Provisional identification of *Haemophilus influenzae* from sputum cultures within 1 h by rapid enzyme tests

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Summary. Possible *Haemophilus influenzae* colonies in cultures of sputum samples are currently identified by tests for dependence on X and V factors. This method requires further overnight culture and may give a relatively high number of false negative results. Identification of suspected *H. influenzae* colonies by a 5-min test for production of indole and β -galactosidase followed by a 1-h porphyrin test was compared with tests for dependence on X and V factors. A commercially produced form of the rapid tests (Haemstrip®, Lab M, Bury, Lancs) was used to test 252 potential haemophilus colonies from cultures of sputum samples on heated blood agar. Colonies that were β -galactosidase-positive after 5 min were considered to be non-*H. influenzae* and those that were β -galactosidase-negative but indole-positive were considered to be *H. influenzae*. At this stage the test had a sensitivity of 99.4% and a specificity of 90.9%. After 1 h, only colonies that were β -galactosidase- and porphyrin-negative were considered to be *H. influenzae*, the sensitivity was then 99.5% and the specificity 100%. Similar results were found with colonies from sputum cultures on selective heated blood agar containing bacitracin. The X and V dependence and Haemstrip results were in 97.6% agreement in a double blind test. Of 100 non-haemophilus colonies tested by Haemstrip, two pseudomonads could have been identified as *H. influenzae* by this method. The high positive predictive value of Haemstrip results depends partly on the initial recognition of potential haemophilus colonies.

Introduction

Identification of possible *Haemophilus influenzae* isolates from cultures of sputum samples depends on the demonstration of growth around disks impregnated with X and V factors placed on the surface of an X- and V-free medium. However, organisms other than *H. influenzae* may be dependent on these factors and the test has several problems, including poor growth of *H. influenzae* on the medium, contamination with other bacteria, and inadvertent inoculation of growth factors with the bacterial inoculum. In our experience, approximately 10% of tests are interpreted falsely or have to be repeated, leading to a further 24-h delay before a result can be given to a clinician.

The development of substrates for enzymes, pre-formed in the micro-organism, that yield chromogenic products has permitted the development of rapid identification systems. In this study such a technique, Haemstrip® (Lab M, Bury, Lancs), was compared with the methods currently used for the identification of *H. influenzae*. Unfortunately, *Haemophilus* is not a well defined genus and what should be the 'gold standard' identification systems with which to com-

pare newer ones are the relatively unreliable X- and Y-dependence tests and the porphyrin test.¹ Because DNA-binding studies have shown that *H. parainfluenzae* is not a clearly defined species,² it was felt that only separation of rapidly growing colonies from clinical samples of sputum into *H. influenzae* or non-*H. influenzae* groups should be attempted.

Materials and methods

Bacterial test isolates

Samples of sputum from approximately 1000 patients with respiratory symptoms were cultured on blood agar (horse blood, 5%, in Blood Agar Base, Oxoid) and heated horse-blood agar (blood agar heated to 80°C for 10 min). After overnight culture in a humidified atmosphere with CO₂ 5%, 252 grey convex colonies that resembled *Haemophilus* spp. and then grew preferentially on heated blood agar or showed enhanced growth on blood agar around a streak of *Staphylococcus aureus* (NCTC 6571, National Collection of Type Cultures, Colindale Avenue, London) were tested by Haemstrip and by the X- and V-dependence tests. At a separate hospital, approximately 450 sputum samples were treated similarly but

with primary culture on selective heated blood agar containing bacitracin 200 mg/L. From these cultures 135 colonies were subcultured as possible *Haemophilus* spp. for identification by Haemstrip and X- and V-dependence tests. In a further part of the study, sputum and pharyngeal flora from symptomatic and asymptomatic hospital patients were cultured. From them, 43 *Haemophilus* strains—*H. influenzae* (17 strains; biotypes I, II, III and VII), *H. parainfluenzae* (25; four biotypes) and *H. segnis* (1) fully identified and biotyped by the methods of Kilian³—were used in a double-blind evaluation of the Haemstrip and X- and V-dependence tests. A selection of 100 non-haemophilus gram-positive and gram-negative organisms isolated from clinical samples and identified by API identification galleries (API Systems, Vervieu, France) or standard methods⁴ were tested by Haemstrip for comparison of results with those obtained with *Haemophilus* spp.

Identification by Haemstrip and X- and V-dependence tests

Haemstrip consists of three impregnated filter-paper sections on a plastic strip. One contains 5-bromo, 4-chloro, 3-indolyl β -galactoside to detect β -galactosidase, the second *p*-dimethylaminocinnamaldehyde for indole, and the third δ -aminolaevulinic acid, which is metabolised to a fluorescent porphyrin by organisms that are not X-dependent.

Colonies were taken by loop directly from the primary isolation plate and inoculated on to the three filter paper sections of Haemstrip. The inoculum was thought to be adequate if visible. One 20- μ l loopful of water was added to each section to ensure that each was damp but that no free water was visible on the surface. The appearance of a blue colour on the indole section within 30 s was considered to be a positive result. Any subsequent colour change in this section was ignored. The Haemstrip was then incubated at 37°C for 5 min in a petri dish containing moist blotting paper to prevent drying. A blue colour at the inoculum site on the β -galactosidase section was considered to be a positive result. The strip was incubated for a further 55 min and then examined by long-wave UV illumination in a darkened room. Pink fluorescence at the inoculum site of the porphyrin strip was considered to be a positive result. Control positive and negative tests were performed at the same time.

X- and V-dependence tests were performed with impregnated paper disks (Medical Wire and Equipment Ltd, Corsham, England) placed on Blood Agar Base and Nutrient Agar (Oxoid) already inoculated with the test organism and the *H. influenzae* control.

Repeated tests. When the results of the two tests were in disagreement (e.g., a β -galactosidase positive organism that was X- and V-dependent, or a β -galactosidase-negative, porphyrin-negative organism that was not X- and V-dependent) or were contaminated then both tests were repeated.

Sensitivity and specificity

The sensitivity of the Haemstrip and X- and V-dependence tests (after the initial test) for the identification of *H. influenzae* was taken as the ratio of the number of *H. influenzae* isolates that were identified by the tests to the number of *H. influenzae* isolates identified by repeated testing for X- and V-dependence. The specificity of Haemstrip was taken as the ratio of the number of isolates identified as not *H. influenzae* to those that were identified as not *H. influenzae* by repeated testing for X- and V-dependence.

Time for positive Haemstrip test results to develop

A selection of 25 strains of *Haemophilus* spp. that were β -galactosidase-positive within 1 h by Haemstrip were re-tested and the times taken to produce visible colour at the inoculum site were noted. Similarly, the times needed by 25 strains that were not X-factor dependent to produce a positive porphyrin test were measured.

Results

Patterns of results obtained with the 252 colonies isolated from non-selective sputum cultures are shown in the table. Seven isolates were incorrectly identified initially by the X- and V-dependence method (patterns 2, 3, 10 and 11) and eight were contaminated or failed to grow on the X- and V-test media (patterns 6, 7, 12, 15 and 17). After 24 h the X- and V-dependence tests had a sensitivity of 93.6% and, in the identification of *H. influenzae*, a positive predictive value (p.p.v.) of 100% and a negative predictive value (n.p.v.) of 77%. The most likely reason for error in this test was contamination of the inoculum with small numbers of V-factor-producing organisms. The Haemstrip method was read initially after 5 min; β -galactosidase-negative, indole-positive organisms were considered to be *H. influenzae*. In the identification of all *H. influenzae* isolates, this test had a sensitivity of 85.7%, a p.p.v. of 99% and a n.p.v. of 90.9% at this stage. If neither indole nor β -galactosidase tests gave positive results, the strip was incubated for a further 55 min for the porphyrin test (a negative result indicated *H. influenzae*); at this stage the sensitivity was 99.4% with a p.p.v. of 99.5% and n.p.v. of 98%. Haemstrip gave a misleading result with two organisms. One isolate (pattern 13) was erroneously identified as *H. influenzae* at 5 min on the basis of positive indole and negative β -galactosidase test results; the positive porphyrin test at 1 h corrected this. The second isolate (pattern 7) was initially recorded as porphyrin-positive but, on re-testing, was found to be porphyrin-negative and X- and V-dependent. The original X- and V-test inoculum of this isolate was contaminated and contamination may also explain the false positive porphyrin result.

Table. Haemstrip and X- and V-dependence test results for 252 colonies from sputum culture that were considered possible *H. influenzae*

Pattern no.	Number of strains	Results of initial test				Results of repeat tests after initial disagreement				Final identity
		Haemstrip			X, V dependence	Haemstrip			X, V dependence	
		β -Gal	Ind	Porph		β -Gal	Ind	Porph		
1	39	—	—	—	X+V	...	...	...	...	HI
2	1	—	—	—	V	—	—	—	X+V	HI
3	1	—	—	+	X	—	—	+	V	?error*
4	2	—	—	+	V	...	...	...	...	Not-HI
5	1	—	—	+	V	...	...	...	...	Not-HI†
6	3	—	—	+	Con/NG	—	—	+	—	Not-HI
7	1	—	—	+	Con/NG	—	—	—	X+V	HI
8	9	—	—	+	—	...	...	...	...	Not-HI
9	176	—	+	—	X+V	...	...	...	...	HI
10	1	—	+	—	V	—	+	—	X+V	HI
11	4	—	+	—	X	—	+	—	X+V	HI
12	2	—	+	—	Con/NG	—	+	—	X+V	HI
13	1	—	+	+	—	...	...	...	...	Not-HI
14	1	+	—	—	X	+	—	—	X	Not-HI
15	1	+	+	—	Con/NG	—	+	—	X+V	HI
16	8	+	—	+	V	...	...	...	...	Not-HI
17	1	+	—	+	Con/NG	+	—	+	V	Not-HI

+, Positive; —, negative; β -Gal, β -galactosidase; Ind, indole; Porph, porphyrin; X, V, dependence on X and/or V factor for growth; Con/NG, contaminated or failed to grow; negative results for X and V tests indicate no dependency for growth on either factor; HI, *H. influenzae*; Not-HI, not *H. influenzae*.

*Strain originally designated X dependent but on repeat testing found to be V dependent, presumed plate reading error.

†Haemolytic strain.

Amongst the 225 strains identified as *H. influenzae*, 184 (82%) were indole-positive; only one (4%) of the 27 non-*H. influenzae* strains was indole-positive. The β -galactosidase test was the least discriminatory (only 10 non-*H. influenzae* isolates gave positive results) and the porphyrin test the most discriminatory, with only a single aberrant result (one non-*H. influenzae* isolate was porphyrin-negative).

Of 135 possible *Haemophilus* isolates from bacitracin heated blood agar, 123 were identified as *H. influenzae* by both methods; in this group, only 53% were indole-positive. With 13 of the strains identified as *H. influenzae* initially by Haemstrip, the X- and V-dependence test had to be repeated because of either dubious reactions or contamination. Five strains were shown not to be *H. influenzae* by both methods. Of seven strains with which there was inconsistency between the two methods, only four remained viable for re-testing. Three were only V-dependent initially but gave negative results in the Haemstrip porphyrin test. On re-testing, all were X- and V-dependent; the fourth strain was X-dependent and gave no positive result on the Haemstrip. Sensitivity, specificity and predictive values were similar to those for isolates grown on non-selective media except for the sensitivity of Haemstrip at 5 min, which was lower at 53%.

With the 43 fully characterised strains in the double blind test, both the X- and V-dependence tests and Haemstrip gave consistently correct identification except with one strain of *H. parainfluenzae* that was

indole-positive and was erroneously identified as *H. influenzae* after 5 min by Haemstrip. However, it was porphyrin-positive at 2 h and was only V-dependent.

With all 25 isolates of *Haemophilus* spp. that gave positive results in the β -galactosidase test, the appearance was clearly positive by 5 min (40% by 3 min; 60% by 4 min). Fluorescence of the inoculum site in the porphyrin test was inoculum dependent and appeared slowly but the result was clearly positive after 1 h in all porphyrin-positive strains tested.

Amongst the 100 strains of other families and genera (*Staphylococcus*, *Streptococcus*, *Enterococcus*, *Listeria*, *Corynebacterium*, *Neisseria*, Enterobacteriaceae, *Pseudomonas*, *Clostridium* and *Bacteroides*) tested with Haemstrip none gave a pattern of β -galactosidase-negative, indole-positive after 5 min and all gave a positive porphyrin test result except for two strains of *Pseudomonas* (*P. paucimobilis* and *P. acidovorans*).

Discussion

In most UK laboratories the routine identification of *Haemophilus* spp. from cultures of sputum samples relies on demonstrating the dependence of the organisms on factors X and V for growth. However, problems of high rates of contamination, lack of growth and incorrect interpretation of results are common. The reliability of Haemstrip to identify *H. influenzae* despite contamination by small numbers of

organisms was very useful; results are obtained quickly because further growth is not required, and interpretation is clear. In tests with other genera, only two strains of *Pseudomonas* spp. gave similar results to *H. influenzae* that could have been misinterpreted. In part, the excellent results obtained in our studies may be related to the experience of the technical staff in choosing only colonies to test that had a high chance of being *Haemophilus* spp. but this would also be true of the good results of the tests for X- and V-dependence. We recommend that the Haemstrip should be used only for testing typical colonies, that grow better on heated than on non-heated blood agar, from sputum cultures. Moreover, we consider that the test should be used only to identify *H. influenzae* and that β -galactosidase- and porphyrin-positive organisms that do not produce indole should not be considered to be *H. parainfluenzae* automatically. This species is considered to be a pathogen by some authors⁵ and may need definitive identification on some occasions.

The use of bacitracin as a selective agent appeared to make little difference to the test results except that a lower proportion of indole-positive isolates was found.

In the diagnostic laboratory, the use of the Haemstrip test was easily mastered and interpretation of results was straightforward, although the lack of positive results with approximately 30% of *H. influenzae* isolates made it difficult to be sure that the inoculum size was adequate or whether the tests were working. We recommend that an *Escherichia coli* control is also inoculated on to a strip, because it gives positive results on each section. It would also be possible to add a phosphatase test, which would give positive results with haemophili, and a β -lactamase test to the strip, which would avoid the problem of all the sections giving negative results and provide further information about sensitivity to antibiotics. The β -galactosidase test was the least discriminatory, only 10 isolates being identified as non-*H. influenzae* after 5 min in our studies. However, with less experienced technical staff the test may be of more value.

Haemstrip results may permit provisional identification of *H. influenzae*, a potential pathogen of the

lower respiratory tract, after overnight culture of primary isolation plates and further research is required to delineate its clinical value. In conjunction with the use of an optochin disk on the primary isolation plate, Haemstrip can permit a useful evaluation of most sputum cultures within 24 h of receipt.

The economic benefit of Haemstrip would appear to result from the speed of results and, hence, the increased economic value of the report to the clinician. The price of the strip is not known at present but is expected to be approximately \$1. This would be lower than that of API 10 S (API-Biomerieux, Vercieu, France), the *Haemophilus-Neisseria* identification panel (American Microscan, Sacramento, USA), Trio Tube (Carr Scarborough Inc., Atlanta, USA), Rapid NH System (Mercia Diagnostics, UK), and Micro ID (General Diagnostics, Morris Plains, USA). On published data, Haemstrip appears to be more accurate and rapid than any of these other test systems that do not contain a porphyrin test,⁶ but it can be used only to identify *H. influenzae*, whereas the other test systems will enable identification of a range of other organisms.

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