Use of the Minitek System for Biotyping *Haemophilus* Species

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The Minitek system was used for characterization and identification of *Haemophilus*. The system was simple to use and provided rapid and clear-cut test results.

Identification of *Haemophilus* is traditionally accomplished by testing the requirement for specific growth factors on a factor-deficient agar medium. Until recently, biochemical characterization of the haemophilus had not been common. Perhaps this is a result of indifference engendered by the lack of adequate testing systems containing proper factors for growth. Reports by Kilian (1, 2), however, have provided a practical basis for biochemical differentiation and have clarified the relationships among the species.

The Minitek system (Bioquest) is a versatile micromethod which utilizes reagent-impregnated disks acting as substrates for various enzymatic reactions. The system has proven flexible and has been adapted for use in identification of the pathogenic *Neisseria* (3, 4). Since there has been an absence of proven biochemical techniques for characterization of the haemophilus, it seemed advantageous to use a system which has proven reliable for classification of another fastidious pathogen. This report presents the findings of preliminary studies to determine the suitability of the Minitek system for characterization and identification of the haemophilus.

The substrates selected for testing are shown in Table 1. A single dextrose-nitrate disk was used to test for dextrose fermentation and nitrate reduction, and the *o*-nitrophenyl- β -D-galactopyranoside (ONPG) disk-containing well was used for the indole test. The disks were dispensed into the wells of the plastic Minitek tray, which accommodates 12 disks each. A milky suspension of the test organism was prepared in *Neisseria* inoculum broth (Bioquest), and using the Minitek pipetter, 0.05 ml of the inoculum was delivered to each of the 12 wells. The *Neisseria* broth was selected as the supportive medium since the demanding nutritional requirements of haemophilus and neisseria are somewhat similar. Each well except ONPG was promptly overlaid with 3 drops of sterile mineral oil, after which the inoculated trays were placed

in the Minitek humidor and incubated overnight at 35°C under ambient air. Trays containing wells inoculated with broth only were used periodically to monitor the broth effect on the substrates.

After recording the reactions of the individual tests, the test for indole was performed by adding two drops of Kovac reagent to the ONPG disk-containing well. The trays were reincubated for an additional 24 h, after which the test for nitrate reduction was performed by adding 1 drop each of the appropriate nitrate reagents to the oil overlaying the dextrose-nitrate disk. The reagents were poked through the oil with a wooden applicator stick, and the reactions were read after 30 s.

A positive test for ornithine decarboxylase was noted by a color change from yellow to red. In all instances, positive tests were strong after 24 h of incubation, and negative tests remained distinctly yellow. Indole production was evidenced by a pink to red color immediately after addition of the reagent, whereas a negative test was a frank yellow. Positive reactions in the urease test (purple) were rapid and often seen within minutes after addition of oil. Negative tests were colorless and compared favorably with urease disks inoculated with broth only. Tests for beta-galactosidase (ONPG) production were seen at 24 h as a change of color from white to yellow. Nitrate reduction was denoted by a magenta color.

Fermentation reactions with the carbohydrate disks were seen as changes from orange-red to yellow. A weak positive reaction was a yellow color of lesser intensity than a positive reaction. In most instances, positive tests with the dextrose, sucrose, maltose, and xylose disks were strong. Those with galactose and levulose (fructose) were often weak, although reactions with these substrates often increased in intensity with further incubation. Negative tests with mannitol, sucrose, and lactose were generally alkaline (red).

TABLE 1. *Biotypes of the Haemophilus using the Minitek system*^a

Test or substrate	<i>H. influenzae</i>			<i>H. parainfluenzae</i>	
	Biotype I	Biotype II	Biotype III	Biotype I	Biotype II
Ornithine	20/20 (100) ^a	0/35 (0)	0/20 (0)	7/7 (100)	5/5 (100)
Indole	20/20 (100)	35/35 (100)	0/20 (0)	0/7 (0)	0/5 (0)
Urease	20/20 (100)	35/35 (100)	20/20 (100)	0/7 (0)	5/5 (100)
ONPG	0/20 (0)	0/35 (0)	0/20 (0)	7/7 (100)	3/5 (60)
Nitrites	20/20 (100)	35/35 (100)	20/20 (100)	7/7 (100)	5/5 (100)
Dextrose ^b	17/20 (85)	35/35 (100)	20/20 (100)	7/7 (100)	5/5 (100)
Mannitol	0/20 (0)	0/35 (0)	0/20 (0)	0/7 (0)	0/5 (0)
Lactose	0/20 (0)	0/35 (0)	0/20 (0)	0/7 (0)	0/5 (0)
Sucrose	0/20 (0)	0/35 (0)	0/20 (0)	7/7 (100)	5/5 (100)
Galactose	20/20 (100)	31/35 (86)	17/20 (85)	7/7 (100)	2/5 (40)
Maltose	2/20 (10)	7/35 (20)	3/20 (15)	7/7 (100)	5/5 (100)
Fructose	5/20 (25)	12/35 (34)	5/20 (25)	7/7 (100)	5/5 (100)
Mannose	1/20 (5)	2/35 (9)	0/20 (0)	5/5 (100)	4/4 (100)
Xylose	20/20 (100)	11/22 (50)	9/9 (100)	0/5 (0)	0/4 (0)

^a Number positive/number tested; figures in parentheses indicate percent positive.

^b Fermentation results after 48 h of incubation.

During pilot studies, tests were performed in duplicate with only one set receiving oil. There was a slight but seemingly definite advantage with oil in that fermentation reactions were sharper and more rapid and reproducible. In addition, key reactions were present after overnight incubation, thereby allowing identification of biotypes at this time. Weak reactions were confirmed with the additional period of incubation. Failure to use oil in the urease test lead to false negative results after 24 h, because the color tended to fade. When under oil, however, reactions remained strong even at 48 h. As a result, oil overlay was adopted for the remainder of the study.

Table 1 shows the results of the study to identify the haemophilus by biochemical means. Twenty strains of *Haemophilus influenzae* (XV-factor dependent) conformed to Kilian's biotype I and were positive in the ornithine, indole, and urease tests. Thirty-five strains were categorized as biotype II and 20 as biotype III. These differed from biotype I in the ornithine decarboxylase test and from each other in the indole test. Fermentation patterns common to *H. influenzae* were in the dextrose, galactose, and xylose tests, although strains of biotype I and III were noticeably more active against xylose than biotype II. Reactions with maltose and fructose were variable.

Seven of the 12 strains of *H. parainfluenzae*

(V-factor dependent) were placed in biotype I and five in biotype II. Aside from not requiring the X factor for growth, characteristics distinguishing these organisms from *H. influenzae* were the ability to produce beta-galactosidase and to consistently ferment sucrose, maltose, fructose, and mannose. Indole was not produced nor was xylose fermented by any of these strains. Biotype I differed from biotype II only in the urease test.

The results of this limited study suggest that, in the absence of standardized procedures and media, the Minitek system is suitable for characterization and identification of the *Haemophilus*. The system is simple to use, provides rapid test results, and represents a reasonable and useful approach to the biochemical typing of the *Haemophilus* species.

LITERATURE CITED

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