

# Rapid Extraction Method with Pronase B for Grouping Beta-Hemolytic Streptococci

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The enzyme, Pronase B, was used to extract the C carbohydrates of beta-hemolytic streptococci for serological grouping. Reference strains of streptococci, groups A through N, were accurately grouped by using this extraction method. Of the beta-hemolytic streptococci isolated from patients' cultures, 1,197 were classified as either group A or not group A by this method. There was 100% correlation with the reference Lancefield method. In contrast, false reactions occurred with the presumptive bacitracin disc method in 4.1% of the group A and 12.7% of the non-group A cultures. The Pronase method proved simple, accurate, and readily adaptable to the clinical laboratory routine.

Since Maxted (8) and Levinson and Frank (6) reported the use of bacitracin sensitivity to differentiate group A streptococci from other beta-hemolytic streptococci, the presumptive bacitracin disc method has been used extensively in diagnostic laboratories. Several reports (1, 2, 12) indicate that the error of this method may be greater than originally conceived and that specific grouping of beta-hemolytic streptococci would be more desirable from a clinical viewpoint.

Serological grouping of beta-hemolytic streptococci requires the use of fluorescent-antibody techniques or the extraction of group-specific carbohydrates and subsequent precipitin tests. Fluorescent-antibody staining is often not feasible for the small clinical laboratory. For routine laboratories with small daily work volume, the Lancefield (5) acid extraction method, although ideal, is cumbersome, and other extraction methods (4, 7, 11) each have their disadvantages. The method reported here has been found to be applicable to the routine diagnostic laboratory and shows promise as a highly specific test for identification of beta-hemolytic streptococci removed directly from the bacitracin sensitivity test plates.

A protease of *Streptomyces griseus* was described by Nomoto (9, 10) and is now commercially available as Pronase B. Chapman (*unpublished data*) used this enzyme to release and purify the group-specific carbohydrates from groups A and C beta-hemolytic streptococci. He also used the enzyme to free group-

specific antigens from groups A through N beta-hemolytic streptococci by using growth from 2- to 5-ml overnight broth cultures. He found 100% correlation with the Lancefield acid extraction method when extraction was made by Pronase B in borate buffer containing calcium chloride.

The Pronase B method was modified subsequently so that organisms grown on sheep blood-agar plates and previously used in testing sensitivity to bacitracin were removed, digested, and serologically grouped. This report describes the efficiency and accuracy of this direct Pronase extraction method and compares it to the reference Lancefield acid extraction method for grouping streptococci. A comparison is also made of the accuracy of the Pronase serological method and the presumptive grouping test using sensitivity to bacitracin.

## MATERIALS AND METHODS

A preliminary evaluation of the Pronase extraction procedure was made using 102 stock reference strains of beta-hemolytic streptococci. The number of strains included in each group were as follows: 24 A, 13 B, 22 C, 13 D, 1 E, 2 F, 22 G, and one each of groups H, K, L, M, and N.

After this evaluation, 1,197 beta-hemolytic streptococci isolated from patients at the University of Minnesota Hospitals from 1968 through 1970 were identified as either group A or not A by using the pronase extraction procedure and only group A antiserum. All group A streptococci were verified, and non-group A organisms were further differentiated

into specific groups by Lancefield technique as described by Frank and Levinson (3). The latter verification was done by a separate streptococcal reference laboratory.

Thirteen medical technologists participated in the Pronase clinical study on a rotational basis.

**Reagents.** Chemically clean glassware and double distilled water were used for all solutions. Reagents were (i) borate buffer with 0.01 M  $\text{CaCl}_2$ . Borate buffer was prepared by combining 525 ml of borate solution (12.404 g of boric acid,  $\text{H}_3\text{BO}_3$ , dissolved in 100 ml of 1 N NaOH and diluted to 1 liter with distilled water), 475 ml of 0.1 N HCl, and 10 ml of 1 M  $\text{CaCl}_2$ . The final pH was 7.4 to 7.5. The buffer was stored at room temperature and remained stable for over 1 year. (ii) Pronase solution. A 20 mg/ml solution of Pronase B (Calbiochem) was prepared in borate buffer. This solution was dispensed as 0.5-ml portions in 13 by 75 mm tubes, corked, and frozen.

**Bacitracin sensitivity.** A Trypticase soy agar plate (BBL) containing 5% sheep blood was inoculated by picking a single isolated beta-hemolytic colony from the initial culture and streaking it down the center of the plate. The inoculum was then spread over the entire plate with a sterile swab. A bacitracin "A" disc (BBL) was placed aseptically in the center of each plate. The plates were incubated at 35 C for 18 to 24 hr. The zone of inhibition around the "A" disc was measured.

**Pronase extraction and grouping.** By using a sterile swab, all growth was removed from each blood-agar plate (bacitracin plate) and suspended in previously thawed tubes of Pronase solution. A positive group A control and a negative control, group G, were included in each set of determinations. The swab was rotated in the buffered enzyme solution to free the organisms from the swab. Finally, the solution was expressed from the swab by rotating it against the side of the tube before discarding. The resultant suspension was very cloudy.

The tubes were then placed in a heating block at 35 or 45 C for 2 hr to enable prompt release of the group-specific carbohydrate by Pronase. This was followed by centrifuging at 2,000 rev/min. When the tubes were removed from the centrifuge, special care was taken so that the sediment was not disturbed.

Grouping was performed by the method of Swift (13) by using capillary tubes with an inside diameter of 0.5 to 0.9 mm. The supernatant fluid containing the specific antigen was introduced into the capillary after the antiserum by carefully tilting the Pronase extract tube to obtain the clear extract.

**Antisera.** Antisera for the pilot study, in which stock strains of beta-hemolytic streptococci of known serological groups (A through N) were used, was obtained from the Center for Disease Control (CDC), Public Health Service, and from Burroughs Welcome.

For the clinical isolates which were identified by the Pronase method as either group A or non-group A beta-hemolytic streptococci, antisera were obtained from three sources. Control sera were supplied by Rockefeller University, courtesy of Rebecca Lancefield, and commercial sera were obtained from

the BBL division of BioQuest and from Difco Laboratories.

The reference streptococcal laboratory which grouped all cultures used in this study by the Lancefield acid extraction method utilized standardized antisera from CDC and antisera prepared by Rebecca Lancefield.

## RESULTS

**Comparison of Pronase and Lancefield reference method.** There was 100% correlation of the Pronase extraction with the Lancefield reference method in grouping 102 stock strains of beta-hemolytic streptococci groups A through N. The Pronase extracts reacted as strongly with the grouping antisera as did the Lancefield extracts; in certain groups, B, C, and D, the precipitin reaction was improved. No difference in reaction was found after incubation with Pronase for 2 hr at 35 or 45 C for any group other than D. The antigen of group D was more difficult to extract, requiring an incubation temperature of 45 C for a period of 4 hr.

In the series of 1,197 clinical isolates of beta-hemolytic streptococci, there were 585 group A and 612 strains which were not group A. The distribution of those identified serologically as not group A is shown in Table 1. There was complete agreement in the serological identification of isolates as group A or not A when the Pronase extraction method was compared with the reference Lancefield method. This complete correlation between the Lancefield reference and Pronase methods is noteworthy since 13 medical technologists did the work on a routine rotational basis.

**Bacitracin sensitivity test evaluation.** The zones of inhibition with bacitracin "A" discs are shown in Tables 2, 3, and 4. The zones of inhibition for all 585 isolates established as group A by both the Lancefield method and Pronase method are shown in Table 2. Twenty-four of these group A isolates had zones of inhibition less than 10 mm in diameter which, according to the manufacturer's directions, would be considered as not group

TABLE 1. *Distribution of non-group A beta-hemolytic streptococci*

Group	No.
B	418
C	59
F	18
G	94
Other	23
Total	612

TABLE 2. Zones of inhibition with an "A" disc for group A streptococcal strains

Zone diameter (mm)	No.
<9	10
9	14
10	38
11	40
12	56
13	107
14	108
15	67
>15	145
Total	585

TABLE 3. Distribution of non-group A beta-hemolytic streptococci with zones of inhibition from 10 to 18 mm

Zone diameter (mm)	No. in group		
	B	C	G
10	24	0	1
11	13	3	0
12	7	1	1
13	5	0	7
14	2	0	7
15	2	0	0
>15	1	1	3
Total	54	5	19

TABLE 4. Evaluation of the sensitivity to bacitracin as a presumptive test for group A streptococci by using the criterion of the manufacturer (BBL)

Serological group	No. of bacitracin-false positive or negative of strains examined	per cent error
A	24/585	4.1
B	54/418	12.9
C	5/59	8.5
F	0/18	0.0
G	19/94	20.2

A. Furthermore, as shown in Table 3, 78 isolates which were not group A by both serological methods, had zones of inhibition around bacitracin "A" discs of 10 mm in diameter or greater. These would have been presumptively identified as group A.

Table 4 shows the per cent error for each group by using the presumptive bacitracin sensitivity method. Whereas 4.1% of group A streptococci were resistant to bacitracin, 12.7% of the non-group A strains were sensitive; the greatest per cent error occurred among the group G organisms. The total error of presumptive grouping with the bacitracin sensitivity test, including false-positive and false-

negative reactions, was 8.5%.

## DISCUSSION

The use of Pronase B for extraction of the C carbohydrates of beta-hemolytic streptococci in conjunction with the capillary precipitin reaction provides accurate reproducible results even when there are many technologists participating in the work on a routine basis. The technical time for performance of serological grouping by this method is greatly reduced, which is a motivational factor for incorporation of serological identification of beta-hemolytic streptococci into routine laboratory procedures. The enzyme Pronase B is readily available commercially and relatively inexpensive. Since it remains stable in frozen portions, variances in work load do not create reagent outdating problems.

Since heavy metal ions inactivate the enzyme (10), care should be taken in choice of water supply for preparation of the enzyme solution. It was to counteract heavy metal toxicity that borate buffer containing calcium chloride was selected as diluent for the Pronase method. Other buffers had been tried in the Pronase method together with different pH values, enzyme concentrations, and time and temperature for extraction. The method, reagents, and procedure finally selected for this study have enabled accurate identifications over a period of several years.

Groups D and K were found to be groupable by the Pronase method. These were not extractable by the *Streptomyces albus* enzyme method of Maxted (7).

The availability of consistently potent commercial antiserum for group A streptococci created the greatest problem. The lot-to-lot variation of group A antiserum produced by the commercial manufacturers described herein was incompatible with the accuracy required in the clinical situation. The only antisera which gave consistent results were those made by Lancefield or provided by CDC.

Commercial antisera for groups other than A were not extensively tried because of the variability in results obtained with different lots of group A antiserum. Since the Pronase extraction method is simple and rapid, other commercially available group A antisera should be evaluated. It would be further helpful if antisera of high quality and reproducibility were also available commercially for the more commonly encountered groups such as B, C, F, and G. Any laboratory would then be able to identify non-group A beta-hemolytic strepto-

cocci as to specific groups by using the Pronase method and provide data of incidence of infection for clinical and epidemiological purposes.

The results indicate that accurate serological grouping of streptococci can be easily performed with the Pronase extraction method. However, since the quality of antiserum available commercially varies lotwise, it must be routinely evaluated with organisms of known serological group.

The Pronase method, as outlined, is completely adaptable to broth cultures or to growth from sheep blood-agar used for bacitracin sensitivity tests. It can thus be used without delay or regrowth of organisms in those instances where there is reason to know precisely the group of the infecting streptococcus or in those instances where the validity of the presumptive bacitracin sensitivity test is questioned.

A need for serological grouping of streptococci was verified by the results of the bacitracin sensitivity testing for the 1,197 beta-hemolytic streptococci studied. The 4.1% of false-negative presumptive tests for group A streptococci is consistent with earlier studies (6, 8) and more recent reports (1, 2, 12). To fail to treat patients due to such an error in diagnosis is less than ideal. With 12.7% of the beta-hemolytic streptococci grouped as A by bacitracin sensitivity, a relatively large group of patients will receive treatment where treatment may be unnecessary.

It was a matter of expediency that the use of the bacitracin disc was introduced and continues to be used as a presumptive identification method. The importance of determining group specificity of beta-hemolytic streptococci has never been discounted. This becomes particularly essential with the recognition that some group A streptococci may be resistant to bacitracin. The Pronase method provides a simple rapid laboratory method for routine grouping of streptococci by using commercially available materials and relatively stable reagents.

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