Antibiotic susceptibilities of *Haemophilus influenzae* in central Scotland

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Objective: To ascertain the incidence of antibiotic resistance in *Haemophilus influenzae* in central Scotland and the β-lactamases produced by these isolates.

Methods: A total of 213 *H. influenzae* isolates from four medical centers in Scotland [Aberdeen (n = 58), Edinburgh (n = 55), Glasgow (n = 64) and Dundee (n = 36)] were tested for susceptibility to a range of antimicrobials including β-lactams, β-lactam/β-lactamase-inhibitor combinations, and a representative 4-quinolone, antifolate and macrolide. Susceptibility testing of the β-lactam/β-lactamase-inhibitor combination amoxicillin plus clavulanic acid was conducted at both 2:1 and 4:1 ratios and with clavulanic acid fixed at a concentration of 2 mg/L. Each strain was further investigated for the presence of β-lactamase activity.

Results: Although the incidence of resistance to amoxicillin was 15%, in the presence of clavulanic acid, this resistance was reduced to 4.2%, 5.6% and 4.2% with the 2:1 ratio, 4:1 ratio and 2 mg/L fixed concentration, respectively. Sixteen percent of the isolates demonstrated immediate β-lactamase production. Isoelectric focusing showed that 77.4%, 16.1% and 6.5% of the β-lactamase-positive strains were found to contain TEM-1, VAT-1 and both TEM-1 and VAT-1 β-lactamases, respectively. A further 29% of the strains were recognized as being β-lactamase-positive after prolonged incubation with nitrocephin.

Conclusions: This study suggests that current testing for β-lactamases may underestimate the prevalence of β-lactamase production in *H. influenzae*.

Key words: *Haemophilus influenzae,* resistance, β-lactamase, Scotland

Haemophilus influenzae is a major cause of systemic disease as well as upper and lower respiratory tract infections [1]. This organism is, however, carried as a commensal in the throat of 80% of healthy individuals, frequently with multiple strains coinciding [2].

A number of studies have reported this pathogen to be increasingly resistant to antimicrobial agents, in particular, to the β-lactams [3-5]. β-lactam resistance in *H. influenzae* may result from changes in the outer membrane porins (OMPs) that reduce cell wall permeability, modification of the drug target or the production of β-lactamases [6,7]; the latter is generally considered to be the most important mechanism of resistance. Among clinical isolates of *H. influenzae*, plasmid-mediated TEM-1 is the most frequently isolated β-lactamase [8]. Previous studies have determined that β-lactamase resistance in this organism may also result from the presence of ROB-1 [9] and, more recently, VAT-1 β-lactamases [10]. Whereas newer-generation cephalosporins or β-lactam/β-lactamase-inhibitor combinations may overcome β-lactamase resistance, these agents may still be compromised by the bacterial intrinsic resistance mechanisms [11].

Because of the increase in the prevalence of resistance in *H. influenzae*, it is important to monitor antimicrobial activity and mechanisms of resistance to ascertain optimal therapy [12]. Amoxicillin plus clavulanic acid has been employed in the treatment of *H. influenzae* infections. There has, however, been some debate as to the most appropriate sensitivity-testing ratio for this combination. The British Society for Chemotherapy states in its guidelines that this

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combination may be tested at either a 2:1 ratio (amoxicillin:clavulanic acid) or with clavulanic acid fixed at 2 mg/L [13]. In comparison, the National Committee for Clinical Laboratory Standards (NCCLS) guidelines recommend that testing be performed only at a 2:1 ratio [14]. In France, testing is conducted with clavulanic acid fixed at a concentration of 2 mg/L [15].

In this study, the susceptibility of *H. influenzae* isolates from four medical centers in Scotland to three different combinations of amoxicillin plus clavulanic acid was examined and the activity compared with a range of cephalosporin agents. In addition, the β-lactamases responsible for mediating resistance in these strains was investigated.

MATERIALS AND METHODS

Bacterial strain collection and identification

A total of 213 *H. influenzae* strains were obtained from four major teaching hospitals in Scotland—Aberdeen Royal Trust Hospital, Ninewells Hospital Dundee, Edinburgh Royal Infirmary and Glasgow Royal Infirmary. Identification of the *H. influenzae* strains was confirmed by the use of x (hemin), v (nicotinamide-adenine dinucleotide; NAD) and xv growth-factor disks (Medical Wire and Equipment Co., Bath, UK) on nutrient agar plates according to the manufacturer's instructions.

Antibiotics

The antibiotics included in the study were: clavulanic acid and amoxicillin (both from SmithKline Beecham Pharmaceuticals, Hertfordshire, UK); cefaclor (Eli Lilly Company, Indianapolis, IN); cefuroxime (Glaxo-Wellcome, Greenford, UK); ciprofloxacin (Bayer, Newbury, UK); trimethoprim (GlaxoWellcome, Greenford, UK); azithromycin (Pfizer Pharmaceuticals, Ringaskiddy, Ireland); and cefixime, piperacillin and tazobactam (all from Lederle Laboratories, Gosport, UK).

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) plates were made with doubling dilutions of each antimicrobial agent in Columbia agar base (Oxoid, Basingstoke, UK) containing 5% w/v defibrinated horse blood. For each antimicrobial agent, the drug concentration range tested was as recommended in the British Society of Antimicrobial Chemotherapy (BSAC) guidelines [13]. An overnight culture of each organism in brain-heart infusion (BHI) broth (Oxoid CM 225) supplem nted with β-NAD (10 mg/L) and bovine hemin (10 mg/L) was diluted in Davis and Mingioli (DM) minimal medium, and approximately 10^3 CFU was inoculated onto the surface of each test plate with a Denley multipoint inoculator. The MIC was defined as the lowest concentration of antibiotic that inhibited visible growth after overnight incubation at 37°C in 5% carbon dioxide (CO_2). The MIC of amoxicillin plus clavulanic acid was performed at ratios of 4:1 and 2:1 (amoxicillin:clavulanic acid), and with 2 mg/L fixed clavulanic acid, and expressed in terms of the amoxicillin concentration.

β-lactamase identification

Each isolate was inoculated onto the surface of a 10-mL Columbia agar base (Oxoid, CM) slope containing 5% w/v defibrinated horse blood and incubated overnight at 37°C in 5% CO_2. Cells were washed from the surface of each slope and suspended in 1 mL volumes of 50 mM sodium phophate buffer (pH 7.0). As described elsewhere [16], sonicated β-lactamase extracts were prepared from the cells and each extract spot-tested for β-lactamase activity with a 50 mg/L solution of nitrocephin. A color change of the nitrocephin solution from yellow to red within 10 min was considered a positive reaction. To identify weak β-lactamase activity, all of the β-lactamase extracts were subjected to prolonged incubation with nitrocephin over a 24-h period. β-lactamase preparations were prepared from known β-lactamase-negative strains and included as negative controls. The β-lactamases were identified by analytical isoelectric focusing and after visualization with nitrocephin (10^{-3} M) compared with known β-lactamases isolated from control strains. The standard β-lactamase-producing strains employed as controls were *Escherichia coli* K12 J62-2 containing plasmid R1 (TEM-1), plasmid RP4 (TEM-2), and plasmid R1010 (SHV-1), and *H. influenzae* encoding VAT-1. (VAT-1 was kindly provided by L. Vali, University of Edinburgh, Scotland, UK.)

RESULTS

Bacterial strains

A total of 213 *H. influenzae* isolates were included in the study: 58 from Aberdeen Royal Infirmary; 36 from Ninewells Hospital Dundee; 55 from Edinburgh Royal Infirmary; and 64 from Glasgow Royal Infirmary. The integrity of each strain as *H. influenzae* was confirmed as described in the materials and methods section.

Susceptibility testing

The MIC of each antibiotic for all isolates has been summarized with MIC_{50} and MIC_{90} values (Tables 1 and 2). The collective MIC data from all centers (Table 1)

Table 1 Minimum inhibitory concentrations (MIC) of a range of antibiotics in *Haemophilus influenzae* from all study centers

Antibiotic	MIC_{50}	MIC_{90}	Range
Amoxicillin	0.5	2	0.06 - > 128
A/C at 2:1	0.5	1	0.06 - > 2
A/C at 4:1	0.5	1	0.06 - > 2
A/C fixed 2 mg/L	0.5	1	0.06 - > 2
Cefaclor	4	8	0.25 - > 16
Cefixime	0.25	0.25	0.25 - 0.5
Cefuroxime	0.5	2	0.25 - 16
Ciprofloxacin	0.008	0.016	0.002 - > 0.032
Trimethoprim	0.125	1	0.015 - > 16
Azithromycin	1	1	0.12 - 4
P/T fixed 4 mg/L	0.008	0.0312	0.004 - > 2

A/C = amoxicillin plus clavulanic acid; P/T = piperacillin plus tazobactam.

show no variation in either the MIC_{50} (0.5 mg/L) or MIC_{90} (1 mg/L) results between the different ratios of amoxicillin plus clavulanic acid. When each of the centers is considered separately (Table 2), only the isolates from Aberdeen exhibited variations in MIC_{50} and MIC_{90} values with the different ratios of the combination, increasing from 0.25 mg/L and 0.5 mg/L to 0.5 mg/L and 1 mg/L, respectively, with the 4:1 ratio. The in-vitro efficacy of the combination was, in all cases, found to be greater than the efficacy of amoxicillin alone.

The MIC_{50} and MIC_{90} values suggest that the prevalence of resistance to each of the other antibacterials tested was slightly higher among those isolates obtained from Dundee than any other center

Table 2 Minimum inhibitory concentrations (MIC) of a range of antibiotics in *Haemophilus influenzae* from individual study centers

	Aberdeen		Dundee		Edinburgh		Glasgow	
Antibiotic	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Amoxicillin	0.5	1	1	2	0.5	4	0.5	2
A/C at 2:1	0.25	0.5	1	1	0.5	1	0.5	1
A/C at 4:1	0.5	1	1	1	0.5	1	0.5	1
A/C fixed 2 mg/L	0.25	0.5	1	1	0.5	1	0.5	1
Cefaclor	4	4	4	>16	4	8	4	8
Cefixime	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Cefuroxime	0.5	1	1	4	0.5	1	0.5	2
Ciprofloxacin	0.008	0.016	0.008	0.016	0.008	0.008	0.008	0.008
Trimethoprim	0.062	0.25	0.25	1	0.062	0.25	0.125	0.5
Azithromycin	0.5	1	1	2	0.5	1	0.5	1
P/T fixed 4 mg/L	0.008	0.016	0.016	0.062	0.008	0.031	0.008	0.031

A/C = amoxicillin plus clavulanic acid; P/T = piperacillin plus tazobactam.

Table 3 Percentage of antibiotic-resistant *Haemophilus influenzae* at standard breakpoint levels

Antibiotic	Breakpoint value (mg/L)[a]	All (n = 213)	Aberdeen (n = 58)	Dundee (n = 36)	Edinburgh (n = 55)	Glasgow (n = 64)
Amoxicillin	1	15.0	10.3	22.2	18.2	12.5
A/C at 2:1	1	4.2	1.7	11.1	1.8	4.7
A/C at 4:1	1	5.6	1.7	11.1	7.3	4.7
A/C fixed 2 mg/L	1	4.2	1.7	11.1	1.8	4.7
Cefaclor						
Low	2	71.8	58.6	83.3	69.1	79.7
High	8	9.9	3.4	27.7	5.5	9.4
Cefixime	1	0	0	0	0	0
Cefuroxime						
Low	1	10.3	1.7	22.2	9.1	12.5
High	4	2.3	1.7	11.1	0	0
Ciprofloxacin						
Low	1	0.5	1.7	0	0	0
High	4	0.5	1.7	0	0	0
Trimethoprim						
Low	0.5	10.3	8.6	25.0	5.5	7.8
High	2	4.7	1.7	5.5	3.6	7.8
Azithromycin	8[b]	0	0	0	0	0
P/T fixed 4 mg/L	16	0	0	0	0	0

[a] As recommended in the BSAC guidelines [13]; [b] As recommended by the manufacturer.

(Table 2). It is apparent that the isolates from each center were largely sensitive to piperacillin plus tazobactam, the other β-lactam/β-lactamase-inhibitor combination tested. The MIC_{50} and MIC_{90} values were less than the breakpoint value for resistance recommended for this combination. Similarly, there was little resistance to the β-lactam cefixime, with MICs of < 0.25 mg/L for 90% of the strains tested. In addition, ciprofloxacin was highly active against all isolates (MIC_{90} = 0.016 mg/L; Table 1).

Table 3 shows the prevalence of antibiotic-resistant *H. influenzae* from all centers and from each center as determined by standard breakpoint levels; in this study, cefixime, azithromycin and ciprofloxacin performed particularly well. Indeed, only a single isolate from Aberdeen exhibited resistance to ciprofloxacin. A total of 15% of the *H. influenzae* were resistant to amoxicillin (1 mg/L). In the presence of clavulanic acid, however, the prevalence of amoxicillin resistance was markedly reduced to 4.2% when the combination was tested at a 2:1 ratio or with clavulanic acid fixed at 2 mg/L. Testing at a 4:1 ratio reduced amoxicillin resistance to 5.6%. The BSAC guidelines recommend both low and high breakpoint values for some agents [13]. A large variation in resistance to cefaclor was observed between the low and high breakpoint values; the prevalence of resistance was 71.8% and 9.9% when 2 mg/L and 8 mg/L, respectively, were used as breakpoint values.

Table 4 Prevalence of β-lactamases in *Haemophilus influenzae* in each center

	All (%)	Aberdeen (%)	Dundee (%)	Edinburgh (%)	Glasgow (%)
TEM-1	24 (77.4)	9 (15.5)	1 (8.3)	9 (16.4)	5 (7.8)
VAT-1	5 (16.1)	4 (6.9)	1 (5.3)	0	0
TEM-1/ VAT-1	2 (6.5)	2 (3.4)	0	0	0

β-lactamase characterization

Each isolate of *H. influenzae* was examined for the production of β-lactamases. Among the *H. influenzae* isolates, 15% responded positively within 10 min to the nitrocephin β-lactamase spot-test. Isoelectric focusing analysis of these β-lactamase-positive preparations identified two different β-lactamase types: those that cofocused with TEM-1 (77.4%); and those that co-focused with VAT-1 (16.1%). Two (6.5%) β-lactamase-positive preparations contained both of these enzymes. Whereas β-lactamases cofocusing with TEM-1 were recognized in isolates obtained from all centers, β-lactamases cofocusing with VAT-1 were only present in *H. influenzae* from Dundee and Aberdeen. In addition, only isolates from Aberdeen contained both enzyme types (Table 4). After prolonged incubation with nitrocephin over a 24-h period, a further 29% of all isolates exhibited a weak β-lactamase activity which, although not specifically investigated, was thought to be chromosomally mediated.

The β-lactam susceptibility of those organisms positive for β-lactamase production within a 10-min period was compared with the non-β-lactamase-producing strains (Table 5). As expected, except for low-level cefuroxime (1 mg/L), the incidence of resistance for each agent tested was higher among the β-lactamase-positive strains. In this particular group, the addition of clavulanic acid markedly increased the efficiency of amoxicillin, decreasing resistance

Table 5 Susceptibility of (β-lactamase-positive and -negative strains to (β-lactam antibiotics in each study center

	Breakpoint value (mg/L)[a]	All (%)		Aberdeen (%)		Dundee (%)		Edinburgh (%)		Glasgow (%)	
		pos (%)	neg (%)	pos (%)	neg (%)	pos (%)	neg (%)	pos (%)	neg (%)	pos (%)	neg (%)
		31 (14.6)[b]	182 (85.4)[b]	15 (25.9)[b]	43 (74.1)[b]	2 (5.6)[b]	34 (94.4)[b]	9 (16.4)[b]	46 (83.6)[b]	5 (7.8)[b]	59 (92.2)[b]
Amoxicillin	1	21 (67.7)	11 (6)	6 (40.0)	0	1 (50.0)	7 (20.6)	9 (100.0)	1 (2.2)	5 (100.0)	3 (5.1)
A/C at 2:1	1	2 (6.5)	7 (3.8)	1 (6.7)	0	0	4 (11.8)	1 (11.1)	0	0	3 (5.1)
A/C at 4:1	1	4 (12.9)	8 (4.4)	1 (6.7)	0	0	4 (11.8)	3 (33.3)	1 (2.2)	0	3 (5.1)
A/C fixed 2 mg/L	1	2 (6.5)	7 (3.8)	1 (6.7)	0	0	4 (11.8)	1 (11.1)	0	0	3 (5.1)
Cefaclor											
Low	2	23 (74.2)	130 (71.4)	11 (73.3)	23 (53.5)	2 (100.0)	28 (82.3)	6 (66.6)	32 (69.6)	4 (80.0)	47 (79.7)
High	8	2 (6.5)	19 (10.4)	1 (6.7)	1 (2.3)	1 (50.0)	9 (26.5)	0	3 (6.5)	0	6 (10.2)
Cefixime	1	0	0	0	0	0	0	0	0	0	0
Cefuroxime											
Low	1	3 (9.7)	19 (10.4)	1 (6.7)	0	0	8 (23.5)	1 (11.1)	4 (8.7)	1 (20.0)	7 (11.9)
High	4	1 (3.2)	4 (2.2)	1 (6.7)	0	0	4 (11.8)	0	0	0	0
P/T fixed 4 mg/L	16	0	0	0	0	0	0	0	0	0	0

pos = β-lactamase-positive isolates; neg = β-lactamase-negative isolates; A/C = amoxicillin plus clavulanic acid; P/T = piperacillin plus tazobactam. [a] as recommended in the BSAC guidelines [13]; [b]total *n* (%).

from 67.7% to < 12.9%. Among non-β-lactamase producers, the incidence of amoxicillin resistance (6%) was also decreased with the addition of clavulanic acid (< 4.4%; Table 5).

DISCUSSION

This study has demonstrated that *H. influenzae* isolates from Scotland remain largely sensitive to a range of antimicrobial agents, including the β-lactams cefixime and cefuroxime, the β-lactam/β-lactamase-inhibitor combinations piperacillin plus tazobactam and amoxicillin plus clavulanic acid, the 4-quinolone ciprofloxacin and the macrolide azithromycin.

Among all centers, the *H. influenzae* isolates were least sensitive to cefaclor (MIC_{50} = 4 mg/L; MIC_{90} = 8 mg/L). Cefaclor has enjoyed extensive use worldwide [17] and studies have reported sustained activity against β-lactamase-producing *H. influenzae* [17,18]. Interestingly, a huge difference in the prevalence of resistance to this agent was identifed, as determined by breakpoint value, when a low breakpoint value of 2 mg/L (71.8%) was used compared with a high breakpoint value of 8 mg/L (9.9%). Although some *H. influenzae* isolates were highly resistant to amoxicillin (range 0.06 mg/L to >128 mg/L), the overall level of resistance to this β-lactam was 15%, indicating that there has been little change in the incidence of amoxicillin resistance among *H. influenzae* in Scotland. Reid and colleagues reported 14.3% ampicillin resistance in *H. influenzae* isolated in Scotland in 1983/1984 [19]. Similarly, in a UK study of *H. influenzae*, 14.6% resistance to ampicillin was identified [4]. The prevalence of amoxicillin resistance is, however, higher than previously reported. In 1987, ampicillin resistance was 7.8% in a study of 23 laboratories in the UK [20] and, in Ireland, 10.9% ampicillin resistance was reported by Howard and Williams [21]. In contrast, other studies have shown an increase in the prevalence of diminished susceptibility to ampicillin [12,17,22]; among Canadian isolates, 29% were ampicillin-resistant.

As reported elsewhere [17], in the present study, the presence of clavulanic acid notably increased the efficacy of amoxicillin. In all centers, the MIC_{90} value of amoxicillin was reduced from 2 mg/L to 1 mg/L when combined with clavulanic acid. Furthermore, when the prevalence of resistance, as determined by breakpoint value, was considered, resistance to amoxicillin among β-lactamase producers (67.7%) and non-β-lactamase producers (6%) was reduced to ≤ 12.9% and ≤ 4.4%, respectively, in the presence of clavulanic acid. Although the mechanism of inhibitor resistance among non-β-lactamase producers was not investigated in the present study, changes in membrane permeability have been found to be associated with resistance to β-lactam/β-lactamase-inhibitor combinations [19]. As would be expected, therefore, this type of resistance would not be circumvented by the use of amoxicillin plus clavulanic acid. In assessing the overall level of resistance, clavulanic acid was more efficacious with amoxicillin when tested at either a 2:1 ratio or with clavulanic acid fixed at 2 mg/L (4.2%) than at a 4:1 ratio (5.6%).

In comparison to the other β-lactam agents tested, cefuroxime resistance appeared to have increased. In a study reported by Burns and coworkers, the MIC_{90} of cefuroxime among ampicillin-susceptible strains was 0.01 mg/L [3] whereas, in the present study, the MIC_{90} of cefuroxime in all centers was 1 mg/L. Cefixime is an oral cephalosporin shown to be stable in the presence of a wide range of β-lactamases. Isolates of *H. influenzae* were inhibited by 2 mg/L of cefixime in a study by Powell and Williams [23]. In the present study, cefixime was inhibitory at 0.25 mg/L. All of the isolates were completely sensitive to piperacillin in combination with tazobactam, a finding which was also recognized in a multicenter study examining the in-vitro activity of this combination [24]. The highest incidence of β-lactam resistance among both non-β-lactamase and β-lactamase producers was to cefaclor. Indeed, the BSAC guidelines [13] state that *H. influenzae* may be intrinsically resistant to this antimicrobial agent.

The prevalence of resistance to trimethoprim was 4.2%. In other studies, similar incidences of trimethoprim resistance in *H. influenzae* have been reported: 4.2% [20]; 5.1% [21]; and 6.8% [5]. A number of other investigations have revealed the 4-quinolone agents — in particular, ciprofloxacin — to be highly active against *H. influenzae* [25]. This agent performed particularly well against the *H. influenzae* isolates currently investigated. Indeed, a single isolate from Aberdeen was resistant to ciprofloxacin with an MIC value of 4 mg/L. Although rare, 4-quinolone resistance has been previously reported in *H. influenzae* and, in particular, from this specific center [26,27]. In the present study, higher levels of overall antibiotic resistance were identified in the organisms isolated from Dundee. The reported values may, however, be distorted by the disproportionate contribution of *H. influenzae* isolates from this particular center.

In the present study, TEM-1 accounted for 78.8% of the β-lactamases identified. This is not surprising as TEM-1 is the most frequently identified β-lactamase among clinical isolates of *H. influenzae*. In 1981, a new β-lactamase in *H. influenzae*, ROB-1, was characterized and has since been isolated in other surveys of this organism [9]. Indeed, this enzyme now accounts for a

significant amount of β-lactamase-positive *H. influenzae* in the community [9,12], although this β-lactamase was not found among the isolates currently investigated. VAT-1, however, a novel β-lactamase [10] specific to *H. influenzae*, was isolated on only eight occasions. It is uncertain whether this β-lactamase will increase in prevalence. The number of β-lactamase-positive isolates increased after prolonged exposure to nitrocephin, suggesting that current testing procedures may underestimate the occurrence of β-lactamase-producing *H. influenzae*. It should be emphasized, however, that such weak β-lactamase-producing strains are not believed to be clinically significant as the MIC of amoxicillin was < 1 mg/L for each of these strains.

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