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Emergence and prevalence of β-lactamase producing *Haemophilus influenzae* in Finland and susceptibility of 102 respiratory isolates to eight antibiotics

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A survey of 102 consecutive clinical isolates of *Haemophilus influenzae* mainly from otolaryngological patients revealed 13 ampicillin resistant ones, while 2 years earlier none were found. All the 13 strains which were resistant according to the broth dilution minimal inhibitory concentration (MIC) could be shown to produce β-lactamase using the chromogenic cephalosporin 87/312. Routine disc diffusion susceptibility testing had under-estimated the resistance and five of the β-lactamase producing strains had been reported as ampicillin susceptible. Amoxycillin and azidocillin were equally active and slightly less active than ampicillin against β-lactamase negative strains. The 13 ampicillin resistant strains were also resistant to amoxycillin and azidocillin, and also the MICs of cephalothin and cephalexin were significantly increased by β-lactamase production, while cefuroxime, erythromycin and chloramphenicol were not affected. A marked inoculum effect was demonstrated with the β-lactam antibiotics, and only a narrow (about tenfold) range of inoculum concentrations distinguished reliably between susceptible and resistant strains. The MICs of the penicillins against β-lactamase positive *H. influenzae* increased markedly with incubation time.

Introduction

Ampicillin resistant *Haemophilus influenzae* type b was first described in several reports in 1974 (Clymo & Harper, 1974; Khan *et al.*, 1974; Schiffer *et al.*, 1974; Thomas *et al.*, 1974; Tomeh *et al.*, 1974; Turk, 1974; Williams & Cavanagh, 1974) and it has since been observed with increasing prevalence in various parts of the world (Howard, Hince & Williams, 1978; Syriopoulou *et al.*, 1978; Ward *et al.*, 1978). This development necessitated periodic surveys of susceptibility to detect the spread of resistance, especially because of the critical methodology of testing *H. influenzae* (Roberts *et al.*, 1974; Thornsberry & Kirven, 1974; Bottone, Brandman & Schneierson, 1976). In such a survey in 1975 we found no ampicillin resistance (Jokipii & Jokipii, 1977), and the first patient with ampicillin resistant *H. influenzae* type b meningitis in Finland was reported in 1977 (Siitonen *et al.*, 1977). The present results show that the resistance has arrived and spread during 1976–1977, and is common in nasopharyngeal strains—previous information has been largely confined to meningitis strains of type b.

In the treatment of life-threatening infections, increasing use of chloramphenicol has been the obvious response to the increasing prevalence of ampicillin resistance.

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In respiratory infections the balance between risks is different, and the reaction to the challenge of ampicillin resistance ought to be modified accordingly. Therefore, we compared six β-lactam antibiotics, erythromycin and chloramphenicol against *H. influenzae in vitro.*

Materials and methods

Bacteria

The study included 102 consecutive different strains of *H. influenzae,* isolated from clinical material and reported as possibly significant pathogens, during the period from October 1977 to June 1978. The sources of the isolates were: nose 42, middle ear 38, throat 10, eye 7, maxillary sinus 4, and blood one. The bacteria were identified by colony morphology, Gram-staining and growth requirements; the presence of capsules or type antigens was not determined. Antibiotic susceptibilities were reported as determined routinely using the disc-diffusion method (Ericsson, 1960) with paper discs (Biodisk, Solna, Sweden) on chocolated Mueller-Hinton agar plates.

β-Lactamase production

Penicillinase production was assayed by placing a paper disc containing the chromogenic cephalosporin 87/312 (Glaxo, lot 811122, supplied by Biodisk, Solna, Sweden) (O'Callaghan *et al.*, 1972; Kattan, 1975) on 48 h growth of *H. influenzae* on chocolate agar. The appearance of a reddish colour during a few minutes' incubation at 36°C indicated hydrolysis of the antibiotic.

Antibiotics

Ampicillin trihydrate (Beecham Pharmaceuticals, batch H/4 12), amoxycillin trihydrate (Beecham, batch C 102), azidocillin sodium (Astra, Södertälje, Sweden, batch CF 13), cephalothin sodium (Lilly, lot 7 HM 36), cephalexin monohydrate (Lääke, Turku, Finland, batch 7374-05), cefuroxime sodium (Glaxo), erythromycin lactobionate (Abbott, Brussels, lot 17-008TZ), and chloramphenicol (Orion, Helsinki) were dissolved in water to a concentration of 2·56 mg of active antibiotic per ml. The solutions were used within a week.

Minimal inhibitory concentrations (MICs)

The broth dilution method was used, and the medium was Mueller-Hinton broth (Difco) supplemented with 5% of peptic digest of blood (Fildes reagent, BBL). To determine the growth curve of *H. influenzae,* five colonies from 48 h chocolate agar were suspended and diluted in the medium to a final volume of 1·5 ml, and appropriate dilutions of the suspensions were made in Mueller-Hinton broth and plated for colony counting both immediately and at 3 h intervals during incubation at 36°C.

The inoculum for MIC determination was prepared as above: a sample was taken during exponential growth, diluted (as indicated in the results section) in Mueller-Hinton broth with 10% Fildes reagent, and 0·1 ml portions of the inoculum suspension were distributed to the wells of microtitre plates. Inoculum density was determined by colony counting for all strains in each experiment. The antibiotics were diluted in unsupplemented broth and distributed 0·1 ml per well. The plates were incubated at 36°C, and the MICs were recorded at 14, 24 and 42 h.

Statistical treatment

The frequency distributions of log MIC were not essentially different from the normal, provided that β-lactamase-producing and non-producing strains were regarded as separate groups. Accordingly, the geometric mean was chosen as the central value, 95% confidence intervals were calculated as the antilog of mean log MIC ± 2 S.D., and statistical significance was assessed for log MICs using Student's *t*-test.

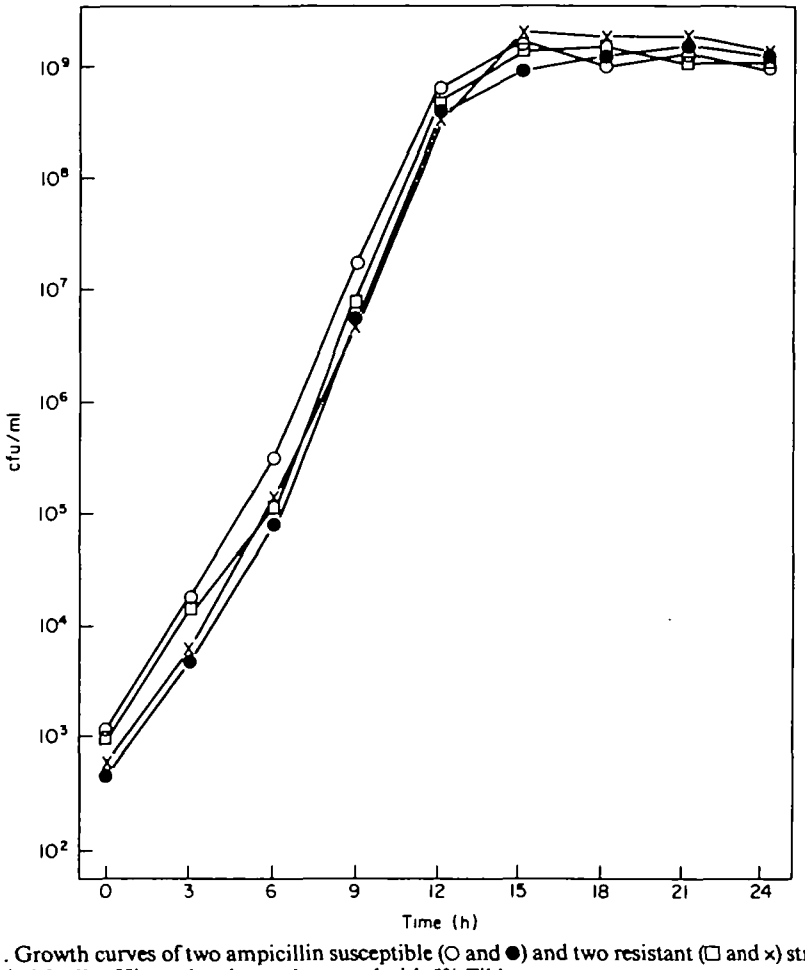

Figure 1. Growth curves of two ampicillin susceptible (○ and ●) and two resistant (□ and ×) strains of *H. influenzae* in Mueller-Hinton broth supplemented with 5% Fildes reagent.

Results

Growth curve

The growth curve of *H. influenzae* in Mueller-Hinton broth supplemented with 5% Fildes reagent was determined for four strains, two ampicillin resistant and two susceptible ones. The exponential phase started before 6 h and ended between 12 and 15 h (Figure 1). The four growth curves were remarkably similar and reproducible, and when inoculum suspensions for MIC determination were

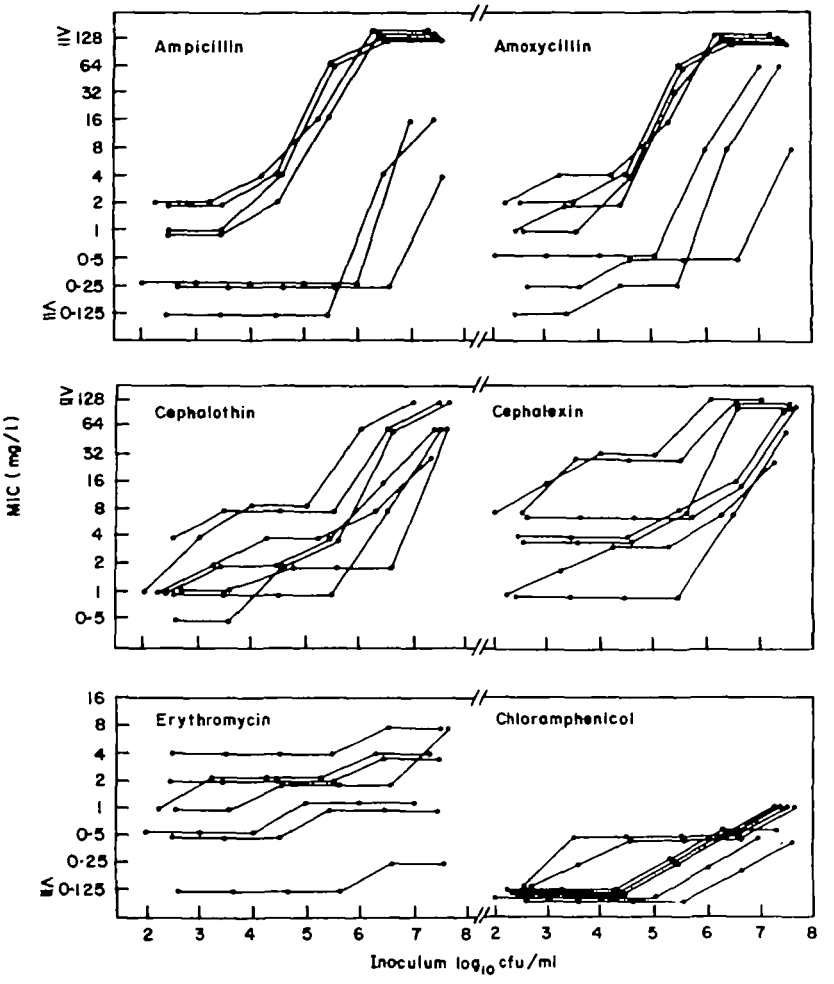

Figure 2. Effect of inoculum density on the MIC of six antibiotics against four ampicillin resistant and three ampicillin susceptible strains of *H. influenzae* after incubation for 24 h. The inoculum was taken from broth during the exponential growth phase.

subsequently prepared in similar tubes, predictable densities of exponential-phase bacteria were obtained between 9 and 12 h.

Effect of inoculum density on MIC

Seven strains of *H. influenzae* in the exponential growth phase were diluted $1 : 10^n$, and the MICs of six antibiotics were determined using six inoculum densities and incubation for 24 h. The selection included four ampicillin resistant and three susceptible strains. Below 10^4 colony-forming units (cfu)/ml the MICs of the resistant strains were only moderately higher than those of the susceptible ones, and as such within the susceptible range (Figure 2). If the inoculum contained more than 10^6 cfu/ml, the MICs of even the susceptible strains rose to the resistant range. Therefore, we decided to accept final MIC results only, when the respective inoculum turned out to have been 3×10^4 to 3×10^5 cfu/ml. Inoculum density profoundly affected the MICs of the two penicillins, ampicillin and amoxycillin, and

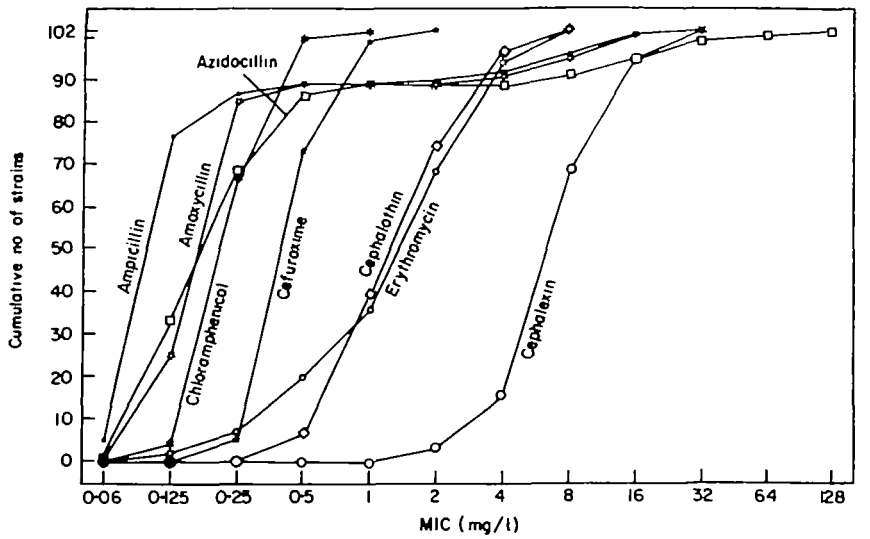

Figure 3. MICs of eight antibiotics against 102 clinical isolates of *H. influenzae*.

the two cephalosporins, cephalothin and cephalexin, while the MICs of erythromycin and chloramphenicol were only moderately dependent on inoculum density (Figure 2). With 1000-fold increasing inoculum, 64- or 128-fold increasing MIC was the rule with the penicillins, and 32-fold increasing MIC with the cephalosporins.

Survey of antimicrobial susceptibility

The susceptibility of 102 consecutive clinical isolates of *H. influenzae* to eight antibiotics was determined. The inoculum density fell in about 10% of cases outside 3×10^4 to 3×10^5 cfu/ml, at which we aimed by prediction from the growth curve. These tests were repeated, until an acceptable inoculum was obtained. The MIC results after 24 h incubation are summarized in Figure 3. The MIC distributions of ampicillin, amoxycillin and azidocillin were bimodal: 89 strains were susceptible and 13 were resistant to a varying degree to each penicillin. Thus, the frequency of ampicillin resistance was 13% among nasopharyngeal strains of *H. influenzae*. Against susceptible strains ampicillin was roughly twice as active as amoxycillin, azidocillin or chloramphenicol, which were roughly twice as active as cefuroxime. Cephalothin, erythromycin and cephalexin were less active.

All the 13 ampicillin resistant strains produced β-lactamase, as determined by the chromogenic cephalosporin disc, and none of the ampicillin susceptible strains did. Of the 13 β-lactamase-producing strains 8 had been reported as ampicillin resistant and 5 as susceptible on the basis of routine disc-diffusion testing. One of the 89 susceptible strains had been reported as resistant.

Effect of incubation time on MIC

The MIC results were recorded at 14, 24 and 42 h. The MICs of the penicillins against β-lactamase negative strains of *H. influenzae* doubled with time in about 20% of cases and did not change in the remaining 80%. Against the 13 β-lactamase-producing strains the average MICs of ampicillin and azidocillin increased 25-fold and that of amoxycillin about 50-fold (Table I), and individual 1000-fold increases were seen several times. The MIC of cefuroxime was doubled in 30% and fourfold

Table I. Effect of incubation time on the MIC of ampicillin, amoxycillin and azidocillin against 13 β-lactamase-producing strains of *H. influenzae*

	Geometric mean MIC (95% confidence interval) (mg/l)		
	Incubation time		
Antibiotic	14 h	24 h	42 h
Ampicillin	1·7 (0·68–5·0)	9·4 (2·1–43)	42 (5·7–310)
Amoxycillin	1·3 (0·39–4·4)	11 (3·3–37)	64 (8·4–490)
Azidocillin	5·2 (1·1–25)	25 (5·2–120)	130 (18–910)

increases were rare. With cephalothin, cephalexin, erythromycin and chloramphenicol the MIC was doubled with time in the majority of cases, fourfold increases were seen in about 25%, and eightfold in less than 10%. Eightfold increases in the MIC of cephalothin and cephalexin were associated with β-lactamase production by the respective strains of *H. influenzae*.

Table II. Effect of β-lactamase production by *H. influenzae* on the MIC of eight antibiotics

	Geometric mean MIC (95% confidence interval) (mg/l)		
	β-lactamase production		
Antibiotic	− (89 strains)	+ (13 strains)	P-value
Ampicillin	0·13 (0·07–0·27)	9·4 (2·1–43)	<0·001
Amoxycillin	0·21 (0·10–0·44)	11 (3·3–37)	<0·001
Azidocillin	0·23 (0·07–0·74)	25 (5·2–120)	<0·001
Cephalothin	1·7 (0·48–5·8)	3· (0·68–17)	<0·005
Cephalexin	8·6 (2·8–27)	14·4 (3·7–57)	<0·025
Cefuroxime	0·58 (0·26–1·3)	0·73 (0·29–1·8)	NS
Erythromycin	1·8 (0·26–13)	1·1 (0·21–6·0)	NS
Chloramphenicol	0·32 (0·14–0·71)	0·29 (0·13–0·67)	NS

P-values are from the comparison of log MIC in Student's *t*-test.
NS = not significant, $P > 0·05$.
The MICs are those after incubation for 24 h.

β-lactamase and MIC of eight antibiotics

As expected, the MICs of ampicillin, amoxycillin and azidocillin were significantly higher (50-100-fold) against the β-lactamase-producing strains than against the non-producing ones, and the MICs of erythromycin and chloramphenicol were not related to β-lactamase (Table II). Amoxycillin and azidocillin were equally active against susceptible strains, and ampicillin was nearly twice as active. Against β-lactamase-producing strains ampicillin and amoxycillin were more than twice as active as azidocillin. Of the cephalosporins, the MICs of cephalothin and cephalexin were significantly increased by β-lactamase production, while those of cefuroxime were not (Table II). The average effect on cephalothin and cephalexin was only approximately twofold, and, therefore, not detectable as bimodality in the frequency distribution of the MICs of the 102 strains of *H. influenzae* (Figure 3).

Discussion

In a survey of respiratory strains of *H. influenzae* isolated in 1975, we found no ampicillin resistance (Jokipii & Jokipii, 1977), and in the present series isolated in 1977 to 1978 the prevalence was 13%. The method of *in vitro* susceptibility testing was the same, and the geometric mean MICs of ampicillin and amoxycillin against susceptible strains differed between the two series by no more than 0·01 and 0·04 mg/l, respectively, serving as a control of the performance of the method. It seems that the resistance had emerged in Finland about 3 years later than in Great Britain and the United States, although *H. influenzae* other than type b have not been intensively studied in the past. All our 102 strains were susceptible to chloramphenicol, and the MIC ranged from 0·125 to 1 mg/l in agreement with previous reports (Thornsberry & Kirven, 1974; Williams & Andrews, 1974; Emerson *et al.*, 1975; Kammer *et al.*, 1975; Goldstein *et al.*, 1977; Watanakunakorn & Glotzbecker, 1979).

Thornsberry & Kirven (1974) emphasized the fact that the MIC of ampicillin is markedly affected by the number of *H. influenzae* in the inoculum and by the time of incubation. These were also our findings: only a narrow range of inoculum concentrations distinguished between susceptible and resistant strains, and incubation for 14 h was too short to reveal resistance in most cases. We used a slightly greater inoculum in 1975 to facilitate the recognition of resistance, which, however, was not found at that time (Jokipii & Jokipii, 1977). The practical significance of these findings is that it is difficult to design a routine method for reliable MIC determination, which stresses the importance of periodic surveys and the direct demonstration of β-lactamase production. The use of the chromogenic cephalosporin 87/312 was simple, rapid and reliable; it discriminated perfectly between susceptible and resistant strains in the present study, and earlier experience has been similar (Kattan, 1975).

We included azidocillin, which is advocated in Scandinavia in *H. influenzae* infections, its sole indication. Against susceptible strains it acted like amoxycillin. It was not unexpected that ampicillin resistant strains were also resistant to amoxycillin and azidocillin. It was perhaps less expected that the MICs of cephalothin and cephalexin were higher in the ampicillin resistant group. A similar finding has been reported, cephalothin being affected more than cephalexin (Emerson *et al.*, 1975), but in another study ampicillin resistance was not reflected in

the MIC of cephalothin (Kammer *et al.*, 1975). Cephalothin is susceptible to Gram-negative β-lactamases and cephalexin slightly less so (O'Callaghan, 1975; Richmond, 1978), and the R-factor-mediated β-lactamase of *H. influenzae* is of the Gram-negative type (Elwell *et al.*, 1975; Sykes, Matthew & O'Callaghan, 1975). Cefuroxime is resistant to β-lactamases (O'Callaghan, 1975; Richmond, 1978), which explains our finding and that of a previous study (Goldstein *et al.*, 1977) that ampicillin resistance did not affect the MIC of cefuroxime. The differential β-lactamase susceptibility of the cephalosporins raises a practical problem in susceptibility testing: one drug may not be enough to represent the family.

The penicillin derivatives have retained a position in the treatment of otitis media, sinusitis and bronchitis, where respiratory strains of *H. influenzae* are the targets, despite the knowledge that a minority of the organisms produce β-lactamase. The present results may have a bearing on the choice between the three penicillins. Although ampicillin is twice as active as amoxycillin against susceptible strains, documented also in previous investigations (Williams & Andrews, 1974; Emerson *et al.*, 1975; Jokipii & Jokipii, 1977), amoxycillin has been generally accepted mainly due to pharmacokinetic properties. However, with amoxycillin the difference between susceptible and resistant strains was smaller and with azidocillin it was greater than with ampicillin. When treatment is started without or before susceptibility testing, this constitutes a new argument for amoxycillin (and against azidocillin); such a minor difference alone may have no clinical significance, but the sum of several minor differences will eventually raise one Haemophilus penicillin above the others. Obviously, none of the aminopenicillins must be used, when resistance is known or suspected.

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