

REVIEW ARTICLE

Antimicrobial resistance in *Haemophilus influenzae*

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Since the early 1970s, recognition of the increasing prevalence of resistance to several antimicrobial agents among clinical isolates of *Haemophilus influenzae* in many countries has brought about changes in prescribing practices. Direct comparison of susceptibility test results from various centres cannot be made without consideration of the factors known to have a crucial effect on the apparent in-vitro resistance of this species to many antimicrobial agents. These include the medium, antibiotic concentration in the disk and, particularly, the size of inoculum used. The variety of zone diameters and minimum inhibitory concentrations (MICs) employed as susceptibility breakpoints in different studies reflects not only the range of methods used but also the lack of agreement on the relationship of in-vitro results to in-vivo efficacy of an antimicrobial agent against *H. influenzae*.

Despite the problems of interpretation of results, it has become clear that isolates of *H. influenzae* of Pittman (capsule) type b merit separate consideration because of their propensity to cause invasive infections. Several national studies have reported a consistently higher prevalence of β -lactamase production among isolates of type b when compared with non-capsulate *H. influenzae* (Doern *et al.*, 1986; Powell *et al.*, 1987; Machka *et al.*, 1988) and some have found that type-b isolates are more likely to be resistant to other antimicrobial agents (Dabernat *et al.*, 1986). Although they are responsible for most cases of invasive disease, this difference is also seen between type-b isolates producing non-invasive disease and the non-capsulate organisms more commonly responsible for less serious infections. Hence, differentiation of type-b isolates from those which are non-capsulate or of other capsule types is an important consideration in any study of the prevalence and nature of antimicrobial resistance in this species and is crucial in the interpretation of the prevalence data.

Resistance to β -lactams

H. influenzae type-b infections which failed to respond to ampicillin therapy prompted some of the earliest reports of resistance to antimicrobial agents in use against this species (Gunn *et al.*, 1974). The prevalence of β -lactamase-producing *H. influenzae* identified from United Kingdom surveys rose from 1.6% in 1977 (Howard *et al.*, 1978) to 18% amongst type-b isolates and 6.2% overall in 1986 (Powell *et al.*, 1987). In Europe, β -lactamase production is most common among type-b (64%) and non-capsulate (26%) Spanish isolates (Machka *et al.*, 1988). Spain has a particular problem in the Barcelona area where 57% of type-b isolates responsible for meningitis are β -lactamase positive and also resistant to chloramphenicol (Campos *et al.*, 1986). A multi-centre study in the USA reported that, overall, 32% of type-b and 16% of non-capsulate clinical isolates were β -lactamase positive (Doern *et al.*, 1988) but noted a geographical variation in prevalence among type-b isolates from <10% to >90%.

Medeiros and O'Brien (1975) established that the enzymes present in some early β -lactamase producers were TEM-like in character. De Graaff *et al.* (1976) found that the transposon coding for β -lactamase production could be located on either large or small plasmids of mol. wts (10^6) 30 and 3 respectively, which were otherwise unrelated in their nucleotide sequences. Several workers, however, have demonstrated that the genes encoding β -lactamase and resistance to several other antimicrobial agents in *H. influenzae* are not always situated on extrachromosomal DNA. Stuy (1980) found that isolates with several different resistance patterns were frequently free of detectable plasmids. Murphy-Corb *et al.* (1984) failed to detect extrachromosomal DNA in 43 of 66 β -lactamase-producing isolates of *H. influenzae*. The plasmid-free isolates conjugally transferred resistance to an ampicillin-susceptible recipient at a lower frequency than their plasmid-containing counterparts but offspring of

both types of donor contained extrachromosomal DNA as single bands (mol. wt 30×10^6). By restriction-enzyme analysis and Southern-hybridisation techniques, entire plasmid sequences were detected within the chromosome of four plasmid-free β -lactamase-producing type-b isolates. Mendelman *et al.* (1985) also found that whereas most (29 of 36) Alaskan β -lactamase-producing *H. influenzae* isolates of type b were plasmid-free, many of the resistant transconjugants obtained harboured extrachromosomal DNA.

The demonstration by Roberts and Smith (1980) that large plasmids in *H. influenzae* are easily overlooked if a single method of lysis is used raised worries that plasmid DNA sometimes co-migrated with chromosomal DNA giving an alternative explanation for probe hybridisations with chromosomal bands. However, Mendelman *et al.* (1985) were able to show in their study, and during an investigation of ampicillin- and chloramphenicol-resistant isolates (Mendelman *et al.*, 1984a), that plasmid probes bearing specific R-determinants hybridised with both the chromosomal and extrachromosomal DNA of those isolates of *H. influenzae* found to contain plasmids.

Whilst the mechanism of cell-to-cell transfer of chromosomal gene sequences coding for β -lactamase production and resistance to other antimicrobial agents has not been fully elucidated, transformation of *H. influenzae* by haemophilus DNA has been shown to be an efficient process involving uptake of transforming DNA into cell-surface membranous structures (transformasomes) which afford some degree of protection against enzymic degradation (Barany and Kahn, 1985). Thus, a hypothesis that implicates transformation in cell-to-cell transfer of chromosomal gene sequences is attractive but it is not known to what extent this process occurs *in vivo*.

Production of a TEM-1 β -lactamase is the predominant, but not the sole, mechanism of ampicillin resistance in *H. influenzae*. In 1981, Rubin *et al.* reported the presence of a novel β -lactamase, later called ROB-1, in a type-b isolate causing meningitis. The enzyme was not detected by the initial cell-suspension chromogenic-cephalosporin assay but did produce a positive result when the test was repeated with a very heavy inoculum. Iso-electric focussing demonstrated that ROB-1 had a very different pI (8.1) from that of TEM-1 (5.4) and slightly different β -lactam-hydrolysis rates. Production of the novel enzyme was lost after curing a similar-sized plasmid identified in two isolates from the patient's blood and cerebrospinal fluid. A possible source for ROB-

1 was suggested when Medeiros *et al.* (1986) discovered that ampicillin-resistant *H. pleuropneumoniae*, a swine pathogen, also produced ROB-1 and that the plasmids encoding the enzyme in both *Haemophilus* species were closely homologous.

Early reports of resistance to ampicillin that was not mediated by β -lactamase production included that found in several non-capsulate isolates from respiratory specimens (Bell and Plowman, 1980) and in sporadically occurring type-b isolates (Markowitz, 1980; Offit *et al.*, 1982). In a survey in the UK in 1986 (Powell *et al.*, 1987) 100 isolates (4.1%) were identified that showed reduced zone diameters (< 20 mm) to 2- μ g ampicillin disks and required ampicillin concentrations between 1 and 64 mg/L for inhibition. These isolates appear to be generally uncommon in Europe (Machka *et al.*, 1988) and the USA (Doern *et al.*, 1988), but it is not possible to gauge accurately the worldwide prevalence of this non-lactamase-mediated ("intrinsic") resistance because of the problems which have become apparent in its detection. Establishment of a resistance breakpoint for these isolates must be referenced to susceptibility-testing methods adopted and is complicated by the observation that they rarely require more ampicillin than 4 mg/L for inhibition (Powell and Williams, 1988).

Attempts to identify the mechanism responsible for this type of resistance have concentrated on differences in penicillin-binding and outer-membrane proteins (PBPs and OMPs) between these isolates and those susceptible to ampicillin. PBP changes noted in a type-b isolate studied by Parr and Bryan (1984) and in transformants derived from four non-capsulate isolates investigated by Mendelman *et al.* (1984b) suggested that such differences played an important role in non-lactamase-mediated resistance. A chromosomal location for the genes determining resistance was postulated. It is unfortunate that interpretation of PBP studies is complicated by the natural variation seen in *H. influenzae*. Whilst both Makover *et al.* (1981) and Serfass *et al.* (1986) identified eight major PBPs in this species, the latter study demonstrated a considerable variation in profiles among ampicillin-susceptible isolates. These differences were even more pronounced among those with non-lactamase-mediated resistance although a strong association with a change in PBP 5 was noted.

Outer-membrane protein profiles of both type-b and non-capsulate isolates have proved to be even more diverse (Loeb and Smith, 1980). Although Reid *et al.* (1987) have recently documented profile differences between ampicillin-susceptible and am-

Table I. Modal MICs of β -lactamase-stable antimicrobial agents against *H. influenzae*

Antimicrobial agent	Modal MIC for 2458 isolates (mg/L)
Amoxycillin and clavulanic acid (2:1)	0.5
Cefaclor	4.0
Cefixime	0.03
Aztreonam	0.06
Imipenem	1.0

picillin-resistant, β -lactamase-negative isolates of the same biotype, no specific protein change has been implicated as being responsible for this phenomenon. Indeed, the variable reduction in susceptibility of isolates of this type (Powell and Williams, 1987a, b) (see tables I and II) to other β -lactams supports the conclusion, inferred by the investigations described, that several different mechanisms may be involved. In addition, the degree of genetic and phenotypic diversity among β -lactamase-negative, ampicillin-resistant, nontypable *H. influenzae*, based on results from multilocus enzyme electrophoresis, auxotroph testing and PBP profiles, has excluded the possibility of a single recent origin for these isolates (Mendelman *et al.*, 1987).

Since the advent of ampicillin resistance in *H. influenzae*, many β -lactamase-stable compounds have proved to be highly active *in vitro* and *in vivo* against ampicillin-susceptible and resistant β -lactamase-positive organisms. The implications of the

finding that ampicillin-resistant, β -lactamase-negative isolates show reduced susceptibility to many of these compounds have yet to be evaluated. The reduction in susceptibility observed is rarely of such a degree that these isolates can be considered resistant on in-vitro testing according to the criteria established for any one agent (Powell and Williams, 1988). Whether differences between the modal MICs of various β -lactams among these isolates and the corresponding modal values for the ampicillin-susceptible population are sufficient to give rise to treatment failures will be most crucial when these agents are used for treatment of infections at sites where their penetration is unreliable or poor.

Chloramphenicol

The rising prevalence of ampicillin resistance, particularly among type-b isolates, promoted the use of chloramphenicol as first-line therapy of invasive *H. influenzae* disease. Although resistance to chloramphenicol has been reported quite frequently since the mid-1970s in both type-b and non-capsulate isolates, its prevalence is still low in many countries. Doern *et al.* (1986) reported resistance in <1% of isolates in the USA, Powell *et al.* (1987) in <2% of isolates in the UK and Machka *et al.* (1988) in <2% of isolates in most European countries with the exception of Belgium (11% in type-b and non-b isolates) and Spain (41% in type b, 22% in non-b isolates).

Early investigations established that resistance to chloramphenicol was almost invariably accom-

Table II. Reduction in susceptibility to β -lactamase-stable agents and relationship to susceptibility to ampicillin in *H. influenzae*

Category (n = number of isolates)	Percentage of isolates requiring an antimicrobial concentration ≥ 2 doubling dilutions above the modal MICs of				
	amoxycillin + clavulanic acid	cefaclor	cefixime	aztreonam	imipenem
Ampicillin-susceptible (n = 2201)	0.2	1	12	2.5	0.3
Ampicillin-resistant, β -lac- tamase-positive (n = 157)	2	2	7	2.5	3
β -Lactamase-negative, * ampicillin MIC 1–2 mg/L (n = 62)	37	40	42	44	8
β -Lactamase-negative, am- picillin MIC ≥ 4 mg/L (n = 38)	87	58	74	68	8

*Reduced susceptibility to ampicillin as indicated by zone diameter <20 mm to 2 μ g ampicillin-containing disk.

panied by tetracycline resistance and both R-determinants were transferred by large plasmids of mol. wt (10^6) 34–46 (van Klinger *et al.*, 1977; Roberts *et al.*, 1980). Roberts *et al.* (1980, 1982) determined that all chloramphenicol acetyltransferases (CATs) mediating resistance in *H. influenzae* collected from many countries resembled the type-II CATs of enterobacteria in their sensitivity to inhibition by 5,5'-dithiobis-2-nitrobenzoate (DTNB) but not all CAT-producing strains of *H. influenzae* possess detectable extrachromosomal DNA. The work of Mendelman *et al.* (1984a) already described provided supportive evidence for frequent chromosomal integration of the chloramphenicol R-determinant. Examination of 42 chloramphenicol-resistant isolates collected during a survey in the UK in 1986 established that, although all of them produced a type II-like CAT constitutively, only five had visually-detectable extrachromosomal DNA as single plasmids (mol. wt 40×10^6), despite the application of three different methods of plasmid extraction to eight isolates (Powell and Livermore, 1988). Moreover, ethidium bromide-curing of plasmids in two isolates originally found to contain extrachromosomal DNA did not affect their original resistance patterns, suggesting that chromosomal integration of the CAT genes is a common phenomenon among UK isolates.

In 1985, Burns *et al.* described four non-capsulate isolates of *H. influenzae* from blood and sputum that did not produce CAT but required chloramphenicol concentrations of ≥ 20 mg/L for inhibition. A decreased rate of chloramphenicol uptake by resistant isolates was demonstrated and, after outer membrane-protein analyses, it was suggested that marked reduction in one protein (mol. wt 40×10^3), shown to function as a porin by Vachon *et al.* (1985), might correlate with chloramphenicol resistance mediated by decreased permeability. Isolates of this type have not been reported from the UK and their prevalence is unknown.

Tetracycline

Tetracycline resistance remains at $<3\%$ in the UK (Powell *et al.*, 1987). Although resistance to tetracycline may occur without resistance to chloramphenicol, a high prevalence is generally seen only in those areas where the latter is high. Jahn *et al.* (1979) found that production and carriage of more than one copy on a plasmid of the transposon encoding resistance to tetracycline followed exposure to this antibiotic and correlated with a higher MIC for both tetracycline and chloramphenicol. The results of hybridisation studies indicated that

the transposon determining tetracycline resistance served as a carrier for the CAT gene and in some isolates the transposon for β -lactamase production had become integrated in a similar fashion.

Marshall *et al.* (1984) reported that 35 of 40 isolates of *H. influenzae* examined showed transferable plasmid-mediated resistance to tetracycline. Hybridisation occurred between a class-B tetracycline-resistance determinant probe, generally found on transposon Tn10, and all 35 isolates which could transfer their resistance as well as two which possessed a non-transferable R-determinant. In contrast with the inducible resistance found in other species carrying Tn10, these isolates of *H. influenzae* showed only constitutive expression of resistance. The same phenomenon has also been observed in *H. parainfluenzae* carrying Tn10 (Levy *et al.*, 1984) and in which it was ascribed to lack of an active repressor molecule.

The remaining three of the 40 isolates examined possessed non-transferable tetracycline resistance and failed to hybridise with any of the four probes derived from other gram-negative organisms. Two of these were also unusual in showing amplification of resistance after incubation with increasing concentrations of tetracycline. Hence, several mechanisms may mediate resistance to tetracycline in this species and studies already described have suggested that there may be frequent integration of the R-determinant in the chromosome.

Anti-folate agents

The prevalence of resistance to trimethoprim in the UK reached 4.2% in 1986 (Powell *et al.*, 1987). Few international studies have reported on resistance to trimethoprim not tested in combination with sulphamethoxazole. Campos and Garcia-Tornel (1987) found that 55% of 83 capsulate and non-capsulate isolates from Spanish children were resistant to five antimicrobial agents including trimethoprim, indicating the presence of a major problem. Prevalence figures, however, for resistance to trimethoprim in combination with sulphamethoxazole from other countries suggest that the Spanish experience is most unusual (Doern *et al.*, 1988).

Kirven and Thornsberry (1978), using a microdilution-broth technique for MIC determination, reported that isolates were either killed at concentrations of trimethoprim near the MIC or were not killed at any concentration tested. When trimethoprim was bactericidal, the combination of trimethoprim and sulphamethoxazole (TMP-SMZ) was also always bactericidal. They also noted a correla-

tion between in-vitro bactericidal activity of sulphamethoxazole and its previously documented efficacy in eliminating nasopharyngeal carriage of some of the type-b isolates studied. Yogeve and Moxon (1982) reported that type-b isolates which were not killed on exposure to TMP-SMZ elaborated significantly more type-b polysaccharide material than organisms sensitive to killing. After removal of the polysaccharide by physical methods, isolates were more easily killed by TMP-SMZ but reverted to their original phenotype after growth which allowed re-accumulation of capsular material.

Although both co-trimoxazole and trimethoprim alone are still used for non-invasive *H. influenzae* infections, particularly in patients hypersensitive to ampicillin, little is known about the mechanisms of resistance to these agents in this species. Since resistance to other antimicrobials documented in *H. influenzae* is frequently mediated by mechanisms which have much in common with those found in the enterobacteria, it has been widely presumed that the anti-folate agents are no exception. However, de Groot *et al.* (1988) have recently examined 10 non-capsulate trimethoprim-resistant isolates, none of which contained visually detectable extra-chromosomal DNA. A DNA probe constructed from a subclone of the trimethoprim R-determinant of one isolate hybridised with the chromosomal DNA of both trimethoprim-sensitive and -resistant *H. influenzae* but not with *Escherichia coli* containing genes coding for dihydrofolate reductases (DHFRs) 1, 2 and 3 or with trimethoprim-resistant strains of *Neisseria*. From an observation that DHFR activity was significantly higher in a trimethoprim-resistant strain than in an isogenic sensitive counterpart, it was suggested that resistance was associated with an overproduction of DHFR—a mechanism occasionally observed amongst enterobacteria (Steen and Skold, 1985). Further elucidation of these observations is awaited.

Erythromycin

Current prevalence of erythromycin resistance in the UK is unknown but with the NCCLS (National Committee for Clinical Laboratory Standards) breakpoint of 8 mg/L, a recent study in the USA reported that 50% of isolates were resistant (Doern *et al.*, 1988) compared with only 6% of French isolates examined by Dabernat *et al.* in 1986. Machka *et al.* (1988) found a variation across Europe from 27% in the Netherlands to 1% in both Austria and the UK.

Fernandes *et al.* (1987) reported that, with

0.5 mg/L and 8 mg/L as susceptible and resistant breakpoints, respectively, in-vitro MIC values correlated well with successful treatment of septicaemia in a mouse model but only when certain media were used and plates were incubated in air. It is clear that the extrapolation of in-vitro susceptibility to the likely clinical efficacy of erythromycin against *H. influenzae* is not straightforward and the usefulness of erythromycin is somewhat dubious.

Rifampicin

Opinion remains divided as to the role of rifampicin in prophylaxis against type-b infections among close contacts of an index case (Granoff *et al.*, 1979; Eskola *et al.*, 1987). Nicolle *et al.* (1982) documented a high prevalence of rifampicin-resistant non-capsulate *H. influenzae* isolated from subjects after prophylaxis and suggested that their failure to find similar type-b isolates was due solely to the overall low prevalence of these strains in their specimens. Doern *et al.* (1988) found that 1% of USA isolates were resistant to rifampicin and Campos and Garcia-Tornel (1987) identified only one such isolate among 83 isolates resistant to ampicillin and chloramphenicol. A high frequency of mutation *in-vitro* to rifampicin resistance was reported among type-b isolates by Mendelman *et al.* (1982) who suggested that the variable degree of resistance observed might indicate that more than one mechanism was involved.

4-Quinolones

The 4-quinolones will undoubtedly be used increasingly for both invasive and non-invasive *H. influenzae* infections and may be of particular use in areas where multi-resistance is a problem. Ciprofloxacin appears to be highly active *in vitro* and effective in respiratory infections where *H. influenzae* is the predominant organism in the sputum (Raoof *et al.*, 1986). Resistance to 4-quinolones is not a problem at present.

Multi-resistant isolates

Type-b and non-capsulate isolates of *H. influenzae* resistant to four or more antimicrobial agents have been reported from many countries but are generally uncommon (0.6% of isolates in the UK survey in 1986). Where multiply-resistant type-b isolates are a local problem, they do not appear to constitute a single clone. The Spanish isolates studied by Campos *et al.* (1987) collected from index cases of

meningitis and carriers in four day-care centres during 1985, varied in OMP and plasmid restriction-endonuclease profiles.

Prospects

Despite the existence of resistance problems, a considerable choice of antimicrobial agents is still available for treatment of the majority of invasive and non-invasive *H. influenzae* infections. Nevertheless, a vaccine which would effectively prevent invasive disease by type-b strains in the age group

most at risk (< 18 months) would circumvent many therapeutic problems arising from antimicrobial resistance amongst type-b isolates (Granoff and Munson, 1986). Meanwhile, the expanding range of orally administered compounds, will facilitate treatment of non-invasive infections caused by type-b and non-capsulate isolates of *H. influenzae* and some of the newer injectable compounds will probably prove to be of particular value for serious infections caused by isolates resistant to many of the previously available antimicrobial agents.

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