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Laboratory Identification of *Haemophilus influenzae*: Effects of Basal Media on the Results of the Satellitism Test and Evaluation of the RapID NH System

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The effects of four different basal media, tryptic soy agar, brain heart infusion agar, nutrient agar, and Mueller-Hinton agar, were investigated with respect to the identification of *Haemophilus influenzae* with a satellitism test in which X and V growth factors were supplied by factor-impregnated filter paper strips. A total of 187 recent clinical isolates of *H. influenzae* were examined. Of these, 179 strains (95.7%) were correctly identified with tryptic soy agar, 173 (92.5%) with brain heart infusion agar, 105 (56.1%) with nutrient agar, and 133 (71.1%) with Mueller-Hinton agar. Failure to obtain a correct identification was usually the result of satelliting growth around V factor-containing strips, possibly due to the presence of trace amounts of hemin in the basal media, or was because of an absence of growth due to nutritional deficiencies in the basal media. All 187 *H. influenzae* strains were also examined with a new biochemical and chromogenic substrate micromethod, the RapID NH system (Innovative Diagnostics Systems, Inc., Decatur, Ga.). A total of 168 (89.8%) strains were correctly identified with this system.

The species identity of *Haemophilus influenzae* strains recovered from human clinical material can be ascertained with any of several different laboratory techniques. These include the porphyrin test (7), determination of X factor (hemin) and V factor (NAD) growth requirements with a satellitism test (3), and determination of fermentation patterns of selected carbohydrates (8).

The porphyrin test is predicated on the observation that hemin-independent *Haemophilus* species possess enzymes necessary for the biosynthesis of hemin, whereas hemin-requiring species such as *H. influenzae* do not (2). The porphyrin test is considered the definitive means for determining the hemin requirement of *Haemophilus* species (7).

X and V growth factor requirements can be determined on agar medium free of both X and V factors by demonstrating satelliting growth around filter paper strips impregnated with hemin and NAD. Potential sources of error with this method are the use of basal media which contain small amounts of hemin and the carry-over of hemin with the plate inoculum (1, 4, 10, 12). Absence of hemin in the basal medium is balanced by the necessity of using a medium which supplies all of the other *Haemophilus* nutritional requirements (5). There has been no published systematic evaluation of the effect of different basal media on the accuracy of identification results obtained with this method.

A third approach to the identification of *Haemophilus* species employs conventional biochemical techniques, in particular fermentation of selected carbohydrates (8). This approach, however, is time consuming and necessitates use of specialized media (9). For these reasons, it has remained largely a reference laboratory exercise. Recently, a 5-h, miniaturized biochemical scheme (RapID NH system; Innovative Diagnostics Systems, Inc., Decatur, Ga.) designed primarily to determine the identity of *Neisseria* and *Haemophilus* species became commercially available. This system has been shown to be an accurate means for identifying *Neisseria gonorrhoeae*, *Neisseria meningitidis* (11; L. M. Kronholm, R. E. Kloosterman, and K. D. McClatchey, Abstr. Annu. Meet. Am. Soc. Microbiol., 1983, C16, p. 314), and *Haemophilus ducreyi* (6) but has not been evaluated with respect to the identification of *H. influenzae*.

The intent of the present study was to investigate the effect of different basal media on the results of the satellitism test and to determine the accuracy of the RapID NH system as a means for correctly identifying *H. influenzae* strains.

A total of 187 *H. influenzae* strains, obtained from a variety of human clinical specimens, were examined. Organisms were identified as *H. influenzae* based on the following characteristics: typical cellular morphology (i.e., pleomorphic gram-negative coccobacilli), oxidase production, absence of growth on tryptic soy agar (TSA) containing 5% defibrinated sheep blood, growth on chocolate agar containing 10 μg of NAD per ml, hemin dependence as demonstrated with the porphyrin test (7), and absence of hemolysis when grown on TSA enriched with 10 μg of NAD per ml and 5% defibrinated horse blood. Presence of type b capsular antigen was determined by slide agglutination with type b-specific antiserum (Burrows-Wellcome Diagnostics, Inc., Research Triangle Park, N.C.).

The satellitism test was performed by first propagating test organisms on enriched chocolate agar overnight at 35°C in 5 to 7% CO_2. Growth from chocolate agar plates was transferred into 1.0 ml of sterile 0.85% NaCl to obtain a suspension equivalent to a 0.5 McFarland turbidity standard. With individual sterile cotton swabs, the surfaces of TSA, brain heart infusion agar (BHIA), nutrient agar (NA), and Mueller-Hinton agar (MHA) plates were streaked confluently. All plates were prepared in the laboratory with individual lots of media purchased from Scott Laboratories, Inc. (Fiskeville, R.I.), stored at 4°C before use, and used within 5 days of preparation. By an aseptic technique, filter paper strips (BBL Microbiology Systems, Cockeysville, Md.) impregnated with X factor, V factor, and both X and V factors were

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placed on the surface of plates containing each of the four media. All plates were incubated for 20 to 24 h at 35°C in 5 to 7% CO_2 and examined for satelliting growth around the strips. Satelliting growth only around strips impregnated with both X and V factors was considered consistent with the presence of *H. influenzae*. Plates in which no satelliting growth was detected were reincubated for a total of 48 h and reexamined. In no case in which growth was absent during the first inspection was growth detected after 48 h. Strains which were misidentified with the satellitism test were confirmed as *H. influenzae* based on fermentation reactions in glucose, sucrose, and lactose (9).

The RapID NH system consists of a plastic micocupule tray containing substrates which permit determination of the following 11 biochemical characteristics: phosphate hydrolysis, nitrate reduction, *o*-nitrophenyl-β-D-galactopyranoside hydrolysis, proline and gamma glutamyl aminopeptidase, resazurin reduction, glucose and sucrose utilization, indole production from tryptophane, urease activity, and ornithine decarboxylation. The first two reactions serve to identify an isolate as belonging to the *Haemophilus* genus. The next six reactions provide species identification. The last three reactions permit biotype determination of *H. influenzae* strains. The RapID NH system was used according to the instructions of the manufacturer. Briefly, a suspension equivalent to a 3.0 McFarland turbidity standard was prepared in 1.0 ml of the inoculating diluent supplied by the manufacturer from an overnight-enriched chocolate agar culture of the test organism. This suspension was used to inoculate the plastic reactant tray. The tray was sealed and incubated in ambient atmospheric air at 35°C for 5 h. Reagents were added to the appropriate reaction cupules. Reactions were interpreted as positive or negative based on color changes, and a four-digit numerical identification code was assigned. The code compendium supplied by the manufacturer was used to determine organism identity. In those cases in which misidentification of an *H. influenzae* strain was apparently due to a single discrepant carbohydrate utilization reaction, conventional carbohydrate fermentation tests, performed by the method of Kilian (9), were applied.

The results of identification with the satellitism test by using different basal media are depicted in Table 1. Among 25 strains of *H. influenzae* type b, 24 (96.0%) were correctly identified on TSA and BHIA. One strain failed to grow on each medium. Seventeen (68.0%) and 18 (72.0%) strains were correctly identified on NA and MHA, respectively. The remaining strains failed to grow on these media. No *H. influenzae* type b strains were misidentified on any of the four media tested. Among 162 strains of *H. influenzae* (not type b), 155 (95.7%) and 149 (92.0%) were correctly identified on TSA and BHIA, respectively. Only rarely did strains fail to grow on these media. Twelve strains (7.4%) were, however, misidentified on BHIA. NA and MHA yielded correct identifications with 88 (54.3%) and 115 strains (71.0%), respectively. Failure to obtain a correct identification on these two media was usually the result of no growth. All of the strains of *H. influenzae* (not type b) which were misidentified on TSA, BHIA, NA, or MHA grew in the vicinity of the V-factor strip, without any apparent X factor requirement. Each of these strains was found to ferment glucose, but not sucrose or lactose, when tested with conventional biochemicals, thus confirming their identification as *H. influenzae*. The aggregate percentages of correct identifications with the different media were TSA, 95.7%; BHIA, 92.5%; NA, 56.1%; and MHA, 71.1%.

TABLE 1. Identification of *H. influenzae* by the satellitism test with different basal media and the RapID NH system

Organism (no.)	Test result	No. of strains yielding indicated test result with:				
		Satellitism test				RapID NH system
		TSA	BHIA	NA	MHA	
H. influenzae type b (25)	Correct	24	24	17	18	23
	Incorrect	0	0	0	0	2
	No growth	1	1	8	7	0
H. influenzae not type b (162)	Correct	155	149	88	115	145
	Incorrect	2	12	2	3	17
	No growth	5	1	72	44	0

The results obtained with the RapID NH system are also seen in Table 1. Of 25 *H. influenzae* type b strains, 23 (92.0%) were correctly identified. Two strains were misidentified, one due to a false-positive sucrose utilization reaction and another due to a false-negative glucose utilization reaction. Among 162 *H. influenzae* strains (not type b), 145 (89.5%) were correctly identified with the RapID NH system. One strain yielded a numerical identification code not found in the code compendium of the manufacturer. A total of 16 strains were misidentified, 2 because of false-positive sucrose utilization reactions, 11 because of false-negative glucose utilization reactions, and 3 because of false-negative resazurin reduction reactions. Collectively then, among a total of 187 *H. influenzae* strains, 168 (89.8%) were correctly identified. All strains which were not correctly identified were tested again with the RapID NH system, and in all cases, the same numerical identification code was obtained.

As demonstrated in this study, determination of X and V factor growth requirements of clinical *Haemophilus* isolates by the satellitism test is limited by the following considerations. It is necessary to use a basal medium which satisfies all of the nutritional requirements of this fastidious group of bacteria (8), but one which is totally devoid of trace amounts of hemin (4–7). NA and MHA were found not to support the growth of numerous *H. influenzae* strains and, thus, cannot be recommended for use in the satellitism test. In contrast, only rarely did test organisms fail to grow on TSA and BHIA. BHIA, however, did yield a significant number of incorrect identifications, probably due to trace amounts of hemin present in the media. For this reason, use of BHIA with the satellitism test should be avoided. The smallest number of discrepant results were obtained with TSA. Among a total of 187 strains of *H. influenzae*, the X and V factor growth requirements of 179 (95.7%) were correctly identified with this medium. Therefore, of the four different media evaluated in this study, TSA was considered superior. Presence or absence of type b capsular antigen did not seem to influence the results of identifications obtained with the satellitism test.

The RapID NH system correctly identified 168 of 187 (89.8%) clinical *H. influenzae* isolates. No differences were observed between capsular type b strains and those lacking type b capsular antigen. The majority of incorrect identifications obtained with the RapID NH system were due to discrepant carbohydrate utilization results. It is possible that by changing the concentration of test substrate, by altering the acid-base color indicator, or by increasing the length of incubation, these incorrect identifications could be eliminated. It should be noted, however, that despite its name, the

RapID NH system should not be considered a rapid means for identifying *Haemophilus* spp. The inoculum size required to perform this test would invariably require an overnight subculture of isolates recovered on primary culture media.

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