Detection of β-Lactamase Activity among Clinical Isolates of Branhamella catarrhalis with Six Different β-Lactamase Assays

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A total of 74 different clinical isolates of Branhamella catarrhalis were examined for their ability to produce beta-lactamase by six different beta-lactamase assays. These included a conventional tube and disk test, in which the chromogenic cephalosporin nitrocefin was used as a substrate; a disk procedure, in which pyridinium-2-azo-p-dimethylaniline cephalexin was used as a substrate; broth and disk acidimetric methods; and a conventional tube iodometric assay. A total of 58 of the study isolates produced beta-lactamase. In all cases, positive results were obtained with the nitrocefin tube and disk assays after 1 min. With the pyridinium-2-azo-p-dimethylaniline cephalexin disk test, 57 of the 58 beta-lactamase-producing strains yielded a positive reaction in 1 min; the remaining strain was positive after 10 min. None of the beta-lactamase-producing strains produced positive reactions by either the broth or disk acidimetric methods after 1 min. With the broth test, 10 min was required for positive test results for 42 strains; 30 min was necessary for 16 strains. By the disk acidimetric procedure, all 58 strains were positive after 10 min. Of 58 beta-lactamase-producing strains, 30 were positive by the iodometric assay after 1 min, 13 strains required 10 min, and 4 strains were detected as being beta-lactamase positive only after 30 min. One beta-lactamase-producing strain remained negative by the iodometric method. Among the 16 strains of B. catarrhalis that lacked beta-lactamase that were examined in this study, no false-positive results were obtained by any of the six assays.

Branhamella catarrhalis is now recognized as an important human pathogen that is associated with a wide variety of different infectious diseases (4). With respect to incidence and morbidity, the most important of these are acute otitis media, maxillary sinusitis, and bronchopulmonary infections. Most clinical isolates of B. catarrhalis produce a beta-lactamase (1, 6, 12, 13, 17). Disease caused by beta-lactamase-producing strains is refractile to therapy with ampicillin and amoxicillin, two antimicrobial agents that are frequently used for empiric therapy of infections such as those with which B. catarrhalis is associated (15, 17, 20). For these reasons, in vitro determination of beta-lactamase production is of clinical value in the management of patients with Branhamella infections. The beta-lactamase of B. catarrhalis is produced in small amounts and remains strongly cell associated (9, 11). Because of this, it is possible that use of conventional beta-lactamase assays to detect activity among clinical isolates of B. catarrhalis could lead to false-negative results. Indeed, in a recent report of the results of a 1983 College of American Pathologists proficiency survey, this was shown to be the case (10). Of 182 laboratories in which acidimetric assays were used, 22% reported negative results with a beta-lactamase-producing survey strain of B. catarrhalis. The false-negative rate among 11 laboratories in which an iodometric test was used was 36%. Similar results were noted in 32 laboratories in which a pyridinium-2-azo-p-dimethylaniline cephalexin (PADAC) assay was used, i.e., a false-negative rate of 31%. In the College of American Pathologists survey, the most accurate beta-lactamase assays appeared to be those in which the chromogenic cephalosporin nitrocefin was used as a substrate (4% false-negative rate among 262 laboratories).

The intent of this investigation was to systematically evaluate several common procedures for detecting beta-lactamase production among 74 clinical isolates of B. catarrhalis. In addition, the in vitro activity of ampicillin and the combination amoxicillin-clavulanate (Augmentin; Beecham Laboratories, Bristol, Tenn.) was determined against all study strains and related to the likelihood of obtaining false-negative results with individual beta-lactamase assays.

MATERIALS AND METHODS

Organisms. A total of 74 clinical isolates of B. catarrhalis were examined in this investigation. A total of 13 strains were obtained from patients seen at the University of Massachusetts Medical Center, Worcester. A total of 20 strains were provided by S. L. Berk, Veterans Administration Medical Center, Johnson City, Tenn.; 40 strains were the gift of R. J. Wallace, University of Texas Health Center, Tyler; and 1 strain was received from A. von Graevenitz, University of Zurich Institute of Medical Microbiology, Zurich, Switzerland. All strains were identified as B. catarrhalis by conventional methods (5). Organisms were maintained at −70°C in skim milk until just before use. Stock cultures were thawed and inoculated onto enriched chocolate agar plates (Scott Laboratories, Inc., Fiskeville, R.I.). Following incubation at 35°C for 18 to 24 h in 5 to 7% CO2, single colonies were subcultured onto a second enriched chocolate agar plate which was incubated under identical conditions. Growth from this plate was used to prepare inocula for all subsequent studies.

Acidimetric beta-lactamase assays. A broth acidimetric beta-lactamase test was performed as described by Esccilla (7). Tubes containing beta-lactamase reagents were examined for a red-to-yellow change indicative of a positive reaction at 1, 10, and 30 min following the addition of the test organism. An acidimetric disk test for beta-lactamase was

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performed by using commercially available beta-lactam reagent disks (Marion Scientific, Div. Marion Laboratories, Inc., Kansas City, Mo.) according to the instructions of the manufacturer. Briefly, a beta-lactam disk was rehydrated with a single drop of sterile 0.85% NaCl (pH 7.0), and then three to five isolated colonies of test organism were transferred onto the surface of the disk by using a wooden stick. The disk was examined after 1, 10, and 30 min for the presence of a purple-to-yellow change as evidence of a positive reaction.

Chromogenic cephalosporin beta-lactamase assays. The conventional tube chromogenic cephalosporin beta-lactamase test, in which nitrocefin was used as a substrate, was performed as described by O'Callaghan et al. (16). At 1, 10, and 30 min following inoculation of a solution containing assay reagents, the suspension was examined for a yellow-to-red change, which was indicative of a positive reaction. In addition, all isolates were examined by using two commercially available chromogenic cephalosporin disk beta-lactamase assays. In one case, disks contained nitrocefin as a substrate (Cefclav disks; BBL Microbiology Systems, Cockeysville, Md.); in the other case, the chromogenic cephalosporin substrate was PADAC (PADAC disks; Calbiochem-Behring, La Jolla, Calif.). In both cases, assays were performed precisely as described above for the acidimetric disk beta-lactamase test. Cefnase disks were examined for a yellow-to-red change as evidence of a positive reaction, while a purple-to-yellow change was taken as evidence of a positive reaction with the PADAC disks.

Iodometric tube beta-lactamase assay. A conventional tube iodometric assay for beta-lactamase production was performed as described by Catlin (2). At 1, 10, and 30 min after the addition of iodine and starch solutions to suspensions of test organism in a penicillin substrate, tubes were examined for complete clearing of the blue coloration as an indication of a positive result.

Ampicillin and amoxicillin-clavulanate MICs. All isolates were characterized with respect to their ampicillin and amoxicillin-clavulanate MICs by using commercially prepared dehydrated microdilution susceptibility test plates (Sensititer, Inc., Lawrence, Mass.). Ampicillin was tested at serial twofold concentration increments that ranged from 0.015 to 256 μg/ml. Amoxicillin-clavulanate was tested at a constant ratio of two parts amoxicillin to one part clavulanate over the same concentration range. Suspensions containing 1 × 10^8 to 5 × 10^8 CFU of the test organism per ml in cation-supplemented Mueller-Hinton broth (pH 7.2) were used to inoculate microdilution plates (100 μl per well). Plates were sealed with a mylar film and incubated at 35°C in ambient atmospheric air for 24 h prior to examination. The lowest concentration of antimicrobial agent tested in which no macroscopic evidence of growth was observed was considered the MIC.

Controls. A beta-lactamase-positive strain and a beta-lactamase-negative strain of Haemophilus influenzae were used as daily controls for all beta-lactamase assays. A stock strain of Escherichia coli for which the ampicillin MIC was 16 μg/ml and the amoxicillin-clavulanate MIC was 0.25-0.125 μg/ml was used as a daily control of ampicillin and amoxicillin-clavulanate MIC determinations.

RESULTS

Among the 74 strains of B. catarrhalis examined in this study, 58 were found to produce beta-lactamase. Each of these 58 strains produced a positive result after 1 min by the conventional tube nitrocefin procedure and by a commercially available nitrocefin disk test. Commercially prepared disks containing a different chromogenic cephalosporin, PADAC, yielded positive reactions after 1 min with 57 of the 58 beta-lactamase-producing strains of B. catarrhalis; in one case a positive result was obtained after 10 min. None of the 58 beta-lactamase-positive strains produced a positive reaction after 1 min by either of the two acidimetric beta-lactamase assays examined in this investigation. By the broth acidimetric assay, 42 strains were positive at 10 min and 16 strains were positive at 30 min. By the acidimetric disk test, in which commercially prepared disks were used, a positive result was noted after 10 min with all 58 beta-lactamase-producing strains. Finally, a conventional tube iodometric assay yielded positive results after 1 min with 30 strains, after 10 min with 13 strains, and after 30 min with 4 strains. One of the beta-lactamase-producing strains never gave a positive result by the iodometric test. Of 74 isolates characterized in this study, 16 were negative by all six of the beta-lactamase assays at all time periods.

In general, the 58 beta-lactamase-positive strains had ampicillin MICs (geometric mean ampicillin MIC, 0.85 μg/ml) that were higher than those for the 16 strains that were found to be beta-lactamase negative by all assays (geometric mean ampicillin MIC, 0.013 μg/ml) (Table 1). In contrast, the amoxicillin-clavulanate MICs for beta-lactamase-producing strains were essentially the same as the amoxicillin-clavulanate MICs for strains that lacked beta-lactamase (geometric mean amoxicillin-clavulanate MICs, 0.06 and 0.024 μg/ml, respectively). The relative activity of ampicillin and amoxicillin-clavulanate, expressed as the ratio of the ampicillin MIC (log_{10}) versus the amoxicillin-clavulanate MIC (log_{10}), for individual strains is depicted in Table 2. Ampicillin was significantly less active against beta-lactamase-producing strains than was amoxicillin-clavulanate, whereas the activity of these two antimicrobial agents was essentially the same against beta-lactamase-negative strains.

As described above, not all beta-lactamase-positive strains were equally reactive in individual beta-lactamase
assays. It was of interest to know if those strains that appeared to be less reactive in a particular beta-lactamase assay, that is, took longer to generate a positive result, could be distinguished based on their ampicillin MICs. With respect to the acridometric broth test, this was indeed the case. The geometric mean ampicillin MIC for the 42 beta-lactamase-producing strains that were reactive after 10 min was 1.21 μg/ml (range, 0.125 to 4.0 μg/ml). In contrast, the geometric mean ampicillin MIC for the 16 strains that were reactive only after 30 min was 0.34 μg/ml (range, 0.06 to 2.0 μg/ml).

**DISCUSSION**

The results of this investigation demonstrate that not all in vitro beta-lactamase assays are comparable when used to detect beta-lactamase activity among clinical isolates of *B. catarrhalis*. If the length of time necessary to detect a positive result is used as an indication of assay sensitivity, the six assays examined in this study would rank in the following order with respect to their sensitivity: tube nitrocefin assay = disk nitrocefin assay > disk PADAC assay > disk acridometric assay > broth acridometric assay > iodometric assay. The fact that, in general, beta-lactamase procedures in which chromogenic cephalosporins were used as substrates appeared to be more sensitive than either acridometric or iodometric assays is consistent with the observation that the *B. catarrhalis* beta-lactamase possesses significantly greater activity against nitrocefin than against either penicillin or ampicillin (9, 18). Penicillin was the substrate used to detect beta-lactamase activity by both acridometric assays examined in this study, as well as by the iodometric procedure. The results of this study are also consistent with observations made in a recent College of American Pathologists proficiency survey in which different assays were assessed as means for detecting *B. catarrhalis* beta-lactamase activity (10). Laboratories in which chromogenic cephalosporin beta-lactamase assays are used with nitrocefin as a substrate reported the lowest rate of false-negative results.

In previous reports it has been demonstrated that the activity of ampicillin and amoxicillin against *B. catarrhalis* is extremely variable (3, 12, 18, 19). Beta-lactamase-negative strains are uniformly inhibited by very low concentrations of this antimicrobial agent. In contrast, while ampicillin MICs for most beta-lactamase-producing strains indicate resistance, i.e., ≥2.0 μg/ml, relatively low ampicillin MICs are found for some strains of *B. catarrhalis* which produce beta-lactamase, i.e., 0.1 to 0.5 μg/ml (3, 18, 19). Indeed, in this study, ampicillin MICs of ≥0.5 μg/ml were observed for 23 strains among a total of 58 beta-lactamase-positive strains of *B. catarrhalis*. Such strains would be considered susceptible to ampicillin if this judgment were made solely on the basis of in vitro determinations. There have been several reports, however, of therapeutic failures when ampicillin or amoxicillin have been used to treat *B. catarrhalis* infections caused by beta-lactamase-positive strains (15, 17, 20). Because of this, it appears that therapeutic decisions are best predicated on the results of beta-lactamase assays rather than determinations of ampicillin or amoxicillin MICs, because the latter may indicate false susceptibility.

The observation that ampicillin and amoxicillin possess extensive activity against some beta-lactamase-producing strains of *B. catarrhalis* can be explained by the results of investigations in which *B. catarrhalis* beta-lactamase has been characterized by isoelectric focusing. Strains of *B. catarrhalis* differ considerably with respect to the pI profiles of their beta-lactamases (11, 18). Two pI patterns, however, seem to be most common in the United States (14). They have been designated Ravasio and 1908, because these were the designations of the type strains from which the pI profiles were first observed (8). It has been suggested that the absolute amount of beta-lactamase produced by 1908 strains is significantly less than that produced by Ravasio strains (12). It is possible that beta-lactamase-positive strains of *B. catarrhalis*, for which the ampicillin or amoxicillin MICs are low, produce the 1908-type beta-lactamase, thus explaining the apparent greater activity of these antimicrobial agents in vitro. It has been shown that strains which produce the 1908-type beta-lactamase typically have larger zones on penicillin and ampicillin of inhibition when tested by a disk diffusion procedure (12). We are currently investigating this hypothesis by determining the beta-lactamase pI profiles of the strains of *B. catarrhalis* examined in this study.

Finally, it should be noted that the combination of amoxicillin-clavulanate was found to have significantly greater activity than ampicillin alone for all of the beta-lactamase-positive strains of *B. catarrhalis* examined in this study. This is consistent with the observation that the activity of the beta-lactamase of *B. catarrhalis* is inhibited by clavulanate (6, 8, 9).

In conclusion, not all in vitro beta-lactamase assays are equivalent with respect to their ability to detect the beta-lactamase produced by *B. catarrhalis*. Among the six assays examined in this study, procedures in which the chromogenic cephalosporin nitrocefin was used as a substrate were superior. Furthermore, determination of ampicillin MICs was found not to be a reliable means for predicting the therapeutic efficacy of this antimicrobial agent. Decisions regarding the use of ampicillin or amoxicillin for the treatment of *B. catarrhalis* infections should be predicated on the results of a suitable beta-lactamase assay.

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**LITERATURE CITED**