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Rapid, Colorimetric Test for the Determination of Hippurate Hydrolysis by Group B *Streptococcus*

STEPHEN C. EDBERG* AND SANDRA SAMUELS

Montefiore Hospital and Medical Center, Division of Microbiology and Immunology, Department of Pathology, New York, New York 10467

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A colorimetric test for the determination of hippurate hydrolysis was developed. Brain heart infusion broth made with 1% sodium hippurate served as the test medium. Hydrolysis was determined by the addition of two chemical developers, M (rhodamine B) and A (uranium acetate). A dark pink color indicated hydrolysis; no color change indicated no hydrolysis. The method was efficacious in either rapid or overnight incubation. One hundred twenty-five strains of group B, 44 strains of group A, 15 strains of group C, and 10 strains of group G *Streptococcus* were tested. By using the Lancefield method as the standard, there was 100% agreement with both the colorimetric and ferric chloride tests for hippurate hydrolysis, and 96% agreement with the CAMP test.

The Lancefield grouping of group B and other streptococci is the best identification procedure for this genus and provides data to which all other methods are compared. Even with recent technical advances (9) the procedure is not readily amenable for large-scale routine use in the clinical microbiology laboratory. As a result, biochemical procedures, with high correlative values to the Lancefield method, are used (3, 4). The detection of hippurate hydrolysis, with over 99% correlation with the Lancefield procedure, is advocated for the presumptive identification of group B *Streptococcus*. Two methods are commonly used to detect hippurate hydrolysis: a ferric chloride method, which detects the end-product benzoic acid (1); and a ninhydrin method, which detects the end-product glycine (6). The former method requires an extended period of incubation, a centrifugation step, and the determination of a somewhat equivocal end point; the latter method is limited by not having growth-medium capabilities and in requiring a nonprotein milieu.

A method was developed to colorimetrically detect the production of benzoic acid from hippurate hydrolysis after either rapid or extended incubation. The method requires no centrifugation and depends upon the addition of two highly stable reagents for color development.

MATERIALS AND METHODS

Isolates. All streptococci were isolated from the General Bacteriology section of the Division of Microbiology and Immunology of Montefiore Hospital and Medical Center, New York. Isolates were from blood, sputum, urine, wounds, throat, cerebrospinal fluid, and vagina.

Lancefield grouping. Lancefield grouping was performed by the autoclave method of Rantz and Randall (8). Group-specific antisera were obtained from Difco Laboratories, Detroit, Mich.

CAMP test. Synergistic hemolysis was determined by the method of Christie et al. (2).

Ferric chloride hippurate hydrolysis. Hippurate hydrolysis was determined utilizing ferric chloride as an indicator as described by Ayers and Rupp (1).

MA hippurate hydrolysis. Brain heart infusion broth (Baltimore Biological Laboratory, Cockeysville, Md.) made with 1% sodium hippurate (Difco Laboratories) served as the hippurate medium. For overnight, or longer, incubation the top of an isolated colony was touched with a standard microbiological wire and immersed in 3 ml of medium. For the rapid test (3 to 4 h) enough inoculum was added to 1 ml of medium to equal a MacFarland 0.5 turbidity standard; incubation was at 37 C.

Two colorimetric developers were prepared. First, a saturated solution of rhodamine B was made by adding rhodamine B (Allied Chemical Co., Morristown, N. J.) to triple-distilled benzene until excess was demonstrated by the formation of turbidity. Insolubilized rhodamine B was removed by filtering three times through standard laboratory filter paper. Care must be exercised to avoid all contact with water during the preparation of this solution. The solution, practically colorless, was found to be stable when stored in brown bottles for up to 6 months. This is the M (major) reagent.

Second, a 1% solution of uranium acetate (Mallinckrodt Chemical Works, New York) was made in distilled water. This is the A (accessory) reagent. Stored in brown bottles it also remains stable for 6 months. Both M and A reagents were used directly from 0.5-ounce (ca. 29.5-ml), droppered, pharmacy dispensing bottles. Bench storage under these conditions was 3 months.

Hippurate hydrolysis was determined by adding

one drop of hippurate medium to a small test tube. The size of the tube is immaterial, but for convenience a 100- by 13-mm tube was used. To one drop of hippurate broth five drops of M reagent was added, and the contents were thoroughly mixed. Two drops of A solution was then added, and the contents were mixed. A bright, dark pink fluorescent color resulted from hydrolysis; no change in color of the hippurate medium denoted a negative test.

RESULTS

The ferric chloride and the rapid and overnight MA tests were run in parallel. The results are presented in Table 1. The CAMP test was also performed. By using the Lancefield grouping procedure as the data base, there was 100% correlation with the detection of hippurate hydrolysis by either the ferric chloride method or the MA procedure. The rapid MA test, using a heavy inoculum, and the overnight incubation, using a small inoculum, were equally efficacious. There was 96% correlation with the 24 h CAMP test and the Lancefield grouping.

DISCUSSION

Next to the more time consuming Lancefield grouping, the detection of hippurate hydrolysis appears to be the best means of identifying group B *Streptococcus*. The determination of hippurate hydrolysis rests on the elucidation of the end product of hydrolysis, either benzoic acid or glycine. Rapid testing is possible (6) because the organism appears to possess inherent enzyme systems.

Rhodamine B interacts with benzoic acid to produce a compound which is fluorescent under ultraviolet, but not under visible, light (7). The addition of a heavy metal, uranium acetate, allows this stable compound to be readily discernible in the visible spectrum. UO_2^{2-} interacts with the carboxylic acids of the quinone form of the fluorescent compound formed by the interaction of rhodamine B and benzoic acid with the development of a bright, dark pink fluorescent color. This reaction series is used to advantage in the development of the MA test for hippurate hydrolysis. As little as 0.1 μg of benzoic acid per ml can be detected with this method. The reagents are stable for months in convenient dropper bottles. The reagents are directly added to one drop of hippurate medium from these bottles; quantitative volumetric measurements are not necessary.

TABLE 1. *Comparison of hippurate hydrolysis procedures*

Organism	No. of strains s	$FeCl_3$	Rapid MA	Overnight MA
Group A *Streptococcus*	44	0[a]	0	0
Group B *Streptococcus*	125	125	125	125
Group C *Streptococcus*	15	0	0	0
Group G *Streptococcus*	10	0	0	0

[a] Numbers represent positive tests.

A note of caution must be added. It was found (Edberg and Samuels, unpublished data) that commercial media (GIBCO, Madison, Wis.) occasionally gives false-positive results. This appears to be due to small amounts of benzoic acid produced from hippurate during the manufacturing process. Although present in a small percentage of lots tested, when using commercially prepared media one should, for each lot, inoculate a known positive and negative control organism.

The MA test has the dual ability of being both a rapid and growth medium method. In addition, since it appears to be considerably more sensitive than the ferric chloride procedure, it may prove efficacious in the semiquantitative procedure of Ferrieri et al. (5). The test is simple to perform and utilizes highly stable reagents. Of 125 strains of group B, 44 strains of group A, 15 strains of group C, and 10 strains of group G *Streptococcus*, there were no false-positive or false-negative reactions.

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