 37°C for 18 h. Assuming a guanosine plus cytosine content of 0.41 mol fraction, these conditions are 20°C below the melting temperature (23, 32). Conjugative resistance transfer was determined by membrane filter matings (30).

Statistical tests. Probability values were determined by chi-square analysis by using an on-line computer statistics program.

RESULTS

Table 2 gives the frequency of isolation of *H. influenzae* from the female genital tract during two prospective surveys of symptomatic males and females and asymptomatic pregnant females. The isolation rate was significantly higher in the symptomatic female population when compared with the asymptomatic pregnant female population ($P < 0.001$). One of the five asymptomatic females colonized with *H. influenzae* developed an episiotomy infection and postpartum fever, and vaginal cultures grew *Haemophilus* species. Also, one of the five infants delivered to colonized pregnant women was symptomatic and received systemic antibiotics after septic workup. Subsequently, this infant developed an eye discharge, and *H. influenzae* was recovered on culture. During the same period of study, one isolate was obtained from 148 urethral swabs from symptomatic males, and this is not different from the rate of colonization in symptomatic females ($P > 0.5$). Other GU tract pathogens were recovered in 5 of the 12 symptomatic patients (two *Neisseria gonorrhoeae*, two *Gardnerella vaginalis*, and two *Trichomonas vaginalis*). Of the 64 strains recovered from clinical specimens submitted for GU tract cultures, 56 were from females (52 cervical) and 9 were from males (7 urethral). Clinical findings in patients from this large group from which *H. influenzae* was recovered included an association with amnionitis in pregnancy and neonatal sepsis, culture-positive gonorrhea, nongonococcal urethritis, vaginitis, and genital ulcer disease.

The distribution of biotypes for the 82 GU tract strains and 122 consecutively isolated respiratory tract strains is given in Table 3. There was a significant difference in biotype distribution by source of isolation ($P < 0.001$), with the major difference noted in the more frequent isolation of biotype IV strains from the GU tract and the more frequent isolation of biotype I strains from respiratory sources.

Because of previous reports of antibiotic resistance in respiratory isolates of *H. influenzae* (9, 12, 21, 31) and the reported relationship between certain *Haemophilus* plasmids and the plasmids of *N. gonorrhoeae* (6, 13, 20, 21), we carried out a preliminary plasmid analysis on both antibiotic-susceptible and -resistant strains of *H. influenzae* from GU tract sources as compared with strains from respiratory sources. Of 82 GU tract strains, 12 were resistant to at least one of the four antibiotics tested (ampicillin, 8 strains; tetracycline, 3 strains; ampicillin-tetracycline, 1 strain). Of 122 respiratory strains,

TABLE 2. Frequency of isolation of *H. influenzae* from female genital tract

Genital tract *H. influenzae*	No. of patients		
	Symptomatic		Asymptomatic pregnant females[a]
	Females[a,b]	Males[b]	
Positive	11	1	5
Negative	563	147	1,719

[a] Chi-square = 14.21; $P < 0.001$.
[b] Chi-square = 0.48; $P > 0.5$.

TABLE 3. Distribution of biotypes of *H. influenzae* by sources of isolation[a]

Source	No. of isolates of biotype:				
	I	II	III	IV	V
GU tract	6	28	31	16	1
Respiratory tract	33	52	31	1	5

[a] Chi-square = 35.31; $P < 0.001$.

12 were resistant (ampicillin, 9 strains; tetracycline, 3 strains). No chloramphenicol- or kanamycin-resistant strains were isolated. The isolation rate for resistant strains by source was not different ($P > 0.2$). Although none of 12 resistant strains from respiratory sources had identifiable plasmids on initial plasmid screen, 10 were able to transfer resistance by conjugative mating to *H. influenzae* strain Rd, and 5 transconjugants had identifiable 30- to 34-megadalton (Mdal) plasmids. Of the 12 resistant strains from GU tract sources, 3 had identifiable plasmids on initial plasmid screen. One 30-Mdal plasmid conferred self-transferrable resistance to ampicillin. Two other plasmids from strains HI4564 and HI80213 were non-self-transferrable and are described below. Five other strains without visible plasmids on initial screen transferred resistance by conjugative matings to *H. influenzae* strain Rd, and two of the transconjugants had identifiable 30- to 34-Mdal plasmids.

A significant difference in plasmid profiles was noted, however, for isolates from the two sources. Of the 82 GU tract isolates, 13 contained one or more plasmids of <10 Mdal, whereas only 3 of the 63 respiratory tract isolates had similar plasmids ($P < 0.05$), as shown in Fig. 1. Six GU strains contained a 1.8-Mdal plasmid similar to that of strain HI80254 (Fig. 1B, lane B), two GU strains contained only a 2.8-Mdal plasmid similar to one of the three plasmids of strain H2 (Fig. 1B, lane C), and three GU strains contained cryptic plasmids of >20 Mdal similar to those of strain HI80018 (Fig. 1B, lane J) and the respiratory strain A3 (Fig. 1A, lane E). All other plasmid patterns were unique to the strain shown. Two of the GU plasmids but none of the respiratory plasmids of <10 Mdal were associated with antibiotic resistance. Strain HI4564 (Fig. 1B, lane I) contained a 5.4-Mdal plasmid which transformed *H. influenzae* strain Rd to ampicillin resistance. Since other <10-Mdal β-lactamase-specifying plasmids of *H. influenzae*, *Haemophilus parainfluenzae*, and *Haemophilus ducreyi* have been shown to be highly related to the 4.7-Mdal gonococcal plasmid pMR0360 (pFA3) (6, 13, 20, 31), we examined by restriction endonuclease digestion the relationship between the plasmid from strain HI4564 (pHI4564) and several of these plasmids. The restriction endonuclease digestion pattern of the previously described plasmids from *H. influenzae*, *H. ducreyi*, and *H. parainfluenzae* is characterized by a 1.44-Mdal *Bam*HI fragment. *H. ducreyi* plasmids contain the entire ampicillin transposon (Tn*A*) sequence and have *Pst*I fragments of 0.44 and 1.75 Mdal within their Tn*A* sequence, whereas the gonococcal β-lactamase plasmids carry about 30 to 40% of Tn*A* and have only a single *Pst*I site. Restriction endonuclease digestion of pHI4564 showed only a single *Bam*HI site, but both *Pst*I fragments were cut from the Tn*A* sequences

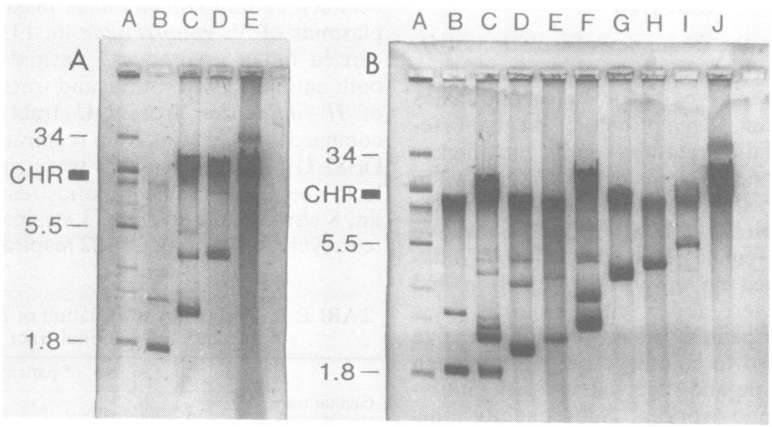

FIG. 1. Electrophoresis in 0.7% agarose of ethanol-precipitated DNA of strains of *H. influenzae* from respiratory (A) and GU tract (B) sources. (A) Lane A, molecular weight standards: RP4, 34 × 10^6 daltons; RSF1010, 5.5 × 10^6 daltons; and pMB8, 1.8 × 10^6 daltons. Lane B, strain HI2215; lane C, strain HI1179; lane D, strain HI3170; and lane E, strain HIA3. (B) Lane A, same molecular weight as (A); lane B, strain HI80254; lane C, strain H2; lane D, strain HI80262; lane E, strain HI80228; lane F, strain HI80213; lane G, strain HI3281; lane H, strain HI2407; lane I, strain HI4564; and lane J, strain HI80218. CHR, Chromosomal DNA.

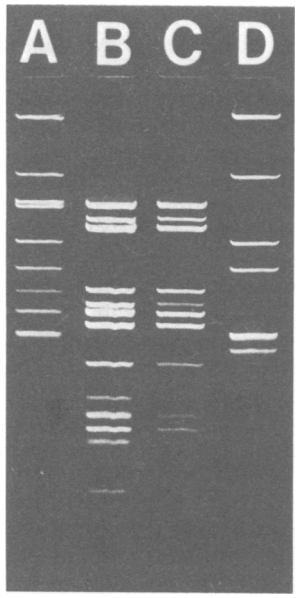

FIG. 2. Electrophoresis of *Alu*I-digested plasmid DNA in 7.5% polyacrylamide gel. Lane A, pHD131; lane B, pJB603; lane C, plasmid of strain HI4654; and lane D, pFA3.

(data not shown). The *Alu*I digestion pattern of pHI4564 and several β-lactamase-specifying plasmids from *H. ducreyi* (pHD131), *N. gonorrhoeae* (pFA3), and *H. influenzae* (pJB603) is shown in Fig. 2. Several plasmids from *H. ducreyi* and *N. gonorrhoeae* have been previously described (1, 5, 6, 29, 35). pJB603 was found in a β-lactamase-producing strain of *H. influenzae* isolated in Winnipeg from the cerebrospinal fluid of a 4-month-old female with meningitis. It is evident that the fragmentation patterns of pHI4564 and pJB603 are identical but differ from the pattern of pHD131, which, other than a complete Tn*A* sequence, is identical to that of pFA3 (6).

Finally, we wondered whether the phenotypically cryptic plasmids visualized by gel electrophoresis (Fig. 1) might represent precursors of the small β-lactamase plasmids found in *H. ducreyi, H. influenzae, H. parainfluenzae*, and *N. gonorrhoeae*. To test this, we transferred plasmid DNA separated by agarose gel electrophoresis to nicrocellulose filters and probed for Tn*A* sequences with RSF1050 and for both Tn*A* and non-Tn*A* sequences with pFA3. The results of these hybridization studies are given in Table 4. Strain H2 carried three phenotypically cryptic plasmids, one of which (2.8 Mdal) was homologous with pFA3 but lacked Tn*A* sequences, as shown by its failure to hybridize with RSF1050. Strain HI2215 also carried a cryptic plasmid of 1.8 Mdal which was also homologous with pFA3 and lacked Tn*A* sequences. The cryptic plasmids in all other isolates showed no homology with pFA3 or RSF1050, whereas, as expected, the β-lactamase-specifying plasmids pJB603, pHI80213, and pHI4564 were homologous with both pFA3 and RSF1050.

DISCUSSION

Several recent reviews of *H. influenzae* infections in adults have indicated that GU tract infections are uncommon (19, 26), and a recent review of *H. influenzae* infections in children indicated that perinatal infections are uncommon as well (8). Additional reports of *H. influenzae* GU tract infections have appeared since the publication of these reviews, including our own report of bacteremic infections in adults (2, 15, 27, 38) and reports of perinatal infections as well (4, 7, 16, 28, 36). Recent studies have also shown that *H. influenzae* can be regularly recovered from the female genital tract (17, 24). Our own prospective studies support these findings. We isolated *H. influenzae* at a frequency of 0.3 to 0.5% from asymptomatic pregnant females and at a higher frequency from symptomatic females. Several authors have suggested that genital tract colonization with *H. influenzae* is due to inoculation with respiratory tract strains (10, 14). Our results, however, suggest that GU tract strains show a different distribution of biotypes, and further studies comparing respiratory and GU tract strains from the same patients are indicated. It is interesting that in the report by Branefors et al. (4), the mother's respiratory isolate was antigenically different from the iso-

TABLE 4. Sequence homology of pFA3 and RSF1050 with strains of *H. influenzae*

Strain	Source[a]	Plasmid sequences homologous[b] with:	
		pFA3	RSF1050
HI603	CSF	+	+
HI4564, HI80213	GU	+	+
HIH2	GU	+	−
HI2407, HI80262, HI80217, HI80254, HI4729, HI4732, HI80200, HI80225, HI80228, HI3281	GU	−	−
HI2215	RESP	+	−
HI1180, HI3170	RESP	−	−

[a] CSF, Cerebrospinal fluid; RESP, respiratory tract.

[b] The presence (+) or absence (−) of homology between plasmids in the indicated strains and pFA3 and RSF1050 was assessed non-quantitatively by filter-blot hybridization. RSF1050 was used as a control to probe for Tn*A* sequences.

late from her neonate. Unfortunately, results with the maternal cervical isolate were not reported.

Our observations with antibiotic-resistant strains of *H. influenzae* from either respiratory or GU tract sources were not surprising. Most of our strains from either source did not show extrachromosomal plasmid DNA on initial screening, but the majority of these strains transferred antibiotic resistance in conjugative matings, and free covalently closed circular plasmid DNA could usually be demonstrated in the transconjugants. Similar observations have been previously described (30, 37). One strain (HI4564), however, contained a 5.4-Mdal ampicillin resistance plasmid similar to a plasmid (pJB603) found in a strain isolated from a patient in Winnipeg with meningitis. The plasmids pHI4564 and pJB603 were identical with respect to their digestion patterns for the restriction endonucleases *Bam*HI, *Bgl*I, *Hin*cII, *Pst*I, and *Alu*I. Their digestion patterns clearly differed from the well-characterized *H. ducreyi* β-lactamase plasmids pJB1 and pHD131, with the exception of fragments cut from within their Tn*A* sequences (J. Brunton and D. Clare, unpublished data). This suggests that the similarity between the two types of plasmids is largely confined to the Tn*A* sequence. This conclusion is supported by electron microscope heteroduplex analysis (M. Meier and J. Brunton, unpublished data). Whether pJB603 and pHI4564 represent an extension of the enteric plasmid pool or the insertion of Tn*A* sequences into a cryptic endogenous replicon remains to be seen.

The large number of cryptic plasmids in GU tract strains of *H. influenzae* was unexpected. Cryptic plasmids of >20 Mdal are uncommon in *H. infuenzae* but have been previously reported and include a plasmid homologous with self-transferrable antibiotic resistance plasmids of the same species. This supports the suggestion that such plasmids arose by transposition of Tn*A* to *Haemophilus* cryptic plasmids (31). The relationship of our cryptic plasmids of >20 Mdal to previously described plasmids has not been determined. The large number of cryptic plasmids of <10 Mdal seen in GU tract strains has not been previously described, presumably because most strains screened for such plasmids have been from respiratory sources. It should be pointed out, however, that the designation of such plasmids as "cryptic" depends on limited phenotypic traits, usually antibiotic resistance, and such plasmids may confer unknown selective advantage to strains containing them.

It is interesting that two strains (HIH2 and HI2215) contained plasmids of 2.8 and 1.8 Mdal, respectively, with sequences homologous with the group of ampicillin resistance plasmids previously described in *H. influenzae, H. parainfluenzae, H. ducreyi*, and *N. gonorrhoeae*. It was thought possible that the apparent homology could be due to non-β-lactamase-specifying Tn*A* sequences derived from further deletion of plasmids such as pFA3 or by a deletion of the β-lactamase-specifying sequences of a plasmid such as pJB1. Accordingly, control experiments were done with the probe RSF1050, which contains the complete Tn*3* sequence. These experiments showed that the cryptic plasmids in HIH2 and HI2215 did not contain Tn*A* sequences. Because these studies were performed by hybridizing probe DNA to whole plasmid DNA, it is difficult to be sure of the quantitative homology between probe and cryptic plasmid. Studies are presently in progress to examine this question.

We have recently shown in a similar manner that 7 of 90 isolates of *H. parainfluenzae* contain cryptic plasmids which are homologous with pFA3 but which do not contain any Tn*A* sequences (J. Brunton, N. Ehrman, and D. Clare, unpublished data). Preliminary studies suggest that there is a range of cryptic plasmids having various degrees of homology with pFA3. The discovery of these homologous cryptic plasmids in *H. influenzae* and *H. parainfluenzae* strongly supports the hypothesis that the small β-lactamase-specifying plasmids originated from the insertion of Tn*A* sequences into a phenotypically cryptic replicon present in *Haemophilus* species. The possibility that the GU tract environment favors both interspecific and intergeneric transfer of plasmids must also be considered.

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