Accuracy of methods used for susceptibility testing of *Haemophilus influenzae* in United Kingdom laboratories

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SUMMARY Antibiotic susceptibility test reports on 1841 strains of *Haemophilus influenzae* from 25 microbiology laboratories were compared with results obtained with the same strains at The London Hospital Medical College. Of strains found to be sensitive to the antibiotics tested, 0.5% were reported as tetracycline-resistant, 1.6% as ampicillin-resistant, and 6.2% as trimethoprim-resistant. Of strains found to be resistant to these antibiotics, 37% were reported as tetracycline-sensitive, 27% as ampicillin-sensitive, and 66.7% as trimethoprim-sensitive.

Factors found to be of significance in improving accuracy of sensitivity reporting included use of chromogenic cephalosporin and low-content antibiotic discs for detection of ampicillin resistance, and use of lysed blood agar rather than chocolate blood agar to detect trimethoprim sensitivity.

Determination of antibiotic resistance in *Haemophilus influenzae* has been shown to be significantly influenced by variations in bacterial inoculum size,¹ growth media,² and antibiotic content of sensitivity test discs.³ New techniques such as detection of beta-lactamase production with chromogenic cephalosporin substrate⁴ have also become commonly used. Antibiotic sensitivity testing of *H influenzae* plays an important part in management of serious infections such as meningitis and septic arthritis, especially in view of increasing resistance of this organism to ampicillin and chloramphenicol.⁵⁻⁷

In a survey of antibiotic resistance in *H influenzae* carried out in 1981,⁵ clinical isolates of haemophili were sent from laboratories in the United Kingdom to The London Hospital Medical College (LHMC). In this paper antibiotic sensitivity test reports for these strains were analysed and compared with results obtained at LHMC; the accuracy of sensitivity testing at each laboratory could then be assessed. Analysis of methods used in laboratories enabled identification of those factors which are of most significance in testing antibiotics against *H influenzae*.

Material and methods

Full details of sensitivity testing methodology at LHMC are contained in an appendix. Twenty-five clinical microbiology laboratories in the United Kingdom sent a total of 1841 isolates of *H influenzae* to LHMC during the three-month period February to April 1981. Each isolate was accompanied by a form with the sending laboratory's antibiotic sensitivity test results. Laboratories also provided details of their sensitivity test methods.

All strains were tested at LHMC using antibiotic discs containing either 10 µg tetracycline, 2 µg ampicillin, 1.25 µg trimethoprim or 100 µg sulphamethoxazole. Strains found to be resistant to tetracycline or ampicillin by the sending laboratory or by LHMC had minimum inhibitory concentrations (MICs) of tetracycline, ampicillin and trimethoprim determined by an agar dilution method, with an inoculum size of 10⁴ colony forming units (CFU); for sulphamethoxazole, a broth dilution method was used, the inoculum being 10⁴ CFU/ml. In addition, ampicillin-resistant strains were tested with chromogenic cephalosporin to detect beta-lactamase production.

Antibiotic resistance was defined as follows: for tetracycline, an MIC of 4 mg/l or greater indicated resistance; for ampicillin, all beta-lactamase-

Table 1 Sensitivity test methods for *Haemophilus influenzae* in 25 laboratories

Laboratory	Sensitivity test agar	Growth factors added	Incubation with added CO ₂	Control organism
A	Oxoid Isosensitest	7% LHB	—	—
B	Oxoid Isosensitest	Chocolated HB	+	—
C	Oxoid DST	0.25% LHB + NAD	+	—
D	TCS chocolate	—	+	—
E	Oxoid DST	5% LHB + NAD	+	Oxford Staph
F	Oxoid Isosensitest	5% LHB	+	Oxford Staph
G	Oxoid DST	10% chocolated HB	+	H influenzae
H	Oxoid Sensitest	5% LHB	+	Oxford Staph
I	TCS chocolate	—	+	—
J	TCS chocolate	—	+	—
K	Oxoid DST	Chocolated HB	+	Oxford Staph
L	Mast SAF	IsoVitallex	+	—
M	—	—	—	—
N	Mast Mueller-Hinton	5% LHB	+	Oxford Staph
O	Oxoid DST	10% HB + 10% serum	+	—
P	Lab M Agar No 2	10% chocolated HB	—	—
Q	Oxoid Agar No 2	Lysed blood + cysteine + thiamine	+	—
R	Lab M Sensitivity test	Chocolated HB	—	Oxford Staph
S	Oxoid DST	Lysed HB	+	Oxford Staph
T	Oxoid Columbia	Chocolated HB	+	Oxford Staph
U	Oxoid Isosensitest	Chocolated HB	+	Oxford Staph
V	Oxoid DST	Chocolated HB	+	Oxford Staph
W	Oxoid Agar No 3	5% chocolated HB	+	H influenzae
X	Oxoid Isosensitest	Chocolated HB	—	—
Y	Oxoid DST	10% LHB	+	Oxford Staph

HB = horse blood

LHB = lysed horse blood

NAD = Nicotinamide adenine dinucleotide

producing strains and beta-lactamase non-producers with MICs of 4 mg/l or more were considered to be resistant. Trimethoprim and sulphamethoxazole-resistant strains had MICs of greater than 4 mg/l and 16 mg/l respectively.

Participating laboratories were assigned code letters from A to Y. Although 1841 isolates of *H influenzae* were received in the 1981 survey, certain antibiotic sensitivity tests were not performed or reported by the sending laboratories. Only those sensitivity test results reported for each strain are included in the figures.

Results

Table 1 summarises the disc sensitivity methods used by each laboratory, showing the type of agar, growth factors added, and incubation with or without added CO₂, and control organism for comparing zone sizes. The methods for detection of beta-lactamase are included in Table 3.

Thirteen out of 25 laboratories used agar containing chocolated horse blood as a sensitivity test medium; the remainder used lysed horse blood (0.25–10%) with or without additional growth factors such as serum, nicotinamide adenine dinucleotide (NAD), cysteine and thiamine. One centre did not routinely perform antibiotic sensitivity testing other than using chromogenic cephalosporin to detect beta-lactamases. Four laboratories incubated sensitivity test plates in air

without added carbon dioxide; their results were not significantly different from other centres.

Reporting of sensitivity results for each antibiotic was as follows:

TETRACYCLINE

Laboratory reports of 1565 tetracycline-resistant and sensitive strains are shown in Table 2. Seven out of 1514 sensitive strains (0.5%) were said to be resistant, the majority from one laboratory (U).

Of the 51 strains confirmed at LHMC to be resistant to tetracycline, 19 (37.3%) were reported to be sensitive by the sending laboratories. The centres using 10 µg tetracycline discs did not detect 11 of 33 resistant strains (33%); those centres using 25 µg and 50 µg discs missed 7 of 10 resistant strains (70%). There is no significant difference in detection rates between these two groups. However, of the centres testing with 10 µg discs, those using Oxoid Isosensitest agar detected 22 of 24 resistant strains (91.7%) whereas laboratories using Oxoid DST agar detected significantly fewer resistant strains (4 of 9, 44.4%); $\chi^2 = 6.13$, $p = <0.01$.

Fifty-seven strains were of intermediate resistance, requiring tetracycline MICs of 1 or 2 mg/l. Of these, 49 (86%) were reported sensitive and 8 (14%) as resistant. There is no correlation between disc size used and reporting of these strains.

AMPICILLIN

Of 1662 strains shown to be ampicillin-sensitive at

Table 2 Reporting of tetracycline susceptibility in 24 laboratories

Laboratory	Disc used (µg)	No tetracycline-sensitive strains	No reported resistant	No tetracycline-resistant strains	No reported sensitive
A	10	193	1	7	0
B	10	50	0	1	0
D	10	46	0	0	—
E	10	58	0	1	1
F	10	36	0	1	0
G	10	41	0	2	2
H	10	73	0	1	0
I	10	40	0	2	2
J	10	6	0	0	—
K	10	18	0	1	0
L	10	87	0	4	0
O	10	58	0	0	—
Q	10	106	0	3	1
R	10	63	0	3	2
T	10	33	0	0	—
U	10	113	5	6	1
V	10	34	0	2	0
W	10	58	1	0	—
X	10	48	0	4	1
Y	10	69	0	3	2
N	25	39	0	0	—
P	25	20	0	0	—
S	25	173	0	7	5
C	50	52	0	3	2
Total		1514	7 (0.5%)	51	19 (37.3%)

LHMC, 27 (1.6%) were reported to be ampicillin-resistant (Table 3). There was no correlation between the antibiotic content of discs used by the sending laboratories and the reporting of resistance in sensitive strains.

Of the strains found to be resistant to ampicillin at LHMC, 28 of 105 beta-lactamase producers (26.7%) were reported as sensitive. Centres using

chromogenic cephalosporin or other methods to detect beta-lactamase identified 57 of 74 beta-lactamase producers (77%), whereas only 11 of 21 (52%) were correctly identified by centres relying on disc sensitivity testing only ($\chi^2 = 6.2$, $p = <0.01$). Amongst laboratories not testing for beta-lactamase production, those using discs containing 2 µg and 5 µg of ampicillin detected 11 of 13 beta-

Table 3 Ampicillin susceptibility testing in 25 laboratories

Laboratory	Disc used (µg)	Beta-lactamase detection method*	Ampicillin-sensitive strains		Ampicillin-resistant strains			
			No sensitive	No reported resistant (%)	Beta-lactamase producers	No reported sensitive	Beta-lactamase non-producers	No reported sensitive
D	2	1	42	1 (2.4)	4	0	0	—
E	2	1	57	1 (1.7)	4	2	0	—
F	2, 10	2	39	1 (2.6)	2	0	0	—
H	2	1	67	1 (1.5)	6	1	1	0
J	2	—	5	0	1	1	0	—
L	2	1	83	0	8	1	0	—
T	2	—	31	4 (12.9)	1	1	0	—
V	2, 10	1	33	0	3	1	0	—
X	2	—	47	0	4	0	1	0
A	5	3	188	3 (1.6)	8	1	2	0
K	5	1	18	3 (16.6)	1	0	0	—
B	10	1	46	1 (2.1)	5	1	1	1
G	10	—	39	0	5	4	0	—
N	10	2	37	6 (16.2)	1	0	0	—
O	10	1	56	0	2	0	0	—
P	10	—	20	0	0	—	0	—
R	10	2, 4	62	1 (1.6)	3	1	1	0
U	10	1	113	0	9	0	0	—
W	10	—	81	0	5	3	0	—
Y	10	—	60	0	2	2	0	—
C	25	1	53	2 (3.8)	2	0	0	—
I	25	—	42	3 (7.1)	4	2	0	—
Q	25	1	108	0	1	1	0	—
S	25	—	165	0	13	4	2	2
M	—	1	170	0	11	2	1	1
Total			1662	27 (1.6%)	105	28 (26.7%)	9	4 (44.4%)

*Beta-lactamase detection methods: 1: chromogenic cephalosporin; 2: Mast Betatest; 3: Clover leaf; 4: Acidometric.

lactamase producers (84.6%); this compares with detection of 4 of 11 beta-lactamase producers (36.4%) by laboratories using 10 µg and 25 µg ampicillin discs ($\chi^2 = 4.03$, $p = <0.05$).

Of nine ampicillin-resistant beta-lactamase non-producers, four were reported as sensitive (44%). Laboratories which did not detect these strains either relied on the chromogenic cephalosporin test alone (with no disc test) or used ampicillin discs containing 10 µg or 25 µg ampicillin. Laboratories that detected these strains used discs containing 2, 5 or 10 µg ampicillin.

Fifty-nine strains were of intermediate resistance—that is, beta-lactamase non-producers with ampicillin MICs of 1 or 2 mg/l. Forty-three of these (73%) were reported as sensitive, and 16 (27%) as resistant. Centres using low content discs (2 and 5 µg) reported significantly more as resistant, compared with centres using high content ampicillin discs ($\chi^2 = 4.82$, $p = <0.05$).

TRIMETHOPRIM

Only results from laboratories reporting individual trimethoprim sensitivities are shown in Table 4; those using cotrimoxazole discs are not included.

Fifty-two of 838 trimethoprim-sensitive strains were reported as resistant (6.2%). One centre (D) accounted for 16 of these strains. Those centres using chocolate blood agar as a sensitivity test medium reported significantly more sensitive strains as resistant (30 of 359, 8.4%) compared with centres using lysed blood or other additives (22 of 479, 4.6%; $\chi^2 = 4.99$, $p = <0.05$).

Of 15 strains found at LHMC to be trimethoprim-resistant, 10 (66.7%) were reported as sensitive; there was no correlation between the accuracy of reporting and the laboratory methods used.

SULPHONAMIDE

The sulphamethoxazole disc and MIC testing at LHMC did not correlate sufficiently closely (correlation coefficient $r_s = -0.55$) for assessment of laboratory performance to be made.

CHLORAMPHENICOL

Although reporting of this antibiotic was not specifically requested, no centres indicated chloramphenicol resistance for any of the 19 strains with MICs of 4 mg/l or more.

Discussion

Analysis of laboratory performance in antibiotic sensitivity testing can help to identify the commonest causes of error and improve the accuracy of the sensitivity test, upon which the clinician may depend for choice of antibiotic therapy.

In this study, participating laboratories were noted to use widely differing sensitivity test methods for *Haemophilus influenzae* (Table 1). However, only a few factors were found to be of significance in terms of identifying resistant strains. There were few false reports of tetracycline resistance (0.5%); in contrast, 37% of the tetracycline-resistance strains were reported as sensitive. The reasons for this are not clear, but variations in concentrations of divalent cations are known to influence the tetracycline sensitivity test⁸ and a significant difference in accuracy of reporting of resistance was found between centres using Oxoid Isosensitest agar and those using Oxoid DST agar. Disc testing of a further 100 tetracycline-sensitive and resistant strains using both of these media could not confirm that there was any significant difference between them in zone sizes around 10, 25 and 50 µg discs.

Detection of ampicillin-resistant strains was

Table 4 Reporting of trimethoprim susceptibility in 14 laboratories

Laboratory	Disc used (µg)	No trimethoprim-sensitive strains	No reported resistant	No trimethoprim-resistant strains	No reported sensitive
E	1.25	60	3	0	—
F	1.25	35	1	0	—
G	1.25	44	0	1	0
H	1.25	74	1	0	—
R	1.25	52	1	4	3
S	1.25	12	0	1	1
U	1.25	121	4	1	1
V	1.25	36	3	0	—
A	2.5	197	5	1	0
D	2.5	37	16	2	2
N	2.5	39	4	0	—
P	2.5	18	3	0	—
X	2.5	51	3	1	1
Y	2.5	62	8	4	2
Total		838	52 (6.2%)	15	10 (66.7%)

shown to be significantly improved with the use of low content ampicillin discs containing 2 or 5 µg of antibiotic, and with the use of beta-lactamase detection methods such as chromogenic cephalosporin. On theoretical grounds, laboratories using beta-lactamase-detecting methods alone will not detect ampicillin-resistant beta-lactamase non-producers; a disc sensitivity test is required. In view of the increasing cost of chromogenic cephalosporin, laboratories may prefer to use a low content ampicillin disc test and reserve beta-lactamase detection methods for those haemophili with a diminished zone of inhibition.

Strains of *H influenzae* showing a low level of resistance to ampicillin and tetracycline were frequently reported as sensitive to the antibiotic. Whether or not infections due to these strains will respond to normal doses of antibiotic is not known.

The results of trimethoprim and sulphonamide sensitivity testing were the least satisfactory. Sensitivity tests with these antibiotics present difficulties with many organisms, including haemophili; antibiotic antagonists are present in certain blood-containing media, and the edges of the inhibition zones around these antibiotics are more frequently indistinct. For example, 10 of the 15 trimethoprim-resistant strains were reported as sensitive. Trimethoprim-sensitive strains were more commonly reported to be resistant by those laboratories using chocolate horse blood agar rather than lysed blood agar; this may be because trimethoprim antagonists are only inactivated by lysis of red cells. A satisfactory method for sulphonamide testing of *H influenzae* has not been described and for this reason we have not included the results in this report.

Sensitivity test methods used by a laboratory should provide accurate and reproducible results yet not require techniques impractical for routine use. The most important requirement is that antibiotic resistance is not missed, as ineffective chemotherapy may be commenced, and result in increased morbidity and mortality. The detection of strains resistant to ampicillin, tetracycline, trimethoprim or chloramphenicol is of particular importance.

Appendix

SENSITIVITY TEST METHODS AT LHMC

Strains

Isolates of *H influenzae* were sent to LHMC from 25 clinical microbiology laboratories in the United Kingdom during a three month period in 1981. Identity of these strains was confirmed using routine bacteriological techniques.

Disc sensitivity testing

Pure cultures of each strain were inoculated into nutrient broth (Oxoid) supplemented with 5% Fildes' extract (Difco). After incubation at 37°C for 5 h, 1/100 dilution in peptone water was prepared and used to seed the surface of Diagnostic Sensitivity Test agar (Oxoid) supplemented with 0.25% lysed horse blood and 10 mg/l nicotinamide adenine dinucleotide (BDH Chemicals Ltd.). Filter paper discs, 6 mm in diameter and containing 10 µg tetracycline, 2 µg ampicillin, 10 µg chloramphenicol, 1.25 µg trimethoprim or 100 µg sulphamethoxazole, were applied to the surface. The plate was incubated in an atmosphere of 95% air and 5% CO₂ at 37°C for 18 h.

Determination of minimum inhibitory concentration

The minimum inhibitory concentrations (MICs) of the five antibiotics used for disc testing were measured for those strains showing a reduced zone diameter (less than 20 mm) to one or more of the antibiotics tested. In addition MICs were measured for any strain reported by the sending laboratory to be resistant to tetracycline or ampicillin, regardless of the result of disc testing at LHMC. An agar dilution method was used for determining MICs of all antibiotics except sulphamethoxazole. Doubling dilutions of each antibiotic were prepared in Diagnostic Sensitivity Test agar (Oxoid), supplemented with 0.25% lysed horse blood and 10 mg/l nicotinamide adenine dinucleotide; final antibiotic concentrations ranged from 0.015 to 128 mg/l. With a Denley multipoint inoculator 0.003 ml of a 1/100 dilution of a 5 h broth culture was applied to the surface of the agar. The number of organisms in the inoculum was approximately 10⁴. The plates were incubated in an atmosphere of 95% air and 5% CO₂ for 18 h.

Minimum inhibitory concentrations of sulphamethoxazole were determined by a broth dilution method using Iso-Sensitest broth (Oxoid) supplemented with 0.25% lysed horse blood and 10 mg/l nicotinamide adenine dinucleotide. In contrast to agar dilution methods, this produced end points that could be read without difficulty using an inoculum of 10⁴ CFU/ml.

The regression lines for each of the antibiotics used are shown in the Figure. The recommended zone diameters for discrimination of sensitive and resistant strains using the above sensitivity test methods are: ampicillin, 2 µg disc: 20 mm; tetracycline, 10 µg disc: 20 mm; trimethoprim, 1.25 µg disc: 25 mm; chloramphenicol, 10 µg disc: 20 mm.

Regression lines for *H. influenzae*.

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