Evaluation of the Rapid Hippurate Hydrolysis Test with Enterococcal Group D Streptococci

MARCIA R. LEE AND GRACE M. EDERER*

Departments of Microbiology and Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota 55455*

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The rapid hippurate hydrolysis test was evaluated with the conventional test, using 17 group A streptococci, 9 non-enterococcal group D streptococci, 108 enterococcal group D streptococci, and 2 strains of *Listeria monocytogenes*. There was complete correlation between the rapid and conventional tests with all organisms except enterococcal group D. The rapid hippurate hydrolysis method was more sensitive with the enterococci; 95.4% were positive with the rapid method, and 9.3% were positive with the conventional method. Thin-layer chromatography (TLC) was performed on all isolates to determine if the end product of hydrolysis, glycine, was indeed present. The TLC results were in agreement with the rapid and conventional methods for group A streptococci, nonenterococcal group D streptococci, and *L. monocytogenes*. TLC results were in total agreement with the rapid hippurate hydrolysis test for the enterococcal group D isolates, thus verifying the accuracy of this more sensitive test. Trace amounts of glycine were found in the substrate, indicating the need for including an uninoculated substrate control as well as stock strains of group A and B beta-hemolytic streptococci (negative and positive controls, respectively) each time the rapid hippurate hydrolysis test is performed.

Facklam and his associates (3) demonstrated the importance of determining hippuricase activity as one of five tests required for the presumption identification of group A, B, and D streptococci. When hippurate is hydrolyzed, the products formed are benzoic acid and glycine. In the method of Facklam et al. (3), a modification of the method of Ayers and Rupp (1), the hippurate hydrolysis product, benzoic acid, is detected by its precipitation in an excess of ferric chloride. Although most streptococci hydrolyzed sodium hippurate in 20 h, for those isolates that were negative, incubation had to be continued for another 24 to 46 h. To reduce the time required for detecting hippuricase activity, Hwang and Ederer (4) devised a rapid test in which the end product, glycine, was detected in 2 h using ninhydrin.

Since only serologically identified groups of beta-hemolytic streptococci were evaluated with the rapid hippurate hydrolysis method (4), it seemed important to determine whether or not correlation could be achieved with streptococci that were nonhemolytic or showed alpha-hemolysis. In a pilot study it became evident that a high percentage of enterococcal group D isolates produced positive results with the rapid hippurate hydrolysis method. These findings

were in contrast to the low incidence of hippuricase activity in alpha- and beta-enterococcal group D streptococci reported earlier (3). The purposes of this study, therefore, were to compare the conventional and rapid hippurate hydrolysis methods using alpha- or nonhemolytic group D streptococci and to confirm the results by using thin-layer chromatography (TLC) to determine the presence or absence of glycine.

MATERIALS AND METHODS

Recent clinical isolates were obtained from the Clinical Microbiology Laboratory of the University of Minnesota Hospitals and the Veterinary Diagnostic Laboratory of the University of Minnesota. These isolates included 108 enterococcal group D streptococci and 9 nonenterococcal group D streptococci. Also included in the study were 17 group A streptococci and 2 strains of *Listeria monocytogenes*. The latter organisms were included since they hydrolyze hippurate and might be confused with beta-hemolytic streptococci. The group D streptococci were presumptively identified by the method of Facklam and associates (3), and the group A streptococci were identified serologically, using fluorescence microscopy (2). All organisms were maintained on tryptic soy agar slants (Grand Island Biological Co. [GIBCO], Grand Island, N.Y.) and subcultured to tryptic soy agar with 5% sheep blood plates (GIBCO) 24 to 48 h prior to testing.

The conventional method for determining hippurate hydrolysis described earlier (3) was used in this study. A 1% concentration of sodium hippurate (Difco Laboratories, Detroit, Mich.) was prepared in heart infusion broth (Difco). The hippurate broth was inoculated with 1 to 2 drops of an overnight broth culture of the organism and incubated for 66 h at 35°C. The cultures were centrifuged, and 0.2 ml of acidic FeCl₃ was added to 0.8 ml of the supernatant, which was then shaken occasionally over a 10-min period. When tests were positive, a heavy cloudy precipitate of ferric benzoate that did not dissolve was formed, whereas with negative tests, the initial precipitate disappeared and the broth became clear.

For the rapid hippurate hydrolysis method (4), a 1% aqueous solution of sodium hippurate (Difco) was prepared and dispensed in 0.4-ml portions, corked, and frozen at -20°C until used. At the time of use, the substrates were thawed, and a large loopful of organism from tryptic soy agar with 5% sheep blood (GIBCO) was emulsified in each substrate to produce a milky suspension. Group A and group B streptococci were included as negative and positive controls, respectively, for each experiment, as was an uninoculated substrate control. The tubes were incubated for 2 h at 37°C in a heating block. The indicator solution for detecting glycine was ninhydrin (3.5 g of ninhydrin in 100 ml of a 1:1 mixture of acetone and butanol). Without removing the tubes from the heating block, 0.2 ml of ninhydrin was added to each tube, and the incubation was continued for 10 min. The tubes were removed and observed, taking care not to mix the reagent and the substrate. A deep purple color was produced when organisms hydrolyzed sodium hippurate. No color change occurred with negative reactions, but if a faint purple was produced, it did not exceed the color reaction in the substrate control.

The 1% sodium hippurate substrate used for the rapid hippurate hydrolysis test was also used for the TLC method; a heavy suspension of the organism was used, as described earlier. The organism-substrate suspension was incubated for 2 h at 37°C in a heating block, removed, and centrifuged, and 2 µl of the supernatant fluid was applied to a cellulose-precoated sheet (MN Polygram Cel 300, Brinkmann Instruments, Inc., Westbury, N. Y.), using 2-µl spotting capillary tubes (Brinkmann Instruments). The samples were applied 1.5 cm from the bottom of the chromatogram at 1.0-cm intervals. On each chromatogram 2 µl of 0.2% aqueous glycine solution was applied as a standard. One group A and one

group B streptococcus were processed for controls with each chromatogram, as were uninoculated substrate controls, one that had been incubated at 37°C and one that had not been incubated. The samples were allowed to air dry, and the chromatogram was developed in a standard Desaga tank filled to a depth of approximately 0.3 cm, using *n*-butanol-acetic acid-water as a solvent. This solution was prepared by adding 20 ml of acetic acid to 100 ml of water in a 250-ml separatory funnel. To this weak acid solution 80 ml of butanol was added, and the mixture was shaken vigorously. The mixture was allowed to separate into layers, and 40 ml of the top layer was removed and introduced into the Desaga tank. The chamber was allowed to become saturated before placing a chromatogram in the tank; the time required for this process was reduced by placing a piece of blotting paper next to one wall to create more surface for vaporization. The chromatogram was placed in the covered tank and developed to 10 cm, removed, and dried on a slide drier at 50°C for about 10 min. The chromatogram was then sprayed to saturation with ninhydrin (7 g of ninhydrin in 100 ml of a butanol-acetone mixture [1:1]). The chromatogram was placed on the slide drier again for 20 to 25 min of drying; development of purple spots indicated the hydrolysis of hippurate to glycine. The *R_f* value for glycine was determined, and the unknowns were evaluated for the presence of glycine.

RESULTS

The results of the conventional and rapid hippurate hydrolysis methods and TLC for detecting hippuricase activity with group A streptococci, nonenterococcal and enterococcal group D streptococci, and *L. monocytogenes* are shown in Table 1. None of the 17 group A streptococci or the 9 enterococcal group D isolates revealed hippuricase activity with any of the three methods. Ten of the 108 strains of enterococcal group D streptococci (9.3%) were hippuricase positive with the conventional method, but when these 108 strains were tested using the rapid hippurate hydrolysis method and TLC, 103 (95.4%) were positive with each method. The results of the rapid method and TLC correlated uniformly when compared with each other; but when the results of these two methods were compared with the results from

TABLE 1. Comparison of conventional and rapid hippurate hydrolysis methods and TLC for detecting hippuricase activity

Organism	No. of cultures	Conventional		Rapid		TLC	
		Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Group A streptococcus	17	0	17	0	17	0	17
Group D nonenterococcus	9	0	9	0	9	0	9
Group D enterococcus	108	10	98	103	5	103	5
<i>L. monocytogenes</i>	2	2	0	2	0	2	0

the conventional procedure, there was agreement with only 15 enterococcal strains (10 positives and 5 negatives). There was complete agreement among the three methods for the detection of hippuricase activity in the two strains of *L. monocytogenes*.

The average R_f value for the glycine standard from 10 different chromatograms was 0.34, and the glycine in the positive tests varied from this value by no more than 1%. The uninoculated hippurate substrate, incubated at 37°C or thawed and applied directly, showed a faint trace of glycine. This faint trace of glycine was easily distinguishable from the depth of color produced when hippurate was hydrolyzed. This small amount of glycine contaminating the sodium hippurate was also detectable, using an uninoculated incubated control, with the rapid hippurate hydrolysis method. The color produced was a faint purple, distinctly different from the deep purple produced by positive reactions.

DISCUSSION

In this study there was uniform agreement of results of the conventional and rapid hippurate hydrolysis methods and TLC for detecting the presence or absence of hippuricase activity in group A streptococci, nonenterococcal group D streptococci, and *L. monocytogenes*; the agreement between the rapid and conventional tests for detecting hippuricase activity with group B streptococci had been shown earlier (4). The rapid hippurate hydrolysis method was shown to be more sensitive in this study for the detection of hippuricase activity in enterococcal group D streptococci, with 95.4% of 108 strains positive versus 9.3% positive with the conventional method. The latter result is in agreement with an earlier report (3). The results of the rapid hippurate hydrolysis test correlated with and were verified by the TLC method.

The two strains of *L. monocytogenes* were included in this study to emphasize earlier recommendations (3) for the identification of beta-hemolytic isolates resembling streptococci. A Gram stain should be performed to determine morphology, and a catalase test should be done. With these precautions, *L. monocytogenes* should never be misidentified as a group B streptococcus. Likewise, if tolerance to 6.5% NaCl and bile-esculin hydrolysis are included with a test for hippurate hydrolysis, a hemo-

lytic strain of enterococcal group D streptococcus would not be misidentified as a group B streptococcus. It should also be kept in mind that some group B streptococci are nonhemolytic (5) and that this battery of tests would provide the correct identification of nonhemolytic strains of this group.

It is interesting to note that a small amount of glycine was present in the sodium hippurate substrate used in this study. It is important, therefore, to test all newly prepared substrates uninoculated and with positive and negative controls. These same quality control measures should also be included with each set of experiments. We are presently evaluating whether or not the powdered sodium hippurate can deteriorate once a bottle is opened. Nevertheless, the small amount of glycine sometimes found in uninoculated substrate and with group A streptococci controls appears to have no effect upon the conventional hippurate hydrolysis test; it likewise has no effect upon the rapid test and TLC, if the proper controls for the tests are included.

Since the rapid hippurate hydrolysis test is not only rapid but also more sensitive for detecting hippuricase activity in enterococcal group D streptococci, those microbiologists who use it will need to keep in mind that most of these isolates will be positive with this rapid method.

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