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Phenotypic Characteristics of *Branhamella catarrhalis* Strains

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Isolates of *Branhamella catarrhalis* from 13 patients with pneumonia, 6 patients with tracheobronchitis, and 8 patients who were colonized with the organism were studied with respect to susceptibility to the bactericidal action of normal human serum (NHS), glass slide hemagglutination (HA) of group O human erythrocytes, β-lactamase production, and susceptibility to selected antimicrobial agents and laboratory drugs. A total of 18 of 27 isolates were serum resistant, 22 of 27 produced HA, and 21 of 27 were β-lactamase positive. Statistically significant correlations were found between susceptibility to NHS and susceptibility to trypsin ($r = +0.47$; $P = 0.01$) and between susceptibility to NHS and HA ($r = -0.48$; $P = 0.009$). Significant correlations were also observed among several pairs of antimicrobial drugs. Analysis of variance showed that mean ampicillin MICs correlated with isolate group ($r = -0.49$; $P = 0.03$) in that the pneumonia isolates had higher MICs. Some phenotypic characteristics appeared to be independent of each other. These data suggest that important differences exist among clinically significant *B. catarrhalis* strains and that these differences may be due to differences in the cell wall envelope of the organism.

Branhamella catarrhalis is now a well-described cause of lower respiratory tract infection. Tracheobronchitis, the most common clinical syndrome in adults, occurs in patients with underlying cardiopulmonary disease. Steroid therapy, malignancy, and immunoglobulin abnormalities appear to be present in a high proportion of patients who develop pneumonia (7, 10, 12).

B. catarrhalis may be normal flora of the upper respiratory tract and has been regarded as "an opportunist which is more frequent commensal than pathogen" (2).

β-Lactamase production, antimicrobial susceptibility patterns, biochemical reactions (6), and isoelectric focusing of β-lactamases (14) have been used to identify some interstrain differences. Recently, restriction endonuclease digestion of chromosomal DNA was used for typing *B. catarrhalis* isolates recovered during an outbreak in an intermediate care unit (16). By using this system, 7 of 13 isolates were identified as identical, but two isolates could not be digested. Hence, additional methods for strain distinction continue to be needed.

We studied selected *B. catarrhalis* isolates recovered from clinical specimens at our institution over the span of 4 years in an attempt to identify interstrain differences. We correlated these differences with clinical data for the patients from whom *B. catarrhalis* was isolated.

The phenotypic characteristics studied were susceptibility to the bactericidal action of 50% normal human serum (NHS), agglutination of human group O Rh^+ erythrocytes, and susceptibility to antimicrobial and laboratory drugs.

MATERIALS AND METHODS

Bacteria. *B. catarrhalis* isolates were obtained from the sputum of hospitalized patients at the Veterans Administration Medical Center in Johnson City, Tenn. The identity of each isolate was confirmed with standard criteria (8). Isolates were stored in methylcellulose at −70°C. For comparison, the reference strain *B. catarrhalis* ATCC 25238 was included in all the observations. Table 1 lists characteristics of *B. catarrhalis* strains used in this study.

Diagnostic categories. A pneumonia-causing isolate was defined by the following: (i) *B. catarrhalis* isolated from a patient with worsening or new onset of cough, dyspnea, tachypnea, and hypoxemia (partial O_2 pressure, <70); (ii) new infiltrate on chest roentgenogram; (iii) Gram stain of expectorated sputum or transtracheal aspirate with >25 polymorphonuclear leukocytes and <10 squamous epithelial cells per low-power field and with gram-negative diplococci predominating; and (iv) culture of sputum or transtracheal aspirate specimen with pure or predominant growth of *B. catarrhalis*.

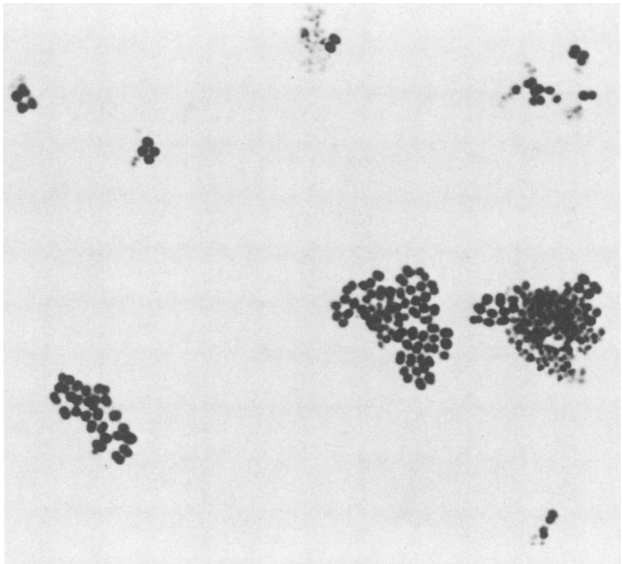

FIG. 1. *B. catarrhalis* ATCC 25238 after growth for 4 h in Muller-Hinton broth with shaking; one drop was air dried and stained with crystal violet. Bacterial aggregates of different sizes are shown. Magnification, ×935.

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TABLE 1. Characteristics of *B. catarrhalis* isolates[a]

Isolate[b]	Isolation date	Results for:		
		β-Lactamase[c]	Hemagglutination[d]	NHS bactericidal assay
P-1	2/83	+	4+	R
P-3	3/83	+	−	S
P-6	12/83	−	−	S
P-14	1/84	+	4+	S
P-21	2/84	+	−	S
P-24	3/84	+	4+	R
P-32	3/84	+	4+	R
P-37	3/84	+	1+	S
P-38	4/84	+	4+	R
P-44	5/84	+	4+	R
P-48	5/84	+	−	S
P-89	3/85	−	4+	R
P-115	5/85	+	4+	R
T-09	3/85	−	4+	R
T-4	4/87	+	4+	S
T-7	4/87	+	1+	R
T-10	4/87	+	−	R
T-12	4/87	+	4+	R
T-25	5/87	+	1+	S
C-1	4/87	−	4+	R
C-2	4/87	+	2+	R
C-9	4/87	+	4+	R
C-11	4/87	+	4+	S
C-16	5/87	−	4+	R
C-20	5/87	+	2+	R
C-26	5/87	−	1+	R
C-30	5/87	+	4+	R
ATCC 25238	1954	−	−	R

[a] P, Pneumonia; T, tracheobronchitis; C, colonization; R, resistant; S, susceptible.

[b] Strains P-1, P-24, and P-37 were recovered from transtracheal aspirates. Strain P-3 is an isolate from blood of a patient with bacteremic pneumonia. All other are isolates from sputum.

[c] Nitrocefin disk (Cefinase; BBL Microbiology Systems, Cockeysville, Md.).

[d] Agglutination of human group O Rh^+ erythrocytes.

The definition of tracheobronchitis-causing isolates included the presence of items (i), (iii), and (iv) above but not of the infiltrate on a chest roentgenogram. Isolates recovered from patients who did not meet the previous criteria were considered colonizing strains. (All of these isolates revealed squamous epithelial cells and <10 leukocytes per low-power field with Gram stain.)

Serum bactericidal assay. NHS was obtained from a single healthy donor and was used in all the assays. Blood was collected under sterile conditions, allowed to clot for 30 min at room temperature, centrifuged at 500 × *g* at 4°C for 15 min, divided into samples, and stored at −70°C until used. The assay procedure described by Apicella et al. (3) was modified as follows. *B. catarrhalis* organisms grown for 16 to 18 h at 37°C in an atmosphere of 5% CO_2 on chocolate agar plates (Scott Laboratories, Inc., Fickeysville, R.I.) were removed and were suspended in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) with 420 mg of sodium bicarbonate per liter, (pH 7.63). Cultures were incubated at 37°C in a shaking water bath with room air for approximately 4 h until they reached the early log phase at an optical density at 550 nm of 0.20. A 0.1-ml portion of the supernatant was carefully removed and immediately diluted in Eagle minimal essential medium containing Earle salts, L-glutamine, and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.45). Colony counts of bacterial suspensions prepared in an identical manner varied between assays because each isolate formed bacterial aggregates of different sizes (Fig. 1). A uniform serial dilution procedure applied to all the isolates gave an inoculum of between 0.6×10^3 and 5.0×10^3 CFU/ml by plate counting.

The assays were performed in sterile glass tubes (12 by 75 mm) containing 0.1 ml of bacterial inoculum and 0.1 ml of serum. The tubes were incubated at 37°C with shaking, and viable colony counts were performed at 0, 30, 60, 90, and 120 min by plating 0.025 ml from each tube onto chocolate agar plates. Controls for each assay included *B. catarrhalis* isolates and heat-inactivated serum (56°C for 30 min). The assays were performed in duplicate for serum-resistant iso-

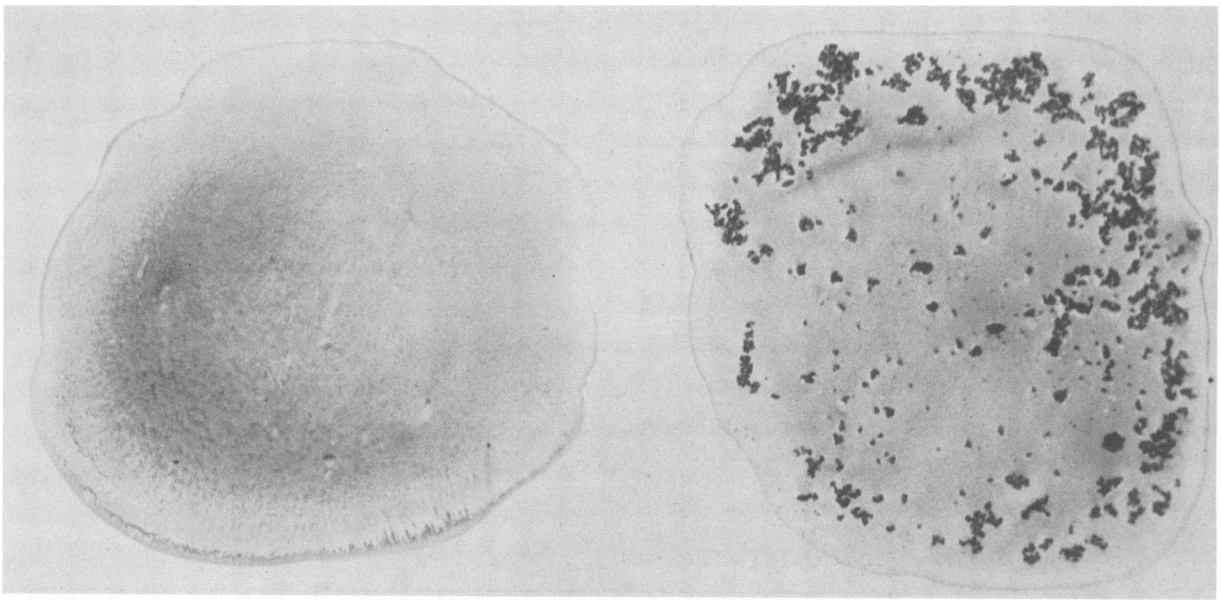

FIG. 2. Glass slide hemagglutination. Left, Strain ATCC 25238, negative; right, strain P-1, positive 4+. Magnification, ×3.

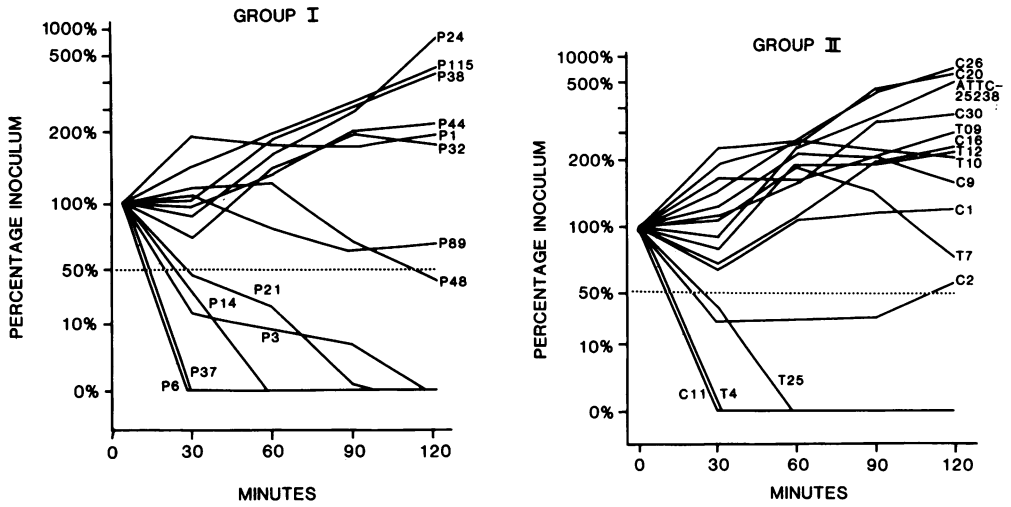

FIG. 3. Susceptibility to NHS of *B. catarrhalis* isolates. Group I, 13 isolates from patients with pneumonia (P); group II, 6 isolates from patients with tracheobronchitis (T), 8 from colonized patients (C), and reference strain ATCC 25238.

lates. Serum-susceptible isolates were tested three or four times, and the endpoints were consistent. Assays were repeated if more than a 20% difference in count existed at 0 min between fresh and inactivated serum plates or when inconsistent growth was found on heat-inactivated serum plates. Organisms that had 50% or less of initial inoculum count at 120 min were considered serum susceptible.

As a control for the use of single-donor serum, a serum-susceptible and a serum-resistant isolate were tested under identical conditions with pooled NHS from five healthy donors. This pooled serum had a normal mean level of 50% hemolytic complement activity (28 U). The results, including killing time of the susceptible isolate, were similar.

Hemagglutination. Erythrocytes from freshly drawn heparinized group O Rh^+ human blood were washed three times with phosphate-buffered saline, pH 7.3, and suspended in a 3% (vol/vol) suspension. On a glass slide (single frosted; Corning Glass Works, Corning, N.Y.), a loopful of confluent bacterial growth scraped from a chocolate agar plate incubated for 16 to 18 h under 5% CO_2 was gently mixed at room temperature (21°C) with 50 μl of erythrocyte suspension. Hemagglutination was read as follows: 4+, coarse clumping of erythrocytes within 30 s; 3+, coarse clumping in 1 min; 2+, clumping perceptible in 1 to 3 min; 1+, fine clumping visible to the unaided eye in 3 to 5 min; and −, no clumping at 5 min (Fig. 2).

Antibiotic and laboratory drug susceptibility testing. MICs were determined by the broth microdilution technique with cation-supplemented Mueller-Hinton broth as previously described (1), with an inoculum of 10^5 CFU/ml.

For disk diffusion susceptibility testing, the method recommended by the National Committee for Clinical Laboratory Standards (15) was followed, except that the inoculum was prepared as described for the serum bactericidal assay. The organisms were inoculated with swabs on fresh (prepared less than 48 h before) Mueller-Hinton agar plates, pH 7.2. Antibiotic disks (Difco) with ampicillin (10 μg), penicillin G (10 U), polymyxin B (300 U), and rifampin (5 μg) were placed in duplicate within 15 min of inoculation. Two laboratory drugs were tested: Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) and trypsin. Triton X-100 (4% vol/vol) was dissolved in distilled water by heating at 60°C for 1 h. A 20-μl portion of this solution was added to 6-mm-diameter blank disks (Difco) in duplicate. Susceptibility to trypsin was assessed by adding 20 μl of a solution prepared with tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin type XIII (Sigma) (20 mg/ml in 0.046 M Tris–0.015 M $CaCl_2$ buffer, pH 9.4) (4, 11) to blank disks in duplicate.

TABLE 2. MICs by broth microdilution

Isolate[a]	MIC (μg/ml)[b]			
	Pen G1	Amp 1	Str	Colistin
P-1	8	2	0.78	2.5
P-3	>8	4	0.78	2.5
P-6	0.032	0.008	0.78	0.63
P-14	8	4	0.78	1.25
P-21	>8	4	0.78	2.5
P-24	4	4	1.56	2.5
P-32	>8	2	0.78	2.5
P-37	4	0.5	0.78	0.63
P-38	>8	4	0.78	1.25
P-44	2	1	0.78	1.25
P-48	2	1	0.39	1.25
P-89	0.016	0.008	0.78	1.25
P-115	4	1	1.56	2.5
T-09	0.25	0.008	0.78	2.5
T-4	8	1	0.78	1.25
T-7	1	0.5	0.78	1.25
T-10	>8	1	0.78	2.5
T-12	>8	4	0.78	1.25
T-25	8	0.25	0.78	1.25
C-1	0.032	0.008	0.78	2.5
C-2	0.5	0.25	0.78	2.5
C-9	>8	0.5	0.78	2.5
C-11	2	1	0.78	1.25
C-16	0.016	0.008	0.39	2.5
C-20	8	1	0.78	1.25
C-26	0.032	0.008	0.78	1.25
C-30	4	0.5	0.78	1.25
ATCC 25238	0.016	0.008	0.78	1.25

[a] P, Pneumonia; T, tracheobronchitis; C, colonization.

[b] Pen G1, Penicillin G; range, 0.001 to 8 μg/ml. Amp 1, ampicillin; range, 0.001 to 4 μg/ml. Str, streptomycin; range, 0.09 to 12.5 μg/ml. Colistin range, 0.08 to 10 μg/ml.

TABLE 3. Disk diffusion susceptibility to antimicrobial and laboratory drugs

Isolate[a]	Zone size (mm) with[b]:					
	Pen G2	Amp 2	Rif	Poly B	Triton X	Trypsin[c]
P-1	10	17	32	17	20	8
P-3	11	20	33	18	18	10
P-6	44	60	34	19	22	15
P-14	13	20	34	18	20	10
P-21	11	15	35	16	24	9
P-24	18	26	37	17	23	8
P-32	12	19	34	19	22	8
P-37	20	26	40	22	26	15
P-38	11	21	36	18	18	8
P-44	20	27	34	18	19	13
P-48	18	21	35	19	23	15
P-89	43	50	33	18	18	9
P-115	20	26	31	18	17	8
T-09	45	53	35	20	17	8
T-4	22	26	33	17	18	9
T-7	19	24	31	17	15	8
T-10	21	26	32	18	13	8
T-12	10	17	32	18	15	9
T-25	19	25	36	19	20	6
C-1	49	62	40	24	23	8
C-2	26	28	34	18	19	8
C-9	21	26	32	17	18	10
C-11	18	23	36	20	18	9
C-16	48	62	37	24	22	8
C-20	17	24	31	17	26	9
C-26	42	48	29	18	14	6
C-30	19	24	33	17	25	9
ATCC 25238	41	43	32	16	16	9

[a] P, Pneumonia; T, tracheobronchitis; C, colonization.
[b] Pen G2, Penicillin G; Amp 2, ampicillin; Rif, rifampin; Poly B, polymyxin B.
[c] The diameter measured was the whole clear zone surrounding the disk even when isolated colonies were observed inside (Fig. 4A and B).

Diameters presented are the averages of three or four subcultures performed on different days.

Statistical analysis of data. The Number Cruncher Statistical System program was used to analyze the data by several techniques. Correlation coefficients were calculated for all the phenotypic characteristics. Analyses of variance between means were obtained for numerical parameters.

RESULTS

Figure 3 shows serum susceptibilities of individual isolates expressed as inoculum percentages. A total of 9 of 27 were serum susceptible, and killing rates among them were variable.

Tables 2 and 3 show the MICs and inhibition diameters by disk diffusion. Correlation coefficients between phenotypic variables are illustrated in Table 4.

A correlation between susceptibility to NHS and MIC of colistin by cross tabulation and chi-square analysis with 2 df did not achieve statistical significance ($P = 0.06$).

Growth inhibition diameters of 10 mm or more with trypsin (Table 3) were present with 6 of 13 isolates in the pneumonia group (Fig. 4A). In contrast, among tracheobronchitis and colonization isolates, only one isolate showed a growth inhibition diameter of 10 mm in response to trypsin, while all others showed a smaller diameter (Fig. 4B). One isolate each from the tracheobronchitis and colonization groups showed complete resistance to the effect of trypsin (Fig. 4C).

Combination of variables in a discriminant analysis revealed that the use of two variables (ampicillin MICs and growth inhibition diameter for trypsin) accurately predicted the classification of 17 of 27 isolates into one of the three clinical groups (pneumonia, $n = 17$; tracheobronchitis, $n = 6$; and colonization, $n = 8$).

DISCUSSION

Our results with serum susceptibility patterns of *B. catarrhalis* show some similarities to two previous studies. Winn and Morse (R. E. Winn and S. I. Morse. Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, B-65, p. 28) found 5 of 15 clinical isolates of *B. catarrhalis* resistant to NHS. Four of these five isolates were felt to be significant pathogens; however, other isolates deemed responsible for clinical disease, including one isolate from blood, were serum susceptible. Details about the methods used in serum bactericidal assays are not available. Chapman et al. (5), using NHS (either individual or pooled) at concentrations from 20 to 80%, found no bactericidal activity against 20 of 21 *B. catarrhalis* isolates. Of the isolates used, 6 were from patients with pneumonia and 13 were from patients with exacerbation of chronic bronchitis. Only one of their isolates from a patient with bronchitis was susceptible to 10% NHS. It seems possible that differences in the methodology are responsible for the greater frequency of serum susceptibility of *B. catarrhalis* in our study. In accordance with the

TABLE 4. Statistically significant ($P < 0.05$) correlation coefficients among 12 phenotypic characteristics for 27 *B. catarrhalis* isolates[a]

Characteristic	*r* for:							
	BL	NHS	Pen G1	Amp 1	Pen G2	Amp 2	Colistin	Rif
HA		−0.4873						
Pen G1	−0.5546							
Amp 1	−0.4962		0.6560					
Pen G2	0.9426		−0.6775	−0.6854				
Amp 2	0.9560		−0.6269	−0.6040	0.9824			
Poly B	0.5330		−0.4029		0.5871	0.6296		0.6933
Trypsin susceptibility		0.4721					−0.4624	
Triton X susceptibility								0.5397

[a] BL, β-Lactamase; NHS, susceptibility to NHS; Pen G1, penicillin G MIC; Amp 1, ampicillin MIC; Pen G2, penicillin G disk diffusion (zone size); Amp 2, ampicillin disk diffusion (zone size); HA, hemagglutination; Poly B, Polymyxin B disk diffusion (zone size); colistin, colistin MIC; Rif, rifampin disk diffusion (zone size). All zone sizes are in millimeters.

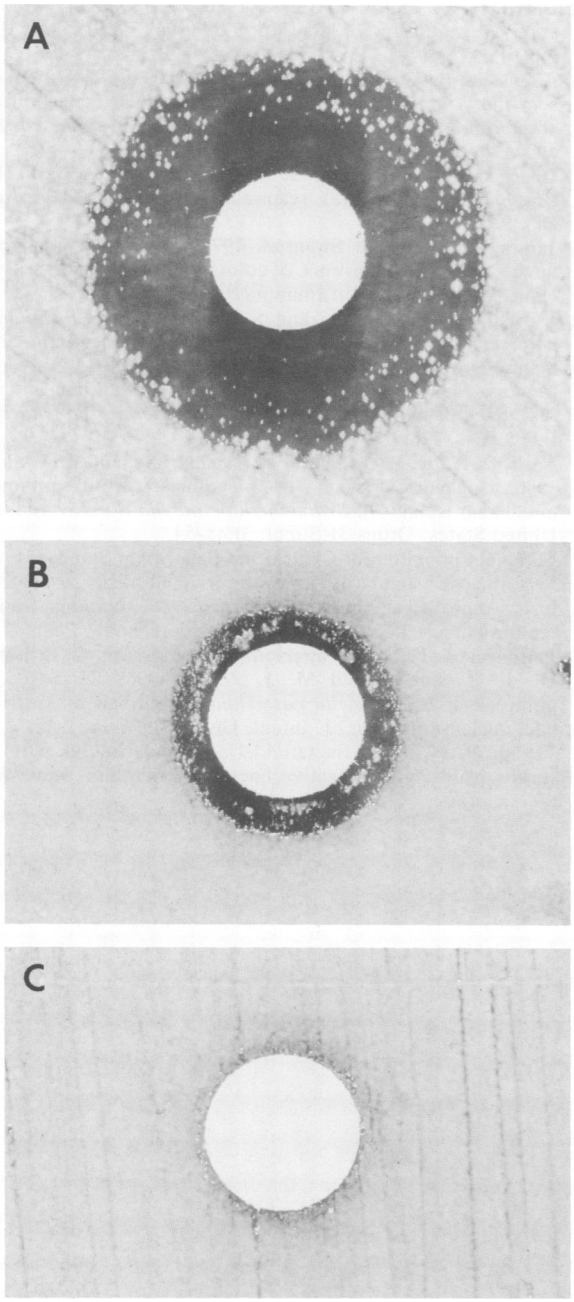

FIG. 4. Semiquantitative disk diffusion assay for susceptibility to trypsin. (A) Susceptible; (B) intermediately susceptible (isolated colonies grew inside the clear zone in both); (C) resistant. Magnification, ×3.4.

method of Apicella et al. (3), the organisms were not washed or centrifuged prior to the bactericidal assays. The diluent was not supplemented with heated fetal calf serum. Smaller volume assays were used, and serial dilution was not used for viable counts. We also include a larger number of isolates recovered from patients with pneumonia.

Our use of NHS from a single donor could have the theoretical advantage that blocking antibody would be less likely than with pooled NHS to affect the characterization of one or more isolates.

The standardization of the inoculum for susceptibility testing with organisms that grew in Mueller-Hinton broth with shaking was derived from the consistent results observed early in this study with serum bactericidal assays. It is well established that the serum susceptibility of gram-negative organisms is influenced by growth conditions and phase of growth. Enhanced serum susceptibility is present in early-logarithmic-phase cultures in comparison with either the lag or stationary phase (17). Shaken liquid cultures yielded greater reproducibility than static broth cultures in our disk diffusion procedures (data not shown).

Chemically dissimilar drugs with different target sites on the bacterial cells have been used by Maness and Sparling (13). They studied the genetic mechanisms of cross resistance of gonococci to antibiotics and laboratory drugs and demonstrated that a low level of resistance to drugs could be lost or restored by a single mutational event in vitro that they related to changes in permeability properties of the cell envelope. Later, in the same laboratory (9), the study of 39 strains of *Neisseria gonorrhoeae* recovered from patients with disseminated gonococcal infection revealed that penicillin susceptibility and serum resistance were independent characteristics of these strains. Strains from patients with disseminated gonococcal infection were found to be phenotypically different from strains recovered from patients with uncomplicated (local) disease.

The assessment of trypsin susceptibility by disk diffusion for comparison of gonococcal culture characteristics was introduced by Swanson et al. (4, 11). This method is a quantitative evaluation of some determinant factors of colony color and opacity. The procedure is less influenced by the subjective factors involved in the assessment of individual colony characteristics. Swanson concluded that gonococci recovered from the female cervix tended to be more resistant to growth inhibition by trypsin than did organisms from the male urethra. It was postulated that the ecological niche of each anatomical site may be of importance in selecting gonococcal populations of various phenotypes. It remains to be established whether the differences in trypsin susceptibility of *B. catarrhalis* isolates that we observed indicate that this organism, like the gonococcus, possesses interstrain outer membrane protein differences.

The finding of higher mean MICs of ampicillin by broth microdilution methods among strains recovered from patients with pneumonia requires additional confirmation. Interestingly, a larger number of isolates susceptible to NHS, negative for hemagglutination, and susceptible to trypsin were found in this group.

The biochemical characterization of one or more hemagglutinins by *B. catarrhalis* needs to be defined. Some morphologic evidence suggests that hemagglutination of human erythrocytes on glass slides is not mediated by classic fimbriae (pili) (J. L. Soto-Hernandez, L. J. Boelen, G. Musil, and S. L. Berk, submitted for publication).

If *B. catarrhalis* colonizing the mucosal surface of the respiratory tract consistently shows phenotypic differences in comparison with organisms that cause invasive disease, it will suggest that not only host factors but also bacterial virulence factors may be involved in the production of disease by this organism. The phenotypic differences may also be helpful in differentiating between colonization, tracheobronchitis, and pneumonia.

In summary, we have identified some phenotypic characteristics of *B. catarrhalis* which appear to be reproducible, stable, and independent of each other. These characteristics may be useful in developing a typing system for *B. catarrhalis*.

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