

Characterization of an Ampicillin-Resistant *Haemophilus influenzae* Type B

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A 28-year-old female in Denver was found in early 1974 to have frontal sinusitis, osteomyelitis, and bacteremia due to *Haemophilus influenzae*, type B. The minimal inhibitory concentration of ampicillin for this organism was 100 $\mu\text{g/ml}$ and the minimal bactericidal concentration was $>100 \mu\text{g/ml}$. It was inhibited by chloramphenicol at 0.4 $\mu\text{g/ml}$. Further studies demonstrated that ampicillin and methicillin were synergistic against this organism. It was shown to produce a diffusible beta-lactamase. Transferase of resistance from this organism to a susceptible *Haemophilus parainfluenzae* and a reciprocal transfer were accomplished. A test for transformation was negative as was a test for reversal of resistance by ethylenediaminetetraacetic acid.

The increase of R factors conferring resistance to particular drugs has always paralleled the extensive use of the corresponding antibiotic. Since its availability in 1963, ampicillin has been widely used as the drug of choice in the treatment of *Haemophilus influenzae* type B infections. If one accepts the reasonable supposition that the R factors best equipped to survive in a given bacterial host will emerge in the long term, the appearance of ampicillin-resistant *H. influenzae* type B was not an unlikely phenomenon.

The purpose of this study was to characterize the first such strain encountered in the Denver area.

CASE REPORT

On 6 March 1974, a 32-year-old white female with known Hodgkin's disease was admitted to a Denver hospital with evidence of acute sinusitis involving the maxillary and frontal sinuses. A trephine procedure and irrigation were carried out on the frontal sinus. She was treated with decongestants and oral penicillin for 4 days. Because of her lack of response, high-dose intravenous penicillin was instituted for 4 more days, but again, she did not respond well, and intravenous chloramphenicol was added on day 8 of therapy. She did well thereafter, and was discharged 21 days later on 29 March. She returned to the hospital 48 h later with shaking chills, fever, and physical findings suggesting an acute frontal sinusitis. Skull films revealed acute osteomyelitis of the frontal bone. Cultures of the nose, sinus drainage, and three blood cultures all grew *H. influenzae* type B.

She was treated with intravenous penicillin and chloramphenicol for 2 weeks and made an uneventful recovery.

The strain of *H. influenzae* type B recovered from her blood forms the subject of this report.

MATERIALS AND METHODS

Bacterial strain. The strain isolated from this patient was identified as *H. influenzae* type B based on its requirement for both hematin (X factor) and nicotinamide adenine dinucleotide (V factor). It was not hemolytic, and agglutinated strongly in *H. influenzae* type B antisera in slide agglutination tests.

Antimicrobial susceptibility tests. Preliminary antimicrobial susceptibility tests were carried out by the method of Bauer et al. (2). An 18-h culture in Fildes broth was diluted to half the density of a McFarland no. 1 standard and plated on chocolate Mueller-Hinton agar containing 5% sheep blood.

Minimal inhibitory concentrations (MICs) for selected drugs were determined by the microtiter method. Serial twofold antibiotic dilutions were made in Fildes broth, and then 0.05 ml of a 10^{-4} dilution of an overnight broth culture in Fildes broth was added to 0.05 ml of the diluted antibiotic in the microtiter plate. The plates were incubated overnight at 35 C. Minimal bactericidal concentrations (MBCs) were determined by plating 0.01 ml from each well to chocolate agar. Complete inhibition of growth was used as the end point, read after overnight incubation at 35 C in ambient air.

Studies for synergism. Killing curves were determined in Fildes broth, using as inoculum a 1:100 dilution of an overnight Fildes broth culture of the organism. The concentration of ampicillin in the mixture was 50 $\mu\text{g/ml}$ and that of methicillin, 10 $\mu\text{g/ml}$. Aliquots were taken at 0 time and at 1, 2, 4, 8, and 12 h after inoculation, serially diluted, plated on

chocolate agar in duplicate, and colonies were counted after overnight incubation at 35 C.

Production and activity of penicillinase. The production and activity of penicillinase by this organism was assessed by inoculating 2 ml of an overnight Fildes broth culture of the ampicillin-resistant *H. influenzae* type B to 8 ml of Fildes broth, incubating at 35 C, and after 1 h adding penicillin to a final concentration of 10 U/ml. After 4 h of incubation, 10^{-3} M zinc sulfate (2.88 mg/10 ml) was added to provide a cephalosporinase cofactor if needed. This mixture was put on a rotary shaker for 5 min, then centrifuged. The supernatant was subjected to membrane filtration (Millipore Corp.), and then checked for sterility. The penicillinase activity of this mixture was assessed by incubating 0.1 ml with 0.5 ml of each penicillin or cephalosporin analogue to be tested, at an initial concentration of 80 μ g/ml. The penicillin content of the mixture was then measured at intervals by applying 20- μ l aliquots to sterile disks, subsequently assayed on plates using a *Bacillus subtilis* spore suspension in Mueller-Hinton agar. The zone sizes were then read on a standard curve simultaneously determined. An ampicillin-sensitive *H. influenzae* type B was used as a simultaneous control organism throughout the entire test.

Transfer study. The transfer study was carried out using a strain of *H. parainfluenzae*, identified by its requirement for nicotinamide adenine dinucleotide, growth without the presence of hematin, lack of hemolysis, and a negative slide agglutination test with *H. influenzae* type B antiserum. Single colonies of the ampicillin-resistant *H. influenzae* and sensitive *H. parainfluenzae* were subcultured separately in Fildes broth and incubated overnight at 35 C. One milliliter of each culture was then inoculated into 5 ml of fresh Fildes broth. The mixture was incubated at 35 C overnight, after which 0.01 ml was spread on Trypticase soy agar (BBL) plates containing 5% yeast extract and 5 μ g of ampicillin per ml. Single colonies growing on this media were subcultured to Trypticase soy and blood agar with a V disk to recheck the hematin and coenzyme requirements. Slide agglutination tests with *H. influenzae* type B antisera were then carried out, as were determinations of the susceptibility to ampicillin as previously described. The same strain of *H. parainfluenzae*, unmated, was used as a control. The reciprocal cross was carried out in a comparable fashion.

Transformations. The method of Juni (6) was used with minor modification. A 4-mm loop containing 0.01 ml of the resistant *H. influenzae* was suspended in 0.5 ml of a sterile solution containing 0.05% sodium dodecyl sulfate in citrated saline (0.15 M sodium chloride and 0.015 M sodium citrate) in a test tube. Organisms were dispersed by agitation, and the suspended cells were heated for 2 h in a 70 C water bath with occasional manual shaking. Samples of 0.1 ml of the suspension were then plated on chocolate agar to test sterility. An 18- to 24-h overnight broth culture of an ampicillin-sensitive *H. parainfluenzae* was then mixed with an equal amount of the deoxyribonucleic acid (DNA) preparation from the resistant *H. influenzae* type B. This was plated after overnight incubation on chocolate

agar containing 5 μ g of ampicillin per μ l.

Test for reversal of resistance by EDTA. The method of Weiser et al. (14) was used. The inoculum was a 10^{-4} dilution of an overnight Fildes broth culture of the ampicillin-resistant *H. influenzae*. A subinhibitory concentration of 1.1×10^{-4} M ethylenediaminetetraacetic acid (EDTA) was incorporated into a microtiter test for ampicillin sensitivity as previously described.

RESULTS

The results of the antimicrobial susceptibility tests are shown in Tables 1 and 2. A high level resistance to ampicillin is demonstrated along with susceptibility to chloramphenicol.

Killing curves were done to detect any synergistic action of a methicillin-ampicillin combination (Fig. 1). Methicillin alone decreased the number of viable *H. influenzae* for 4 h, suggesting that a proportion of the population was susceptible, but after that time the count begins to increase. Ampicillin alone and the methicillin-ampicillin combination decreased the viable count at almost identical rates up until 8 h. After this time, the number of *H. influenzae* in the ampicillin-only flask began to increase, whereas the number in the combination continued to decrease.

Penicillinase activity of this strain is shown in Fig. 2. Penicillin G and ampicillin were rapidly inactivated. Carbenicillin was also inactivated, albeit more slowly. Methicillin and cephalothin were stable for 90 min, after which cephalothin began to inactivate slowly. Control

TABLE 1. Disk tests on *H. influenzae* type B strain "Young"^a

Drug	Zone diam (mm)
Ampicillin	Nil
Methicillin	Nil
Carbenicillin	Nil
Sulfadiazine	Nil
Cephalothin	Nil
Tetracycline	21.0
Sulfa-trimethoprim	20.4

^a Kirby-Bauer Method, Mueller-Hinton agar with 5% chocolate sheep blood.

TABLE 2. MIC and MBC tests on *H. influenzae* type B strain "Young"^a

Drug	MIC (μ g/ml)	MBC (μ g/ml)
Ampicillin	100	>100
Chloramphenicol	0.2	0.2
Methicillin	12.5	12.5
Kanamycin	3.1	3.1
Control <i>H. influenzae</i> type B		
Ampicillin	0.4	0.4

^a 10^{-4} dilution overnight broth culture in Fildes broth.

antibiotic solutions and a parallel test using an ampicillin-sensitive *H. influenzae* type B showed no effect on drug concentrations during the test period.

In Table 3 the results of mating the ampicillin-resistant *H. influenzae* type B from this patient with an ampicillin-sensitive *H. parainfluenzae* are shown. We were successful in the transfer of resistance in this attempt and also in the reciprocal cross-match. However, five strains of ampicillin-resistant *H. influenzae* type B organisms acquired originally from the Center for Disease Control, Atlanta, Ga., were tested for transference with negative results. Using the same technique, we also attempted to transfer ampicillin resistance from our original strain to one strain each of *Streptococcus pneumoniae* type III, *Proteus mirabilis*, *Escherichia coli*, and group A meningococci. These

attempts were uniformly unsuccessful as was our attempt many passages and several months later to repeat the original experiment. The attempt to demonstrate transformation yielded negative results as did the attempt to reverse the ampicillin resistance of the organism by incorporating EDTA in subinhibitory concentrations in MIC titrations. The MIC remained at 100 $\mu\text{g/ml}$.

DISCUSSION

Our characterization of this strain of ampicillin-resistant *H. influenzae* type B correlates with other recent investigations. It is now clear that ampicillin resistance is mediated by the production of a beta-lactamase (7, 15). Farrar and O'Dell have found that this beta-lactamase resembles those produced by other gram-negative bacilli (5). The substrate profile reported by them and the one described in this paper are almost identical. Medeiros and O'Brien have further identified this enzyme as a TEM-type beta-lactamase (8). Neu has shown that most high-level (1,000 $\mu\text{g/ml}$ or greater) ampicillin-resistant *E. coli* produce epimerally mediated beta-lactamase and are not susceptible to the synergistic action of a penicillinase-resistant penicillin with ampicillin (9). He has also shown that in those strains with low-level resistance (8 to 250 $\mu\text{g/ml}$) 78% are susceptible to penicillin synergy. It is interesting that this strain of *H. influenzae* with an MIC of 100 $\mu\text{g/ml}$ to ampicillin did demonstrate ampicillin-methicillin synergy. Ellwell et al. have now

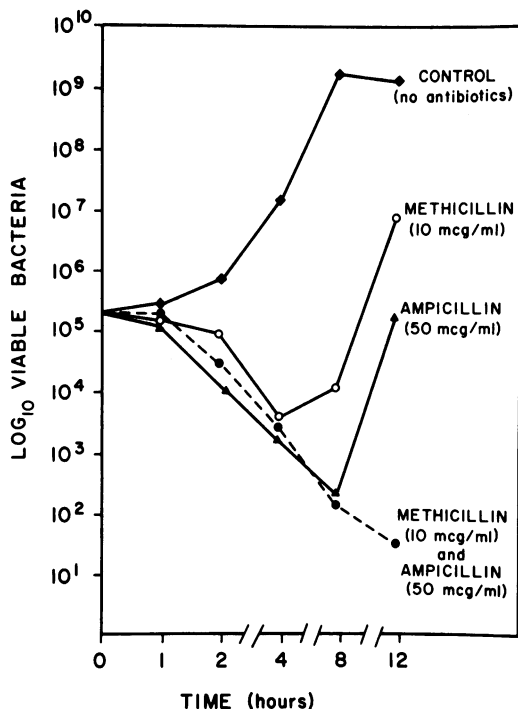


FIG. 1. Synergistic effect of methicillin and ampicillin on a strain of ampicillin-resistant *H. influenzae* type B.

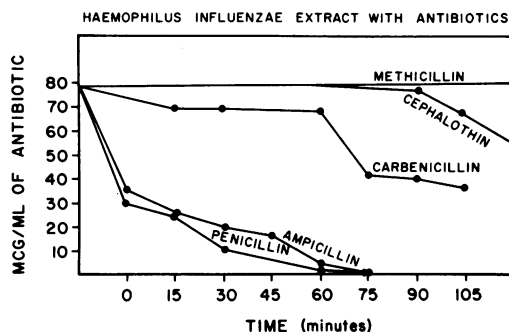


FIG. 2. Inactivation of antibiotics by ampicillin-resistant *H. influenzae* extract.

TABLE 3. Mating experiments

Crosses	Strain	MIC of ampicillin ($\mu\text{g/ml}$)
Ampicillin-resistant <i>H. influenzae</i> B \times ampicillin-resistant <i>H. parainfluenzae</i>	Mated <i>H. parainfluenzae</i>	100
	Control <i>H. parainfluenzae</i>	0.4
Ampicillin-resistant <i>H. parainfluenzae</i> \times ampicillin-sensitive <i>H. influenzae</i> B	Mated <i>H. influenzae</i> B	100
	Control <i>H. influenzae</i> B	0.4

shown that ampicillin-resistant strains of *H. influenzae* contain plasmid DNA carrying the structural gene for beta-lactamase (4). Thorne and Farrar have demonstrated transference of ampicillin resistance to an ampicillin sensitive *H. influenzae* type B and have shown that this was probably mediated by conjugation (12). This is in accordance with the results reported in this study demonstrating that resistance could be transferred from an ampicillin-resistant *H. influenzae* type B to an ampicillin-sensitive *H. parainfluenzae*. Smith has reported that optimum frequency R-factor transfer occurs when mating takes place upon a filter while matings taking place in broth even with a transfer frequency of 10^{-4} are often sterile (10). With Thorne's transfer frequency of 4.6×10^{-5} inability to repeat the original experiment in broth is not unexpected. Sykes et al. have also recently demonstrated transference of ampicillin resistance from *H. influenzae* to an ampicillin-sensitive *H. parainfluenzae* (11).

It has been long known that *H. influenzae* is susceptible to transformation (1); therefore, although the presence of a TEM-type beta-lactamase has commonly been mediated by R factors (3), an attempt was made to demonstrate transmission of cell-free DNA. This was unsuccessful. Thorne and Farrar found that transfer of ampicillin resistance between strains of *H. influenzae* remained unchanged in the presence of deoxyribonuclease I, making transformation unlikely as the mechanism of transfer (12).

This strain of *H. influenzae* demonstrated no reversal of ampicillin resistance when tested with EDTA as has been shown with some gram-negative organisms resistant to penicillin (14). Medeiros and O'Brien (8) have reported that ampicillin-resistant *H. influenzae* type B organisms have a greater permeability to ampicillin than *E. coli*.

The sudden and rather dramatic appearance of ampicillin-resistant *H. influenzae* type B in widely scattered areas all over the United States beginning in December 1973 represents an intriguing epidemiological phenomenon. It is of some interest that these strains have not yet become more widely prevalent. We, for example, have encountered very few strains in the Denver area, in spite of what is now routine disk testing of *H. influenzae* type B isolates in most of the clinical laboratories in the area. Physicians taking care of patients with *H. influenzae* type B disease, must certainly keep the possibility of ampicillin resistance clearly

in mind. Clinical laboratories are well advised to monitor the ampicillin susceptibility of *H. influenzae* type B isolates on a continuing basis using a reliable method such as that described by Thornsberry and Kirven (13).

Although *H. parainfluenzae* is generally regarded as a nonpathogenic inhabitant of the upper respiratory tract it has been associated with subacute bacterial endocarditis. It will be of interest if ampicillin-resistant variants of this organism spontaneously appear, since this study shows it to be an in vitro possibility.

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