

Rapid Speciation of *Haemophilus* with the Porphyrin Production Test Versus the Satellite Test for X

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The detection of porphyrins produced from δ -aminolevulinic acid was evaluated for use in speciation of the *Haemophilus*. Two hundred and forty-eight clinical isolates of nonhemolytic *Haemophilus* were tested concurrently for porphyrin production and for X and V requirements. The porphyrin production test was more rapid (4 h) and more accurate than the satellite test.

Determination of the hemin (X) requirement of *Haemophilus* species using satellite methods is notoriously troublesome. Problems involving misidentification of *Haemophilus parainfluenzae* as *Haemophilus influenzae* and vice versa have been reported by Evans and Smith (5, 6), Klein and Blazevic (9), and Biberstein and Gills (1).

In 1974, Kilian (7) described two rapid tests that avoid the problems commonly encountered when testing for the X requirement. Both tests are based upon the ability of the non-hemin-requiring *Haemophilus* to synthesize heme precursors from δ -aminolevulinic acid (ALA). These methods were evaluated to see if they would be practical in the routine clinical laboratory.

MATERIALS AND METHODS

Clinical isolates of *H. influenzae* and *H. parainfluenzae* were used. The organisms were grown on either chocolate or selective horse blood agar (3). The horse blood agar also enabled determination of hemolysis.

X and V requirements were determined on brain heart infusion (BHI) agar supplemented with 0.2 μ g of β -nicotinamide adenine dinucleotide per ml (Sigma) and 1.0 μ g of thiamine hydrochloride per ml (Eastman). This is a modification of the medium of Evans et al. (4), which we have found to give equivalent results. A colony was picked from the primary isolation plate and was spread evenly over the surface of the BHI medium with a sterile swab. Commercially prepared X and V strips (BBL) were placed on the inoculated plates at opposite sides. The X-free base medium contains enough V factor to support the growth of *H. influenzae* but not enough to support the growth of *H. parainfluenzae* which requires 5 to 25 times more V than does *H. influenzae* (6). After overnight incubation at 35°C, nonhemolytic organisms were identified as *H. influenzae* if growth occurred only around the X disk; nonhemolytic organisms growing only around the V disk were identified as *H. parainfluenzae*. Occasional strains of *H. parainfluenzae* with a minimal

requirement for V may grow over the entire plate in this method.

The substrate for Kilian's tests (7) consisted of 0.03352 g of ALA hydrochloride (Sigma), and 0.01972 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 100 ml of 0.1 M Sorensen phosphate buffer, pH 6.9. The substrate was dispensed into unsterile tubes (13 by 100 mm) and was stable for at least 9 months at -20°C.

One of Kilian's tests detects the production of porphyrin. A heavy suspension (milky) of organisms was made in 0.5 ml of substrate using overnight growth from either chocolate or selective horse blood agar; either a loop or a swab was used to prepare the suspension. The tubes were incubated in a heating block at 35 to 37°C for 2 to 4 h. The tubes were observed for red fluorescence (positive test) against a white background under a Wood lamp (long wavelength). The room lights were dimmed to read the tests. A positive test indicated a lack of requirement for X factor.

The second test, for porphobilinogen (PBG), was prepared in the same manner as the porphyrin test. After the appropriate incubation period, a few drops of Kovacs reagent were added to the tubes. Development of a red aqueous layer indicated a positive test (lack of X requirement).

RESULTS

In preliminary trials with the PBG test it was difficult to differentiate a positive test for PBG from a positive test for indole. Many of the *Haemophilus* strains produced indole. Therefore, extensive testing was carried out only with the porphyrin test.

Of the 248 isolates tested, 74 were *H. influenzae* and 174 were *H. parainfluenzae* (Table 1). Only two organisms gave discrepant results in the parallel test systems. Both organisms, isolated on chocolate agar, were type b *H. influenzae*, which appeared to require only V factor upon initial testing although the porphyrin production test was negative. Repeat tests for X and V requirement with the inocula taken from the area surrounding the X strip on BHI agar demonstrated the organisms' requirement for X

TABLE 1. Identification of clinical isolates of nonhemolytic *Haemophilus* using XV requirements versus the rapid porphyrin test

Organism	Total no.	No. identified by:	
		XV requirements	Porphyrin test ^a
<i>H. influenzae</i>	74	72	74
<i>H. parainfluenzae</i>	174	176	174

^a All tests were read within 4 h of incubation.

factor. Inocula taken from the area of growth around the original V strips proved to be nonviable.

All positive porphyrin tests were positive within 4 h. The heavier the inoculum, the more rapidly the red fluorescence was detected. If the inoculum was not milky, however, a false-negative reaction sometimes occurred. Occasionally, false-negative tests were observed if a culture older than 24 h was used for the test.

DISCUSSION

The problems resulting from use of satellite methods to identify *Haemophilus* species are due to several factors. According to Evans and Smith (5), on proteose-peptone agar supplemented with β -nicotinamide adenine dinucleotide (V), 11 of 36 strains (30%) of *H. parainfluenzae* were misidentified as *H. influenzae* when horse blood was used as the source of X factor. Apparently the horse blood served to detoxify the medium, because the same 11 organisms grew on an identical base medium to which sodium oleate had been added. On the other hand, when the base medium was nutrient agar rather than proteose-peptone agar, 14 isolates (32%) of *H. influenzae* appeared to lack an X requirement, probably due to traces of hemin or related compounds in the base. Klein and Blazevic (9) also described the latter problem and found BHI agar to be superior to tryptic soy agar as a base for testing the X requirement. Carryover of X in the inoculum is yet another source for error (1, 10) and was a problem with the two strains of *H. influenzae* type b in our study. We have also had difficulties with fastidious strains of *H. parainfluenzae* isolated from respiratory specimens. These are difficult to grow on subculture, and their poor growth has resulted in false identification as *H. influenzae*. The modified BHI medium used in this study eliminates the growth problems of these strains, but like any satellitism test, it can lead to misidentification due to carryover of X factor in the inoculum.

Kilian's rapid tests are superior because they

use a chemically defined substrate, ALA. Briefly, two molecules of ALA condense to form the pyrrole-ringed compound PBG. Next, four molecules of PBG combine to form uroporphobilinogen, which subsequently undergoes side chain substitutions to form coproporphyrinogen and then protoporphyrin IX, the immediate precursor of the heme moiety. Through auto-oxidation, uroporphyrin and coproporphyrin are formed as by-products of the reaction.

The first test described by Kilian depends on the detection of the pyrrole ring of PBG, which reacts readily with Kovacs reagent. Kilian himself raises two objections to the test for PBG: (i) lack of sensitivity, and (ii) destruction of the test system once Kovacs reagent is added. Kilian claims the indole formed by some *Haemophilus* does not interfere with the test because PBG is water soluble, whereas indole is alcohol soluble. However, in preliminary trials we found preformed indole often forced us to perform additional extractions of the aqueous layer to prove the reactant to be PBG rather than unextracted indole. Therefore, the PBG test was not considered satisfactory for routine use in the clinical laboratory. That the indole was present in the initial inoculum was demonstrated by adding Kovacs reagent to a heavy, saline suspension of organisms taken directly from either chocolate or selective horse blood agar plates.

The second test described by Kilian depends on the detection of the porphyrins, uroporphyrin, coproporphyrin, and protoporphyrin produced from ALA. These porphyrins exhibit red fluorescence when exposed to ultraviolet light from a Wood lamp (wavelength, approximately 360 nm). The test is easily performed, and positive results may be observed as early as 2 h. The test is suitable for routine use in the clinical laboratory as a rapid test if the following caveats are observed: (i) the inoculum must be heavy, the heavier the better; (ii) the culture must be fresh, not more than 24 h old; (iii) observation for fluorescence must be made in a darkened room or black box; and (iv) one must be sure a *Haemophilus* organism is being tested. Failure to follow any of the above directions may cause erroneous results.

One may object to using porphyrin production as a substitute for the conventional satellitism test. It is conceivable that an organism lacking an enzyme late in the heme synthesis pathway (therefore requiring X) could produce the intermediates detected in the porphyrin test. However, such an organism has not been shown to exist in nature (7, 8). It has been demonstrated that some X-dependent strains of *Haemophilus* require protoporphyrin rather

than hemin, but neither ALA, PBG, uroporphorinogen, nor coproporphorinogen will serve as a growth substrate for these strains (2, 10). Therefore, the porphyrin production test, due to its rapidity and accuracy, is superior to the conventional satellite test for the X requirement.

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