

Haemophilus influenzae: Antibiotic Susceptibility

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INTRODUCTION

In 1972 the first isolate of *Haemophilus influenzae* that was resistant to ampicillin was reported (63). It was recovered from a child with meningitis who had failed to respond to therapy. This was an ominous event since disease caused by this organism had been treated effectively with ampicillin for over a decade. Reports of ampicillin-resistant *H. influenzae* isolated from various places around the world began to appear almost simultaneously (21, 52, 84, 108, 113, 114, 120), and it soon became clear that the development of resistance in this organism would present a serious therapeutic dilemma to the clinical community.

As scientists began to unravel the events which we believe led to the acquisition of resistance by *H. influenzae*, a rare opportunity was presented to explore the epidemiology of transmissible antibiotic resistance in a previously susceptible species. My goals in writing this review are (i) to trace the development of antibiotic resistance in *H. influenzae*, (ii) to discuss the mechanisms which appear to be important in mediating resistance, (iii) to explore newer antimicrobial agents and their role in the treatment of disease caused by *H. influenzae*, and (iv) to analyze the various methods for testing the susceptibility of this organism to antibiotics.

HISTORICAL PERSPECTIVE: DEVELOPMENT OF RESISTANCE

Ampicillin Resistance: Mechanisms and Genetic Basis

H. influenzae represents a significant cause of disease in both children and adults. Although the organism is relatively resistant to penicillin G, most other early antibiotics were somewhat more effective in treating infection. During the 1950s, tetracycline and chloramphenicol were widely used. However, after its introduction in 1961, ampicillin rapidly became the antibiotic of choice. There were few reports of therapeutic failure in serious infection when ampicillin was used. In all instances there were reasons other than resistance of the organism which accounted for the failures, and *H. influenzae* was considered uniformly susceptible to the antibiotic.

Beginning in 1972, reports of clinically significant ampicillin resistance began to appear. Investigation of the resistant strains proceeded quickly, and it soon was evident that resistance was associated with the presence of a beta-lactamase enzyme (52, 121) which was cell associated and produced constitutively. The enzyme showed strong hydrolytic activity against penicillin G, ampicillin, and cephaloridine but had little effect on methicillin or cephalixin. The hydrolysis of cephalothin and carbenicillin was intermediate. This substrate profile suggested that the *Haemophilus* enzyme was similar to the type IIIa beta-lactamase common in R-factor-carrying strains of *Escherichia coli* (85) and referred to as TEM-1. The isoelectric focusing point was shown to be identical to that of the TEM-1 enzyme (104, 121), and further characterization demonstrated immunologic identity as well (104). The TEM-1 beta-lactamase has been found in the majority of ampicillin-resistant isolates of *H. influenzae* from around the world. It is the most common cause of ampicillin resistance in this species.

The genetic basis of beta-lactamase-mediated resistance in *H. influenzae* is well characterized. Thorne and Farrar first demonstrated that ampicillin resistance could be transferred from resistant strains to susceptible recipients through a conjugative mechanism (109). Elwell and colleagues (24, 31) identified two types of plasmid in resistant strains. One was a 30-megadalton conjugative plasmid and the other was a 3.0-megadalton plasmid. Although the plasmids were similar in guanine-plus-cytosine content (0.39 versus 0.40 M), they shared homology only in the gene sequence coding for ampicillin resistance. Further, the gene segment which encoded ampicillin resistance was homologous with the gene for TEM-1 beta-lactamase found on R factors of enteric origin.

The deoxyribonucleic acid (DNA) sequence which codes for TEM-1 is a 3.0-megadalton segment widely distributed among many different bacterial species. It has been found in organisms from widely separated geographic areas and on plasmids of many different incompatibility groups. The sequence is much larger than the gene for the enzyme, and it undergoes a form of transformation called transposition (41).

Transposition is the process by which a discrete sequence resident on one replicon is inserted into a separate DNA replicon, either chromosome or plasmid. Unlike conventional transformation, transposition occurs in the absence of homology between donor and recipient replicons. Thus, transposition allows the dissemination of discrete genes which code for antibiotic resistance to bacterial plasmids and chromosomes with vastly different DNA sequences and consequently to a wide variety of genetically unrelated bacteria. The discrete segment of DNA is called a transposon, and the specific sequence for TEM-1 is called transposon A, or TnA.

Two hypotheses have been advanced to explain the origin of ampicillin resistance plasmids in *H. influenzae* (32). The first proposes that a previously uncharacterized group of plasmids was transferred from the enteric pool into *H. influenzae* by conjugation. The second hypothesis proposes that a plasmid carrying the TnA sequence was transferred into *H. influenzae* and was unable to survive. The plasmid disappeared, but prior to its elimination the TnA sequence was rescued by transposition onto a cryptic replicon (plasmid) in the *Haemophilus* recipient.

Most evidence supports the latter hypothesis. The resistance plasmids found in *H. influenzae* are distinctly different from those found in the majority of enteric bacteria. For example, the guanine-plus-cytosine content of the *Haemophilus* plasmids is 0.39 to 0.40 M, a value close to that of the *Haemophilus* chromosome, but substantially different from those of enteric bacteria. The TnA sequence appears to be the only homologous region shared by both *Haemophilus* and enteric plasmids. Further, significant homology exists between a variety of large resistance plasmids from *Haemophilus* spp., including plasmids which carry different resistance phenotypes. A 27-megadalton phenotypically cryptic plasmid has been identified in a strain of *H. influenzae* (57) which shares 80% homology with the large ampicillin resistance plasmid, but lacks the TnA sequence. It appears that it may represent a "core" plasmid onto which resistance transposons can insert (55).

The large plasmids of *Haemophilus* spp. are conjugative, and it is likely that conjugation represents the most important mechanism for transferral of resistance. Although transformation is known to occur in *Haemophilus* spp., the efficiency of this mechanism of genetic exchange is very low in the absence of homologous plasmid DNA sequences (1). However, the conjugative resistance plasmids are not infrequently integrated into the chromosome (102). Under such circumstances, the efficiency of conjugation is considerably decreased, but the stability of the plasmid is greatly enhanced. Thus, when the plasmid exists as an integrated element, transformation may play a more important role in transfer.

The small resistance plasmids in *Haemophilus* spp. are not capable of conjugative transfer, although they can be mobilized by a conjugative plasmid distinct from the large resistance plasmid (25). The small plasmids are interesting from a number of perspectives. They typically carry approximately 40% of the TnA transposon found on the larger plasmids (56). They occur frequently in *H. parainfluenzae* as well as in *H. influenzae* and several other *Haemophilus* species, including *H. ducreyi* (2). Homology exists between the small resistance plasmids and some of the small, phenotypically cryptic plasmids which have been identified among various species of *Haemophilus*, and a similar origin, i.e., transposition of the TnA sequence onto endogenous plasmids, has been proposed (2, 13). In addition, significant homology

exists between some of the small resistance factors of *Haemophilus* and similar plasmids in *Neisseria gonorrhoeae* (87). The gonococcal plasmids carry the same TnA transposon coding for TEM-1. Their occurrence was reported just after the discovery of ampicillin-resistant *H. influenzae*, and it is likely that they had a common origin.

As increasing numbers of isolates have been studied, there have been a variety of plasmids characterized, suggesting that multiple transformations are occurring over time which alter the genetic character of resistance. Recently, resistance plasmids of a novel type were described in two clinical isolates of *H. influenzae* (82). These plasmids are 10 kilobases in size and do not share homology with any of the other resistance plasmids apart from the TnA sequence. They do, however, share some homology with gonococcal resistance plasmids. The opportunity to track the genetic transformations in these organisms has provided us with an accurate, albeit frightening, example of how easily genes for resistance can move within a susceptible population of bacteria.

Although the TEM-1 beta-lactamase is the most common enzyme associated with ampicillin resistance in *H. influenzae*, another beta-lactamase has been reported (91). This enzyme was also detected as a result of therapeutic failure. It does not react under standard assay conditions with the chromogenic cephalosporin nitrocefin, which is commonly used to detect the TEM-1 beta-lactamase. The newly described enzyme has a different isoelectric focusing point and substrate profile from TEM-1. It is plasmid associated; however, no report as to its transmissibility has been made. The significance of this enzyme is unknown, since there have been no further reports of its occurrence.

The acquisition of the TEM-1 beta-lactamase does not confer the same level of resistance to *H. influenzae* as it does to enteric bacteria, although resistance is clearly within a significant clinical range for the organism. This is probably due to the limited nature of the permeability barrier of the organism in contrast to that of enteric bacteria (22). Both penicillins and cephalosporins are able to diffuse easily across the outer membrane, allowing high concentrations of antibiotic in the periplasmic space. This characteristic has important implications for susceptibility testing, which will be discussed later.

Non-beta-lactamase-producing strains of ampicillin-resistant *H. influenzae* are also known to occur (45, 60, 112). The mechanism for this form of resistance is probably alteration of one or more penicillin-binding proteins (PBPs). PBPs are proteins which are present in bacterial cell membranes and which have the capability of forming covalent bonds with penicillin and related antibiotics. The formation of such bonds is believed to be the event which initiates the bactericidal effect of the beta-lactam antibiotics. Several of these *H. influenzae* strains have been characterized. In all *H. influenzae* isolates studied to date (69, 79, 95), ampicillin resistance is associated with decreased affinity of PBP 5 and variably with decreased affinity of PBP 4.

The genetic basis of this form of resistance is poorly understood. The altered genes are carried on the chromosome and thus are not as readily transmissible as the TnA gene sequence. The genes for the altered PBPs in *H. influenzae* have recently been cloned (59), which should allow a more complete delineation of the mechanisms involved.

It is likely that the alterations arise as a result of mutation which affects the binding site of the proteins. Intrinsic resistance in *Haemophilus* spp. confers resistance to a

number of beta-lactam antibiotics in addition to ampicillin. The antibiotics so affected probably share a common target site with ampicillin. Intrinsic resistance typically imparts only an intermediate level of resistance. PBPs are the enzymes involved in cell wall synthesis, and there is probably a biologic price exacted when mutation occurs. Thus, a major alteration which might generate high-level resistance may be associated with too high a biologic price to the organism.

Prevalence of Ampicillin Resistance

Between the recognition of beta-lactamase-mediated resistance in 1972 to 1974 and today, the prevalence of ampicillin-resistant *H. influenzae* has continued to increase. Most laboratories reported that <5% of their isolates produced beta-lactamase prior to 1978 (122). However, the incidence of beta-lactamase-producing encapsulated strains had increased to 14% by 1981 in Great Britain (81). Ampicillin resistance was reported to occur at a rate of 12.7% among all isolates in Helsinki, Finland, where 5 years earlier no ampicillin resistance had been found (44). A similar increase has been reported in the United States. The incidence of beta-lactamase-producing isolates from blood and cerebrospinal fluid from children in Houston, Tex., rose from 12% in 1977 to 29% in 1979 (62). The incidence increased from 5.3 to 15.6% in Boston, Mass., between 1975 and 1977 for isolates from a variety of clinical sources (105). Perhaps most striking, 4.2% of cerebrospinal fluid isolates recovered in Colorado produced beta-lactamase in 1977, but by 1981 that value had risen to 31.3% (43). In a recent collaborative study, the overall rate of beta-lactamase production was 15.2% among a total of 3,356 clinical isolates obtained from 22 medical centers distributed throughout the United States (29). Twenty-one percent of encapsulated type b isolates produced beta-lactamase. The highest rates of resistance were observed among isolates from infants and young children and from blood and spinal fluid.

The prevalence of ampicillin-resistant non-beta-lactamase-producing *H. influenzae* is unknown. Thornsberry and Kirven reported that 10% (2 of 20) of resistant isolates had this phenotype among strains they evaluated in 1974 (112). In a collaborative study by Doern et al. (29), 4.3% of the isolates tested as resistant to ampicillin, using a disk diffusion susceptibility test method, and as beta-lactamase negative. However, in repeat testing of those isolates which were still available, 6 of 12 were found to produce beta-lactamase. The issue is further complicated since current methods of susceptibility testing may not accurately predict the presence of this type of resistance (68).

Exposure to ampicillin may increase the likelihood of colonization with a resistant strain of *H. influenzae*. Syriopoulou et al. (105) observed that ampicillin-resistant isolates of *H. influenzae* occurred more frequently in ill children who had received prior beta-lactam therapy. Kaplan et al. (51) reported that invasive disease due to ampicillin-resistant *H. influenzae* was twice as likely to occur in children who had received antibiotics within 1 month of admission. A study of colonization rates among healthy day-care children in central Massachusetts demonstrated a significant correlation between the isolation of an ampicillin-resistant strain and treatment with ampicillin or amoxicillin within the previous 3 months (99). This observation may have important implications for the repeated use of these antibiotics to treat respiratory tract infections in children.

Chloramphenicol Resistance: Mechanisms and Genetic Basis

Chloramphenicol-resistant strains of *H. influenzae* were reported shortly after ampicillin resistance due to beta-lactamase production was recorded (53, 117). There rapidly followed reports of strains resistant to both chloramphenicol and ampicillin (70, 97, 116). Chloramphenicol resistance is mediated in most cases by an acetyltransferase which catalyzes the diacetylation of the antibiotic with acetyl coenzyme A (88, 117). The product is no longer active in inhibiting procaryotic protein synthesis.

The genetic sequence which codes for chloramphenicol acetyltransferase (CAT) is a transposon which can insert into the same core plasmid which carries the TnA sequence (55, 88, 117). All strains evaluated are also resistant either to tetracycline alone or to both tetracycline and ampicillin. The genes for resistance are all located on the same R factor.

A second mechanism for resistance to chloramphenicol has also been reported. Chloramphenicol is accumulated in *H. influenzae* via an energy-dependent transport mechanism (16). A two-step process for accumulation has been hypothesized. The first step involves the passage of the antibiotic across the outer membrane through a specific pore, followed by active transport across the cell membrane. Resistance to chloramphenicol has been associated with decreased accumulation of the antibiotic. Using isogenic transformants, Burns et al. (15) demonstrated the simultaneous loss of a 40-kilodalton outer membrane protein with the acquisition of resistance. They postulate that resistance in *H. influenzae* strains which lack CAT is mediated by a change in the relative permeability barrier of the outer membrane to the antibiotic.

The prevalence of chloramphenicol-resistant isolates remains relatively low in most parts of the world. For example, in a collaborative study in the United States by Doern et al. (29), the incidence of chloramphenicol resistance among isolates was only 0.6%. About half of these isolates were also resistant to ampicillin. In a survey of 1,841 strains from 25 laboratories in the United Kingdom, only 1.4% of the isolates were chloramphenicol resistant (81). In this instance, approximately one-third of the strains were also resistant to ampicillin and all of the isolates were resistant to tetracycline.

Perhaps more alarming is a recent series of reports from Barcelona, Spain (17-19), where there has been an explosive increase of multiply resistant strains causing invasive and noninvasive infections. For example, from 1981 to 1984, 60% of *H. influenzae* strains recovered from patients with meningitis were resistant to ampicillin, 65.7% were resistant to chloramphenicol, and 57% were resistant to both. Previous antibiotic therapy did not seem to influence acquisition and subsequent infection with these multiply resistant strains. However, there is also a high rate of asymptomatic colonization in Barcelona and other parts of Spain (19). Since the genes for resistance to both antibiotics are carried on the same plasmid, the possibility of a similar occurrence in other countries always exists.

NEWER ANTIMICROBIAL AGENTS AND THEIR ROLE IN TREATMENT OF *H. INFLUENZAE* INFECTION

With the emergence of resistance to both ampicillin and chloramphenicol, alternative therapy is essential for infection caused by *H. influenzae*. The need for effective antibiotics includes both oral and parenteral alternatives, since most patients with local infections are treated as outpatients.

It is important for the laboratorian to be aware of the specific antibiotics which might be used as alternatives to ampicillin and chloramphenicol, since this knowledge may influence susceptibility test strategies. There are many studies reporting the in vitro susceptibility of resistant *H. influenzae* to a variety of antimicrobial agents (Table 1). However, all of these studies must be viewed carefully, since in vitro testing for this organism is fraught with difficulties. Media, inoculum, and atmosphere of incubation will all influence results significantly, and at present no standard method is universally agreed upon.

There are several alternatives for oral therapy. Amoxicillin combined with the beta-lactamase inhibitor clavulanic acid is active against beta-lactamase-producing strains. However, this combination is not more effective against ampicillin-resistant non-beta-lactamase-producing strains than is ampicillin. Cefaclor has some activity against beta-lactamase-producing strains, but not all isolates are susceptible, and many fall into the moderately susceptible range (26, 98). Susceptibility to this agent should be determined before it is considered for therapeutic use. Erythromycin is somewhat less active than cefaclor (26), and multiple problems exist in testing and interpreting results for this antibiotic. Until these issues are resolved, erythromycin should not be tested against *H. influenzae* in the routine clinical laboratory.

Sulfamethoxazole-trimethoprim has been reported to have good in vitro activity against both ampicillin-susceptible and beta-lactamase-producing strains, but testing must be performed with thymidine-free media containing thymidine phosphorylase for test results to be accurate (26).

Among the newer agents available for parenteral therapy, several of the cephalosporins are the most promising. Ceftriaxone, ceftizoxime, ceftazidime, and cefotaxime are all highly active against resistant strains (3, 7, 96, 100), including non-beta-lactamase-producing isolates (unpublished observations). With experience, one or more of these may even replace ampicillin as the drug of choice for susceptible strains. Cefoxitin, cefamandole, and cefuroxime should not be considered for therapy of serious infection nor should they be routinely tested in the laboratory, primarily because the clinical experience has not paralleled in vitro test results (64).

TABLE 1. Relative activity and inoculum effect of various antibiotics for beta-lactamase-producing strains of *H. influenzae*

Antibiotic	Relative activity ^a	Inoculum effect ^b	Reference(s)
Ampicillin	—	+	67, 106
Cephalothin	—	+	96, 106
Cefamandole	+	+	9, 106, 107
Cefoxitin	+	—	14, 96
Cefaclor	++ ^c	—	26, 98
Amoxicillin/clavulanic acid	++	—	26
Cefoperazone	+	+	14, 96
Ceftriaxone	+++	—	3, 7, 100
Ceftizoxime	+++	—	3, 7, 100
Ceftazidime	+++	—	100
Cefotaxime	+++	—	3, 7, 96, 100
Moxalactam	++	+	14, 96
Aztreonam	++	—	100
Imipenem	+	—	42, 100
Chloramphenicol	++	—	107

^a —, No activity; +, limited activity; ++, moderately active; +++, highly active.

^b —, Absent; +, present.

^c Approximately 10% of strains are resistant (26).

SUSCEPTIBILITY TESTING

The methodology for in vitro susceptibility testing of *H. influenzae* remains controversial. Standard conditions which have been devised for other microorganisms cannot be used because of the relatively fastidious nature of this species. It has been necessary, therefore, to develop modifications of standard methods since the susceptibility of strains is no longer predictable.

A number of factors have been shown to alter test results. These include the basic growth medium used for testing, supplements added to the basic medium, the use of an agar versus broth system, and the inoculum size tested. Studies attempting to define the sources of variation are often difficult to interpret because several of these variables may be operative simultaneously. In addition, there appear to be strain-to-strain differences, and isolates with different mechanisms of resistance are influenced differently by each variable. In the remainder of this review, I will attempt to address each of these issues.

Media and Additives

H. influenzae will not grow on the standard media used for susceptibility testing. Ideally, test conditions should reflect in vivo conditions as closely as possible. The organism should be growing at an optimal rate, and media should be free of substances which might influence test interpretation. Consequently, the first step in developing test methods should be to identify the combinations of supplements and basic media which will yield optimal growth for the majority of isolates with the least interference. There have been more than 30 broth and agar media evaluated for their ability to support the growth of or for susceptibility testing of *H. influenzae* (Table 2). An extensive evaluation of growth media and supplements was recently reported by Bergeron and his colleagues (10). They found that brain heart infusion broth (BHIB), Mueller-Hinton broth (MHB), and tryptic soy broth (TSB) will each allow maximal growth after 24 h of incubation when supplemented with 5% Fildes enrichment. They further compared the minimal inhibitory concentrations (MICs) of eight antibiotics for their beta-lactamase-positive and -negative strains, using the three basal media supplemented with 5% Fildes. Although they were able to demonstrate medium-dependent differences in the endpoints among the antibiotics tested, the majority of the interpretive categorizations remained the same. Exceptions were erythromycin and trimethoprim-sulfamethoxazole. Similar variation was noted by D'Agostino and Tilton (23) when they compared BHIB and Levinthal broth.

There is little information regarding the influence of various supplements on MIC determinations. Bergeron et al. (10) were able to demonstrate a number of significant differences when they evaluated four commonly used supplements tested in MHB. Perhaps the most notable observation was supplement influence on chloramphenicol. The median MICs for the 10 strains tested ranged from <0.06 µg/ml with 1% hemin-1% IsoVitaleX to >128 µg/ml with 5% Fildes. The authors failed to state whether their strains produced CAT. However, major differences in interpretive categorization occurred depending upon the supplement used.

One potential source of medium-related variation is the inherent cloudiness of the medium, which can make reading an endpoint difficult. Mueller-Hinton medium with added supplement C (yeast autolysate and hematin) is a transparent medium which will support the growth of *H. influenzae* and

is simple to prepare. It has been used successfully in broth and agar dilution testing as well as for disk diffusion assays (46, 48). However, it has only been evaluated for differentiating ampicillin-susceptible strains from beta-lactamase-producing strains. The reliability of this medium in predicting resistance to other antimicrobial agents has not been established. In addition, at least one lot of supplement C has been associated with spurious resistance to ampicillin (118).

Another source of variation in MIC interpretation is the formation of stable spheroplasts by some strains of *H. influenzae* upon exposure to cell wall-active antibiotics (11, 86). Formation of spheroplasts gives rise to slight turbidity,

which makes endpoint determination in both broth and agar media difficult. A low-osmolality medium (103) and microscopic determination of endpoints (124) have both been used successfully to eliminate the effect of spheroplast formation with such strains. The role of spheroplasts of *H. influenzae* in disease and response to therapy is unknown, although there are some limited data demonstrating a better relationship between clinical response and an endpoint determination which eliminates spheroplasts (103, 124).

The most recent guidelines from the National Committee for Clinical Laboratory Standards (NCCLS) recommend the use of cation-supplemented MHB with the addition of laked horse blood and nicotinamide adenine dinucleotide (NAD) for dilution testing of *H. influenzae* (73) and Mueller-Hinton agar with 1% hemoglobin or 5% horse blood and a factor X and V supplement for disk diffusion testing (74). The effect of these additives has not been thoroughly evaluated. The disk diffusion assay has been shown to accurately predict resistance of beta-lactamase-producing strains to ampicillin and penicillin and chloramphenicol resistance in CAT-producing strains.

Perhaps the most promising medium for susceptibility testing of *H. influenzae* has recently been reported by Jorgensen et al. (49). The growth medium, referred to as *Haemophilus* test medium by the authors, is composed of an MHB or Mueller-Hinton agar (MHA) base to which bovine hematin, yeast extract, and beta-NAD is added. This medium yields optimal growth within 8 h of inoculation and is comparable to other media that have been recommended. *Haemophilus* test medium is optically clear and endpoint determinations can be made easily. The comparison between *Haemophilus* test medium and the media currently recommended by the NCCLS for broth dilution, agar dilution, and disk diffusion susceptibility testing was favorable for the 10 antibiotics evaluated.

Effect of Inoculum Size

Although many bacteria exhibit an inoculum effect when tested for antimicrobial susceptibility, results for beta-lactamase-producing strains of *H. influenzae* seem to be particularly influenced by the inoculum used. This rather dramatic effect upon endpoint determination when increasing numbers of organisms are tested was first documented by Medeiros and O'Brien in 1975 (67). They noted that, although ampicillin resistance could be accurately predicted for beta-lactamase-producing strains when an inoculum of 10^4 colony-forming units (CFU)/ml was used, resistance was of a much lower order than that seen for enteric bacteria with the same TEM-1 enzyme present. Increasing the inoculum size to 10^6 CFU/ml significantly increased the MIC of ampicillin for the resistant strains. This observation was largely overlooked until Smith and his colleagues again documented the effect of inoculum size on susceptibility testing in 1979 (106).

The reason for this exaggerated inoculum effect in *H. influenzae* is very likely linked to the permeability of the organism. Unlike the enteric bacteria, the outer membrane of *H. influenzae* is relatively permeable. Consequently, beta-lactamase produced by the organism may not be retained in the periplasmic space as it is in enteric bacteria and antibiotic may diffuse through the outer membrane more easily. It has been hypothesized that a critical cell mass is necessary to overcome this permeation in sufficient time to inactivate antibiotic (67) and thus allow resistance to be expressed.

TABLE 2. Media for growth of *H. influenzae*^a

Medium ^b	Reference(s)
Broth	
MHB + 10% Fildes	111, 123
MHB + 5% Fildes	30, 110
MHB + 5% Fildes + 1% supplement C	98
MHB + 5% supplement C	47, 67
MHB + 2.5% supplement C	46
MHB + 2% hemoglobin + 1% IsoVitaleX	30
MHB + 1% supplement B	77
MHB + 5% lysed horse blood + 0.25% NAD	54
BHIB + 5% Fildes	37, 46
BHIB + 2.5% supplement C	39
BHIB + 1/1000 (1 mg per ml) of NAD + 33% horse blood	78
BHIB + 1/300 (1 mg per ml) of NAD + 33% horse blood	101
BHIB + 5% horse erythrocytes	47, 66, 92
BHIB + 2% defibrinated sheep blood	115
BHIB + 1% hemin + 0.2% NAD	94
BHIB + 1% hemin + 2% IsoVitaleX + 1% L-histidine	65
BHIB + 0.8% hemin + 0.5% NAD	38
TSB + 10% Fildes	123
TSB + 5% Fildes	65
TSB + 5% rabbit blood + 1% IsoVitaleX	65, 75
Dextrose phosphate broth (1 part Fildes + 1 part 20% yeast extract)	103
Schaedler broth + 5% Fildes	111
GC broth + 1% supplement B	77
Agar	
MHA + 1% IsoVitaleX + 5% hemoglobin	30, 110
MHA + 1% IsoVitaleX + 2% hemoglobin	72
MHA + 1% IsoVitaleX + 1% hemoglobin	8, 9
MHA + 1% IsoVitaleX + 5% cooked rabbit blood	40
MHA + 5% Fildes + supplement C	98
MHA + 2% Fildes	83
MHA + 5% supplement C or horse blood	5
MHA + 5% sheep blood	30
BHIA + 5% horse erythrocytes (Levinthal)	6, 36, 61, 66, 115
TSA + 2% Fildes	123
TSA + 1% hemoglobin + 1% IsoVitaleX	34
GC agar base + 2% hemoglobin + 1% IsoVitaleX	47, 30
ASM medium + 2% IsoVitaleX + 8% rabbit blood	75

^a Adapted from Bergeron et al. (10).

^b BHIA, brain heart infusion agar; TSA, tryptic soy agar.

The clinical significance of this in vitro phenomenon remains somewhat controversial. However, the number of bacteria in the cerebrospinal fluid of children with meningitis typically reaches between 10^5 and 10^7 CFU/ml (35). Some clinical data support the use of a higher inoculum for susceptibility testing. For example, cefamandole appears to be relatively active against beta-lactamase-producing strains of *H. influenzae* when the inoculum size used in testing is 10^4 CFU/ml (110). When the inoculum size is increased to 10^6 CFU/ml, most strains test as resistant (9, 106, 107). Early clinical trials with cefamandole yielded treatment failures when beta-lactamase-producing strains were isolated (5). Similar experience has been noted with other antibiotics which behave in the same manner. It thus seems wise to use a higher inoculum density, particularly when testing beta-lactamase-producing strains.

Inoculum is not as significant in testing beta-lactamase-negative, ampicillin-susceptible strains of *H. influenzae*. However, little has been documented regarding beta-lactamase-negative, ampicillin-resistant isolates. The limited evidence which exists suggests that these isolates are less influenced by inoculum than are the beta-lactamase-producing strains (68). Results may vary sufficiently to warrant the testing of a higher inoculum.

Not all antibiotics are influenced by varying the test inoculum. Table 1 summarizes antibiotic activity of a number of alternative agents against beta-lactamase-producing strains and indicates whether an inoculum effect has been observed. Chloramphenicol is among the antibiotics for which inoculum does not influence results. In addition, several of the newer cephalosporins are highly active when tested against both low and high inoculum densities.

Test Methods

A surprisingly limited number of studies have been reported documenting the comparability of different methods for susceptibility testing of *H. influenzae*. Broth macrodilution testing should be considered the standard. However, this method is cumbersome and not generally applicable to use in the clinical laboratory. Jorgensen and his colleagues (46-48) have provided a valuable series of comparisons of various methods, using Mueller-Hinton medium with added supplement C. Their results suggest that microdilution compares favorably with macrodilution as well as with agar dilution. However, it must be noted again that a limited number of antibiotics (ampicillin and chloramphenicol) were tested and that all of their resistant strains were enzyme producing. Bergeron et al. (9) compared agar dilution and microdilution testing using brain heart infusion agar or BHIB supplemented with 1% IsoVitaleX (BBL Microbiology Systems) and 1% hemin. Results between the two methods were comparable for ampicillin, chloramphenicol, cefamandole, and moxalactam. On the other hand, D'Agostino and Tilton (23) compared microdilution and agar dilution, using brain heart infusion medium supplemented with 5% Fildes. They reported significant variation in results for ampicillin, chloramphenicol, and gentamicin between the two methods, although high-level resistance to ampicillin was accurately predicted by both methods.

The use of disk diffusion testing for *H. influenzae* is appealing because of its simplicity. However, reliability of zone-interpretive criteria for *H. influenzae* is controversial. The NCCLS recommends that strains tested by their standardized method with zone diameters of ≤ 19 mm surrounding 10- μ g ampicillin disks be considered resistant. Those

with zone diameters of >19 mm should be considered susceptible (74). Although several investigators have validated this breakpoint for ampicillin-susceptible and beta-lactamase-producing resistant strains (28, 48), the categorization of ampicillin resistance in non-beta-lactamase-producing strains is less satisfactory (49, 68, 80).

Mendelman et al. (68) reported that 8 of 18 ampicillin-resistant strains which lacked beta-lactamase were classified as falsely susceptible by the NCCLS guidelines. They proposed the use of a 2- μ g ampicillin and 2- μ g ampicillin/10- μ g clavulanic acid disk as an alternative method for classifying resistant and susceptible strains.

Jorgensen and his colleagues (49) have suggested a modification of the NCCLS zone-interpretive criteria for ampicillin. Based upon error rate-bounded analysis of MICs and zone diameters for a large series of recent isolates, they propose that strains with a zone of inhibition of ≥ 22 mm around a 10- μ g ampicillin disk be considered susceptible and those with <22 -mm zones be designated resistant. The use of 10- μ g cloxacillin disks has also been reported to separate isolates exhibiting one of the two mechanisms of resistance (80). However, susceptible strains are more frequently identified as resistant by this method than is desirable.

For the present, it seems advisable to conform to the standardized method outlined by the NCCLS for susceptibility testing of ampicillin, including the recommended zone-interpretive criteria. *H. influenzae* isolates recovered from sterile body fluids and which fall within 2 to 3 mm of the NCCLS breakpoint probably warrant dilution testing. A beta-lactamase test should be included in the evaluation of susceptibility to ampicillin, and any strain which yields a positive test result should be designated resistant.

The NCCLS zone-interpretive criteria for chloramphenicol are the same as those used for nonfastidious rapidly growing bacteria (i.e., ≤ 12 mm = resistant and ≥ 18 mm = susceptible). At least two recent studies have questioned the validity of these breakpoints. Doern et al. (27) and Jorgensen et al. (49) both recommend that the interpretive criteria be changed for *H. influenzae* such that isolates with a zone diameter of <25 mm would be classified as resistant. It would appear that the MIC/zone diameter comparisons reported from each group were generated by using the same collection of isolates. Their conclusion, nonetheless, seems valid.

NCCLS guidelines (74) state that *H. influenzae* can be tested against a number of additional antibiotics and that the zone interpretive criteria established for rapidly growing bacteria can be applied. In many cases, only susceptible strains have been available for comparison, and it is unknown whether resistance would be accurately predicted by using the NCCLS recommendations. Further, Jorgensen's results (49) suggest the need for modification of zone-interpretive criteria for amoxicillin-clavulanate, tetracycline, and cefaclor, in addition to ampicillin and chloramphenicol. For the present, disk diffusion testing should be limited to antibiotics for which zone interpretive criteria have been established specifically for *H. influenzae*.

Certain antibiotics should not be tested by disk diffusion methods until better guidelines have been established. For example, the NCCLS zone-interpretive criteria for erythromycin are clearly inadequate for *H. influenzae* (74), and medium variation still represents a significant problem in testing sulfamethoxazole. As a final caveat, zone-interpretive criteria recommended by the NCCLS cannot be applied when media other than the Mueller-Hinton-based agar are used in the disk diffusion assay.

DIRECT METHODS FOR DETECTING AMPICILLIN AND CHLORAMPHENICOL RESISTANCE

With the development of resistant strains came the need to quickly predict ampicillin and chloramphenicol resistance. Several methods have been described for detection of both beta-lactamase and CAT. All of these methods rely upon direct testing of the organism once it has been recovered in culture.

TEM-1 beta-lactamase can be reliably detected by three techniques. The first of these to be described adapted an acidometric capillary tube method which had been used successfully for detecting beta-lactamase production by *Staphylococcus aureus* (89). The method, which was modified slightly by Thornsberry and Kirven (112), is based upon the detection of a pH change which occurs when penicillin is hydrolyzed to penicilloic acid by the enzyme. Although the penicillin solution must be freshly made for this assay, it has been validated by a number of investigators (33, 93, 119) and offers an accurate and relatively convenient method for detecting beta-lactamase production.

A second approach uses an iodometric assay for the presence of the enzyme (20, 58, 90). This assay is based upon the fact that the intact penicillin molecule does not bind iodine, but penicilloic acid, the product of enzyme cleavage, does. A starch indicator, which will combine with free iodine and yield a dark purple color, is used to detect the presence of iodine which has not reacted with penicilloic acid. Thus, the absence of color formation is indicative of a positive test. This method has been adapted to a filter paper technique (58).

A novel chromogenic cephalosporin substrate called nitrocefin has also been used for detection of beta-lactamase activity in *H. influenzae*. The antibiotic was described in 1972 by O'Callaghan and co-workers (76). It has a highly reactive beta-lactam ring which is readily hydrolyzed by a variety of beta-lactamases. In its active form, nitrocefin is bright yellow. Upon cleavage of the beta-lactam ring, it turns red. This approach will accurately predict the presence of the TEM-1 beta-lactamase in *H. influenzae* (50, 71). Nitrocefin testing has been applied directly to spinal fluid of patients suffering from *H. influenzae* meningitis (12). Although a negative test result cannot be used to assure susceptibility of the infecting strain, a positive test appears to be a highly reliable indicator of infection with a beta-lactamase-producing organism.

No assays are as convenient for the detection of CAT, although at least three methods have been described. One assay relies upon the spectrophotometric detection of coenzyme A, which is generated as the end product of the acetyltransferase reaction (88). Although reliable, this method takes approximately 2 days to complete and requires a spectrophotometer and highly trained personnel. A colorimetric assay has been described (4) which eliminates the need for specialized equipment and can be completed within approximately 1 h. However, this assay also requires a series of relatively complicated reagents and does not offer a suitably practical alternative to the clinical laboratory. A commercially available reagent-impregnated disk test for detecting CAT is also available. Although simple to perform, this screening test should not be used in its current configuration. It has been demonstrated to generate numerous false-negative and equivocal results (27).

Finally, it must be remembered that these assays will only identify strains whose mechanism of resistance involves an enzyme. Ampicillin- and chloramphenicol-resistant strains have been described which do not rely upon an enzymatic

mechanism, and these strains would be negative in all of the assays described above. Thus, a positive result can be regarded as highly accurate in predicting resistance, but a negative result cannot be viewed with the same assurance in predicting susceptibility.

Susceptibility testing for *H. influenzae* must be approached thoughtfully if it is to be routinely performed by the clinical laboratory. Beta-lactamase-mediated ampicillin resistance and CAT-mediated chloramphenicol resistance are accurately predicted by a variety of methods including direct testing. For other antibiotics and other mechanisms of resistance, the reliability of various methods, media, and inocula is far less clear. The inoculum effect must be remembered, and the inclusion of a high inoculum density (5×10^5 to 1×10^6 CFU/ml) should be considered. Should the laboratory elect to provide testing, it is imperative that quality control be performed with strains which represent both beta-lactamase- and non-beta-lactamase-mediated ampicillin resistance. As more experience is gained with the nonenzymatic mechanisms of resistance, our current approach may require revision.

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