Antibiotic Susceptibility of Beta-Lactamase-Producing Strains of *Branhamella* (*Neisseria*) *catarrhalis*

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All 11 clinically significant isolates of *Branhamella catarrhalis* examined in this study were found to produce β-lactamase. The enzyme was apparently not plasmid associated since extrachromosomal deoxyribonucleic acid was not detected in any of the strains. The β-lactamase activity of all strains was significantly inhibited by the β-lactamase inhibitors clavulanic acid and CP 45899. Based on comparisons of relative susceptibility to various β-lactam antibiotics, it was inferred that the β-lactamase of *B. catarrhalis* was significantly more active against penicillin congeners than against cephalosporin congeners. Most strains were not inhibited by readily achievable serum concentrations of the penicillinase-sensitive penicillins, penicillin G, ampicillin, and amoxicillin. Methicillin was equally ineffective. With rare exceptions, most strains of *B. catarrhalis* were inhibited by achievable serum concentrations of seven cephalosporins (cephalothin, cepharopin, cephaloridine, cephalixin, cephamandole, cefaclor, and cefuroxin) and one cephamycin (cefoxitin). All strains were uniformly resistant to clindamycin but were inhibited by achievable serum concentrations of erythromycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole. Comparison of geometric mean minimum inhibitory concentrations of all antimicrobial agents tested suggested that *B. catarrhalis* was most susceptible to cefoxitin, erythromycin, and tetracycline.

*Branhamella* (*Neisseria*) *catarrhalis*, previously considered a harmless, upper respiratory tract commensal of humans (9), is now recognized as the etiological agent of significant disease in humans (7). It has been implicated in a wide variety of infections. (G. V. Doern, M. J. Miller, R. E. Winn, submitted for publication), including conjunctivitis, otitis media, sinusitis, endocarditis, meningitis, septicemia, and pneumonia, particularly in patients with underlying compromised pulmonary function. Although previously defined as being uniformly susceptible to penicillin (5), several recent studies demonstrated that clinically significant isolates of *B. catarrhalis* were resistant to penicillin by virtue of their ability to produce β-lactamase (8, 10, 12–14). Due to the paucity of reported clinical antibiotic trials and in vitro susceptibility studies, little additional information exists regarding the antimicrobial susceptibility of *B. catarrhalis*. The present study was undertaken in an attempt to define the nature of the β-lactamase produced by *B. catarrhalis* and to elucidate the antimicrobial susceptibility pattern of this organism.

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MATERIALS AND METHODS

Organisms. The 11 clinically significant isolates of *B. catarrhalis* and other organisms employed in this study are described in Table 1.

β-Lactamase determinations. β-Lactamase production was determined by the iodometric penicillin-G agar overlay method of Baldwin et al. (1) with organisms grown on GC agar (Difco) with or without 1 μg of penicillin G per ml for 16 h at 37°C in 10% CO₂ and by the rapid acidoimeter tube method of Escamilla (6) with organisms grown on IsoViteX-enriched (BBL Microbiology Systems) chocolate agar for 16 h at 35°C in 10% CO₂. A 10-μg/ml solution of penicillin G (GIBCO Laboratories, Grand Island, N.Y.) was used as a substrate in the iodometric penicillin-G agar overlay method. A 120-μg/ml solution of penicillin G (Pfizer Inc.) was used as a substrate in the rapid acidoimeter tube method.

Isolation of plasmid DNA. Cultures of *B. catarrhalis* and *N. gonorrhoeae* were grown to a density of 10⁹ colony-forming units per ml in glucose-containing liquid medium as previously described (11). To prevent the loss of plasmids, penicillin G (1 μg/ml) was added to all medium used to grow β-lactamase-producing strains.

Two independent isolation procedures were used to purify supercoiled plasmid deoxyribonucleic acid (DNA). The first procedure was essentially as described by Baron et al. (2). In the second technique, a modification of the Hirt lysis procedure was employed (7). The cells were concentrated 50- to 100-fold by
extracted several regions for the case ride, pH were at pronase final to a (10 mg/ml) bromide
Polaroid type 105 stained broniude rotor at 50.1
lysed were gently by bromide buffer were acetate-0.5
dium
mM
g g
Gel electrophoresis. Vertical 1% agarose (Bio-Rad) gels with 40 mM Tris-hydrochloride-5 mM sodium acetate-0.5 mM EDTA (pH 8.0) electrophoresis buffer were run at 3 V/cm for 9 to 16 h. The gels were stained in electrophoresis buffer containing ethidium bromide (0.5 pg/ml) for 1 h and photographed with Polaroid type 105 film with a mineralight short-wave lamp (Ultra Violet Products, Inc., San Gabriel, Calif.) and a Kodak 23 A orange filter.

**Agar dilution MICs.** Agar dilution minimum inhibitory concentrations (MICs) were determined essentially by the method described by Washington and Barry (15). *B. catarrhalis* was propagated on chocolate agar enriched with 1% (vol/vol) IsoVitalexX for 16 h at 35°C in 10% CO2. Several colonies were then removed and suspended in 5.0 ml of Mueller-Hinton broth. After incubation at 35°C for 6 to 8 h, the cell suspension was diluted in Mueller-Hinton broth to yield an optical density equivalent to a 0.5 McFarland standard. With a Steers replicator device, 0.001 ml of this suspension was transferred to the surface of Mueller-Hinton agar plates containing 5% chloretated sheep blood and serial twofold dilutions (0.02 to 80 μg/ml) of the appropriate antibiotics. In this manner, approximately 10 viable log-phase colony-forming units were present in each inoculum spot. The inoculated plates were incubated at 35°C in 10% CO2 and examined for growth after 20 h. MICs were defined as the lowest concentration of a given antibiotic that inhibited visible surface growth.

The antibiotics tested and the solvents in which they were initially dissolved were as follows: penicillin G (Pfizer), methicillin, cephalixin (Bristol), cephalothin, cefaclor, cephaloridine, cephalaxin, cephamandole (Lilly), cefotaxin (Merch, Sharp and Dome), cefuroxime (Glaxo Research Ltd.), clindamycin (Upjohn), tetracycline (Lederle), and trimethoprim (Burdoughs-Wellcome) in glass-distilled water; ampicillin (Bristol) and amoxicillin (Parke-Davis) in 0.1 M phosphate buffer, pH 8.0; erythromycin (Abbott) and chloramphenicol (Parke-Davis) in 70% ethanol; and sulfamethoxazole (Burdoughs-Wellcome) in 0.1 N lactic acid.
Inhibition of β-lactamase by clavulanic acid and CP 45899. Inhibition of β-lactamase activity by clavulanic acid (Beecham Pharmaceuticals) and CP 45899 (Pfizer, Inc.) was determined by comparing broth dilution MICs of penicillin G, ampicillin, methicillin, cephalexin, and cefoxitin against three strains of B. catarrahalis (318, A1262, A1246) in the presence and absence of each β-lactamase inhibitor. Bacteria were propagated on chocolate agar enriched with 1% (vol/vol) IsoVitaleX for 16 h at 35°C in an atmosphere containing 10% CO₂. Inocula were prepared by suspending several colonies in Mueller-Hinton broth containing 5% (vol/vol) Fildes enrichment (MH-F), incubating for 6 to 8 h at 35°C, and then diluting in MH-F to an optical density equivalent to a 1.0 McFarland standard. This suspension was further diluted 1:200 in MH-F to yield a final inoculum of approximately 10⁹ viable log-phase bacteria per ml. Portions (1 ml) were transferred to 10-ml volumes of MH-F containing serial twofold dilutions (0.02 to 80 μg/ml) of appropriate antibiotics either alone or in the presence of a 10-μg/ml final concentration of clavulanic acid or CP 45899. Tubes were incubated at 35°C for 16 h and examined visually for the presence of turbidity. MICs were defined as the lowest concentration of antibiotic in which turbidity was not observed. Incubation of tubes for 24 or 48 h did not alter results.

RESULTS

β-Lactamase production. All 11 clinically significant isolates of B. catarrahalis produced β-lactamase when tested by the isometric penicillin-agar overlay method. The clear zones surrounding the colonies were narrow, suggesting that the β-lactamase was essentially cell-associated. No increase in zone diameter was observed after the growth of these isolates on GC agar (Difco) containing 1 μg of penicillin G per ml. ATCC 8176 did not produce detectable β-lactamase activity by this procedure. When tested by the rapid acidimetric tube assay, these isolates required incubation times of 60 to 75 min for development of the color change indicative of β-lactamase activity. β-Lactamase activity was not evident with shorter periods of incubation. ATCC 8176 remained negative after incubation for up to 100 min.

Extrachromosomal DNA. Agarose gel electrophoresis of the supercoiled DNA from two strains of N. gonorrhoeae revealed the presence of plasmids. N. gonorrhoeae strain CS-7 contained the cryptic 2.6 × 10⁶-dalton plasmid. The β-lactamase-producing N. gonorrhoeae strain 76-073389 contained 2.6 × 10⁶-dalton, 4.4 × 10⁶-dalton, and 24.5 × 10⁶-dalton plasmids. No plasmids were detected in any of the strains of B. catarrahalis examined. Electron microscopy revealed the presence of covalently closed circles with preparations from N. gonorrhoeae strains CS-7 and 76-073389. No circles were observed with preparations from B. catarrahalis (G. Wiesehahn, personal communication).

Antibiotic susceptibility. The MICs of 17 antibiotics for the 11 β-lactamase-producing strains of B. catarrahalis and ATCC 8176 are presented in Table 2. The geometric mean MICs of each antibiotic for the clinical isolates are also shown. A reasonably narrow range of MICs (≤16-fold difference) was observed with the cephalosporins, cephalexin, erythromycin, clindamycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole. A wider variation was observed with penicillin G, ampicillin, amoxicillin, and methicillin.

The geometric mean MICs of all 17 antibiotics for the clinical isolates were uniformly higher than the MICs of the corresponding antibiotic for ATCC strain 8176. This variation was most significant with penicillin G, ampicillin, amoxicillin, methicillin, and clindamycin. The only exceptions were one strain which was susceptible to penicillin and three strains which were susceptible to methicillin. Conversely, nearly all strains were susceptible to each of the other antibiotics tested, the sole exception being a single strain which was not susceptible to cephalaxin. ATCC 8176 was uniformly susceptible to all antibiotics except clindamycin. Based on analysis of individual and geometric mean MICs, the 11 clinical isolates of B. catarrahalis examined in this study were most susceptible to cefoxitin, erythromycin, and tetracycline.

Inhibition of β-lactamase by clavulanic acid and CP 45899. Inhibition of β-lactamase activity was examined by comparing broth dilution MICs of penicillin G, ampicillin, methicillin, cephalexin, and cefoxitin for three strains of B. catarrahalis (strains 318, A-1262, and A-1246) in the presence and absence of clavulanic acid and CP 45899 (Table 3). The broth dilution MICs of these five antibiotics determined in the absence of β-lactamase inhibitors were equivalent to the MICs of these antibiotics when determined by agar dilution as described above. Penicillin MICs for all three strains were significantly diminished (94 to 98%) by both clavulanic acid and CP 45899. Similarly, both inhibitors depressed the MIC of ampicillin for strains 318 and A-1262. Strain A-1246, a strain with a relatively low ampicillin MIC (0.32 μg/ml) in the absence of inhibitors, was not affected by clavulanic acid or CP 45899. The methicillin, cephalexin, and cefoxitin MICs for all three strains were not altered by either clavulanic acid or CP 45899.
TABLE 3. Effect of clavulanic acid and CP 45899 on the MIC of five antibiotics for clinically significant isolates of B. catarrhalis

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Inhibitor*</th>
<th>MIC</th>
<th>Reduction in MIC</th>
<th>% Reduction in MIC</th>
<th>MIC</th>
<th>Reduction in MIC</th>
<th>% Reduction in MIC</th>
<th>MIC</th>
<th>Reduction in MIC</th>
<th>% Reduction in MIC</th>
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<tbody>
<tr>
<td>Penicillin</td>
<td>None</td>
<td>0.32</td>
<td>98</td>
<td>100</td>
<td>0.32</td>
<td>98</td>
<td>100</td>
<td>0.32</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Clavulanic acid</td>
<td>CP 45899</td>
<td>0.32</td>
<td>98</td>
<td>0.32</td>
<td>97</td>
<td>0.32</td>
<td>94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>None</td>
<td>0.64</td>
<td>87</td>
<td>94</td>
<td>0.16</td>
<td>97</td>
<td>0.32</td>
<td>0.32</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clavulanic acid</td>
<td>CP 45899</td>
<td>0.16</td>
<td>97</td>
<td>0.16</td>
<td>97</td>
<td>0.32</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin</td>
<td>None</td>
<td>0.02</td>
<td>5</td>
<td>5</td>
<td>0.32</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clavulanic acid</td>
<td>CP 45899</td>
<td>0.32</td>
<td>5</td>
<td>0.32</td>
<td>5</td>
<td>0.32</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>None</td>
<td>2.5</td>
<td>50</td>
<td>50</td>
<td>0.16</td>
<td>50</td>
<td>0.32</td>
<td>0.16</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clavulanic acid</td>
<td>CP 45899</td>
<td>0.16</td>
<td>50</td>
<td>0.16</td>
<td>50</td>
<td>0.32</td>
<td>0</td>
<td></td>
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</tr>
</tbody>
</table>

* Clavulanic acid or CP 45899 was added at a final concentration of 10 µg/ml.
* A 50% reduction in MIC represents a single twofold (one-tube) change which is within the experimental variation of this test and thus cannot be considered significant.

Several investigators have reported that clinically significant isolates of B. catarrhalis produce beta-lactamase (8, 10, 12-14). In the present study, 11 of 11 strains of B. catarrhalis...
implicated as the etiological agents of significant human disease were found to produce β-lactama-
se. β-Lactamase was not detected after short incubation times by a rapid acidimetric assay. This
assay is useful in detecting constitutive, extracellular β-lactamases such as those produced by Haemophilus influenzae and Neis-
seria gonorrhoeae. Only after prolonged periods of incubation (60 to 75 min) in the presence of
penicillin was the B. catarrhalis β-lactamase detected. Conversely, the iodometric penicillin-
agar overlay method clearly detected β-lacta-
mas production by all clinical isolates of B. catarrhalis. This assay includes preincubation of
test organisms in the presence of penicillin for
10 min. No difference was observed when organ-
isms were grown in the presence of penicillin.
These results are consistent with the notion that
the β-lactamase of B. catarrhalis is strongly
pharmacologically such that minimal amounts of
enzyme are released into the surrounding envi-
ronment when organisms are suspended in fluid
media.

Since extrachromosomal DNA was not found in
any of the strains of B. catarrhalis, it was
concluded that the β-lactamase of B. catarrhalis
was not plasmid mediated, but rather of chro-
omosomal origin. This is consistent with the ob-
servations of Hoi-Dang Van et al. (8) who con-
cluded that the β-lactamase of the single B.
catarrhalis strain that they examined was also
not plasmid mediated. Furthermore, attempts to
transfer the β-lactamase phenotype from B. ca-
tarrhalis to other bacteria by means known to
transfer plasmid DNA have been unsuccessful
(14).

The substrate specificity of the β-lactamase of
the 11 strains of B. catarrhalis examined in this
case could be inferred from the differential
susceptibility of these organisms to various beta-
lactam antibiotics. Three penicillin-sensitive
penicillins were significantly less active (i.e.,
demonstrated higher MICs) than seven cepha-
losporins and one cephamycin. These results are
consistent with the observations of Hoi-Dang
Van et al. (8) and Percival et al. (14). In contra-
distinction to the β-lactamase of N. gonorrhoeae
(4, 8), our data suggest that the β-lactamase of
B. catarrhalis possesses minimal or no cepha-
losporinase activity. If this is so, then β-lacta-
mas inhibitors such as clavulanic acid and CP
45899 would depress the penicillin-sensitive
penicillin MICs, but would not affect the MICs
of the penicillin-resistant penicillins, cepha-
losporins, or cephamycin. Indeed, both clavu-
lanic acid and CP 45899 markedly lowered pen-
icillin and ampicillin MICs to B. catarrhalis
while not significantly altering the MICs of
methicillin, cephalothin, or cefoxitin. Similarly,
Ninane et al. (12) found that clavulanic acid
rendered three β-lactamase-producing clinical
isolates of B. catarrhalis very susceptible to
amoxicillin. When three patients with presumed
B. catarrhalis pneumonia who had failed to
respond to either ampicillin or amoxicillin were
covered with BRL 25000, a drug containing both
amoxicillin and clavulanic acid, clinical cures
were rapidly achieved.

Several investigators (8, 14) have observed
multiple bands of β-lactamase activity when ex-
tracts of B. catarrhalis were analyzed by isoe-
lectric focusing in polyacrylamide or agarose
gels. It is not known whether these bands rep-
resent β-lactamases with different substrate pro-
files or merely isoenzymes with different isoelec-
tric points. Results of the present study suggest
that the former might be true. Not only were
wide variations observed in the levels of resis-
tance to penicillin-sensitive penicillins, but the
effect of β-lactamase inhibitors on these antibi-
otics was strain dependent. For example, strain
A 1246 was significantly less resistant to ampi-
cillin (MIC = 0.32 μg/ml) than penicillin (MIC
= 10 μg/ml). The penicillin MIC was reduced
97% by clavulanic acid and 94% by CP 45899.
The ampicillin MIC was not affected by either
β-lactamase inhibitor. The possibility arises that
among the B. catarrhalis β-lactamases, there
exists a heterogenous population of enzymes
that vary with regard to their activity against
the penicillin-sensitive penicillins. Such heter-
ogeneity would be manifest in strain-to-strain
variation in susceptibilities to these antibiotics.

Since B. catarrhalis is apparently not suscept-
ible to the penicillin-sensitive penicillins, due in
large part to the elaboration of β-lacta-
mas, it was of interest to know what alternative
antibiotics might be used in treating human
infections caused by this organism. Based on the
results of MIC determinations of 17 antibiotics
against 11 clinically significant isolates of B.
catarrhalis, cefoxitin, erythromycin, and tetracy-
cline were found to be most effective. The
generic mean MICs of these agents were 0.5,
0.4, and 0.4 μg/ml, respectively.

Finally, it should be emphasized that the B.
catarrhalis ATCC 8176 is significantly different
from the strains of B. catarrhalis described in
this study which were associated with human
disease. As previously reported (G. V. Doern
and S. A. Morse, submitted for publication),
ATCC 8176 possessed several growth character-
istics (growth on nutrient agar at 22°C and ab-
sence of growth on Thayer-Martin medium) dif-
ferent from those of numerous clinically signifi-
cant strains. Similarly, in the present study,
ATCC 8176 was uniformly more susceptible to
all 17 antibiotics tested when compared with 11
clinical isolates of *B. catarrhalis*. Furthermore, the ATCC strain did not possess β-lactamase activity, whereas the clinical isolates did. The possibility exists that the ATCC strain is representative of nonpathogenic, commensal strains of *B. catarrhalis* and that isolates from clinical material represent a distinct subvariety of this bacterium which in addition to enhanced virulence possesses altered growth and antibiotic susceptibility characteristics. This hypothesis awaits further investigation.

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**LITERATURE CITED**
