Methods for the routine characterisation of isolates of *Haemophilus*

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SUMMARY Methods and their evaluation are described for the routine characterisation of *Haemophilus* spp isolates using a test for dependence on V factor and a test for the conversion of delta-amino-laevulinic acid (ALA) to porphyrin in which the ALA is incorporated into a solid medium. A method is also described whereby the difference in the size of the inhibition zones around discs of ampicillin and of amoxycillin/clavulanate can be used to detect the production of beta-lactamase.

As a result of problems with the determination of the requirement for haemin (X factor) by strains of *Haemophilus* spp, Kilian¹ introduced a method based on the ability of strains independent of haemin to convert delta-amino-laevulinic acid (ALA) into protoporphyrin. This paper describes the use and evaluation of this reaction, when the ALA is incorporated into a solid medium, in the routine characterisation of isolates.

The production of beta-lactamase by haemophili is at present mainly detected by two methods—the hydrolysis of a chromogenic cephalosporin² or the detection of pH change on the hydrolysis of penicillin as in the paper strip test.³ The method described here uses the observed differences in the diameters of the zones of inhibition of growth around ampicillin discs and amoxycillin clavulanate discs, where the clavulanate protects the amoxycillin from hydrolysis, to distinguish between strains that produce beta-lactamase and those that do not.

Material and methods

BACTERIAL STRAINS

Thirty-two strains of *Haemophilus* spp were kindly provided by the National Collection of Type Cultures. There were as follows: *H influenzae* NCTC 8465, 8466, 7279, 8467, 8433, 8143, 8469, 8502, 8134, 8135, 4560, 4842, 8468, 8470, 10479, 8455, 8472; *H haemolyticus* NCTC 10659; *H parainfluenzae* NCTC 7857, 10558, 10665, 8479, 10794, 10670, 10671, 10672; *H paraphrophilus* NCTC

10556, 10557; *H aphrophilus* NCTC 5886, 5906, 5907, 5908. In addition, 219 clinical isolates were obtained from our laboratory. All strains were stored until required at -70°C as a turbid suspension in peptone water.

METHOD FOR IDENTIFICATION

The media for the determination of species are poured into two-compartment Petri dishes with one compartment containing 9 ml of Blood Agar Base No 2 (Oxoid) with 0.1% dextrose and with 2% (vol/vol) of haemin solution (0.1% (wt/vol) haematin hydrochloride in 0.1 M Na₂HPO₄ solution, autoclaved at 121°C for 15 min) added just before pouring. The other compartment contains 9 ml of 10% heated horse blood (chocolate) agar in Blood Agar Base No 2 with 2% (vol/vol) of ALA solution (3.6% (wt/vol) ALA (BDH) with 1% (wt/vol) MgSO₄·7H₂O in distilled water sterilised by membrane filtration) which is added just prior to pouring. One plate is used for every batch of isolates tested. One colony of the organism is emulsified in 3 ml of peptone water and loopfuls are applied with 4 mm diameter sterile loops to single marked spots on both halves of the Petri dish. Up to 10 strains may be accommodated on one plate. A strain of *H parainfluenzae* is included, as a positive control for the ALA medium, with each batch. The plate is incubated for 18 h at 37°C in an atmosphere containing 10% CO₂ and is then examined for the presence of growth on the medium which contains haemin and for red fluorescence of the bacterial growth on the medium containing ALA when examined under a Wood's lamp. The species of the isolate can be determined by reference to Table 1.

Table 1 Interpretation of results from the media for identification

	Porphyryns produced	Porphyryns not produced
Growth on haemin-containing medium	—	<i>H aphrophilus</i>
No growth on haemin-containing medium	<i>H parainfluenzae</i> <i>H paraphrophilus</i>	<i>H influenzae</i> <i>H haemolyticum</i>

EVALUATION OF THE MEDIA USED FOR IDENTIFICATION

Unautoclaved yeast extract was prepared by heating 50 g of fresh baker's yeast with 100 ml of 0.2 M KH_2PO_4 solution at 80°C for 20 min. After centrifugation the supernatant was prefiltered through filter paper, sterilised by passing through a 0.2 μm cellulose acetate membrane and then stored, until required, at -20°C.

Petri dishes were prepared containing 20 ml of the ALA medium described above. Three sets of plates were also prepared containing 20 ml of Blood Agar Base No 2 with 0.1% glucose and either 0.4 ml of unautoclaved yeast extract alone, 0.4 ml of haemin solution alone or both together, added just before pouring.

Strains were characterised in batches of 20. They were applied by means of a Multipoint Inoculator A400 (Denley-Tech Limited) as suspensions in sterile saline. The size of each inoculum was about 2×10^5 organisms. One each of the above four plates was inoculated. The plates were then incubated for 18 h at 37°C in an atmosphere containing 10% CO_2 . Requirement for X factor or yeast extract (V factor) was detected as a lack of, or a marked reduction in, growth on the plates containing yeast extract or haemin respectively as compared with the control plate containing both factors. The production of porphyrin from ALA was detected as described above.

DETECTION OF BETA-LACTAMASE PRODUCTION

The sensitivity tests are carried out on 10% heated horse blood agar with Blood Agar Base No 2. The

inoculum should give a semiconfluent growth and the test is carried out with the test strain and a fully sensitive control strain on either side of discs containing 25 μg of ampicillin and 20 μg amoxycillin/10 μg clavulanate (Augmentin (R)). The zones of inhibition are measured after 18 h incubation at 37°C in an atmosphere containing 10% CO_2 .

EVALUATION OF THE METHOD FOR DETECTION OF BETA-LACTAMASE

This was carried out using the method described above on three groups of organisms, all isolated in this laboratory.

(i) The first group consisted of 17 strains of *H influenzae*, negative for the production of beta-lactamase by the method of Slack *et al.*³ and for which the minimum inhibitory concentrations of ampicillin were equal to or less than 0.5 $\mu\text{g}/\text{ml}$ when tested on heated blood agar.

(ii) The second group consisted of 15 strains of *H influenzae* and two strains of *H parainfluenzae* all of which were found to produce beta-lactamase by the method of Slack *et al.*³

(iii) The third group consisted of five strains of *H influenzae*, negative for the production of beta-lactamase and for which the minimal inhibitory concentrations of ampicillin were greater than 2 $\mu\text{g}/\text{ml}$ when tested on heated blood agar. Also for these strains the minimum inhibitory concentrations of cefuroxime were raised at greater than 2 $\mu\text{g}/\text{ml}$.

Results

MEDIA FOR IDENTIFICATION

The results for the NCTC strains, when tested with the media for identification are shown in Table 2. It can be seen that, except for the four strains of *H aphrophilus*, all strains independent of haemin gave a positive fluorescence on the ALA medium. Of the 219 clinical isolates none were independent of V factor and 24 were independent of haemin. All of these 24 strains gave a positive fluorescence on the ALA medium.

DETECTION OF BETA-LACTAMASE

The diameters of the zones of inhibition are given in

Table 2 Results on testing the NCTC strains

	No of strains	No positive for:		
		ALA to porphyrin	X requirement	V requirement
<i>H influenzae</i>	17	0	17	17
<i>H haemolyticus</i>	1	0	1	1
<i>H parainfluenzae</i>	8	8	0	8
<i>H paraphrophilus</i>	2	2	0	2
<i>H aphrophilus</i>	4	0	0	0

Table 3 Inhibition zone diameters (mm)

	Zone diameter around ampicillin disc			Zone diameter around amoxycillin/clavulanate disc		
	Mean	Range	SD	Mean	Range	SD
Sensitive strains (17 × <i>H influenzae</i>)	29.41	27–36	2.37	26.71	24–34	2.28
Beta-lactamase producing strains (15 × <i>H influenzae</i> 2 × <i>H parainfluenzae</i>)	16.65	14–20	1.73	25.24	24–27	0.97
Other strains* (5 × <i>H influenzae</i>)	24.80	23–29	2.50	23.00	17–28	4.00

*For these strains the minimum inhibitory concentrations of both ampicillin and cefuroxime were $\geq 2 \mu\text{g/ml}$ but they did not produce beta-lactamase.

Table 3. It can be seen that the zone sizes for those organisms which produced the beta-lactamase were reduced around the ampicillin discs when compared with the zones around the amoxycillin/clavulanate discs. The smallest difference between the zone diameters was 5 mm in the case of two of the strains. For both groups of organisms which did not produce beta-lactamase the zone sizes were found either to be slightly greater around the ampicillin disc or to be equal around both of the discs.

Discussion

This study suggests that a test with a solid medium containing ALA is a suitable replacement for a test for dependence on haemin. The results for both types of test were in agreement for all strains except for the type strains of *H aphrophilus*. The detection of porphyrin production from ALA worked best with heated blood agar, rather than blood agar, and this was also the medium giving the most abundant growth. Haemin is freely available in this medium suggesting that, for *H parainfluenzae* and *H paraphrophilus*, either that haemin in the medium cannot be used or that the enzymes in the conversion of ALA to porphyrin may be constitutive. *Haemophilus aphrophilus*, giving a negative result in this test but a positive result in Kilian's,⁴ may be able to use haemin contained in the medium. When this test is used together with a test for dependence on V factor, as described in the method, the vast majority of isolates can be divided into two categories. The first category comprises *H parainfluenzae* and *H paraphrophilus* and the second *H influenzae* and *H haemolyticus*. If further characterisation is required a test for haemolysis will give positive results for all isolates of *H haemolyticus* and many isolates of *H parainfluenzae*.

The test for the detection of beta-lactamase can

be carried out as part of a routine sensitivity test although the medium used here, heated blood agar, is not suitable for the determination of sensitivities to trimethoprim and sulphonamides. The control organism should be fully sensitive in order to monitor the ampicillin and amoxycillin contents of the discs. In addition, a strain which produces beta-lactamase should be tested occasionally to monitor the clavulanate content. The inoculum size is very important when testing the susceptibilities of *Haemophilus* to beta-lactam antibiotics and this test will probably be reliable only with a semiconfluent inoculum. A positive result for the presence of beta-lactamase is obtained when the diameter of the zone of inhibition is greater around the amoxycillin/clavulanate disc than around the ampicillin disc and in this evaluation the difference in zone diameters was always equal to or greater than 5 mm. For the control strain, and for any strain negative for beta-lactamase, the zone around the amoxycillin/clavulanate disc should be equal to or slightly less than the zone around the ampicillin disc.

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