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Identification of Streptococci: Use of Lysozyme and *Streptomyces albus* Filtrate in the Preparation of Extracts for Lancefield Grouping

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A combination of lysozyme and *Streptomyces albus* filtrate has been shown effective in extracting group-specific antigen for all commonly occurring serologically groupable streptococci. A prospective comparison of this method with that of Rantz and Randall (1955) for grouping 761 clinical isolates has confirmed its accuracy, which in our hands exceeded that of the latter more complicated method of serogrouping. Its rapidity and simplicity and the relatively low cost of the reagents involved make it practical for routine use in clinical bacteriology laboratories.

While evaluating the fluorescent antibody method of serogrouping streptococci in smears of colonies scraped from blood agar plate cultures, we observed that a significant number of strains of group D streptococci either failed to stain or stained poorly with the homologous conjugate (20). Subsequent studies in our laboratory (Watson, Moellering, and Kunz, unpublished data) demonstrated that the staining of group D isolates was markedly improved by growth of the organisms in the presence of penicillin or exposure to lysozyme. These studies also showed that treatment with lysozyme was effective in preparing extracts for microprecipitin tests of group D and F but not of group A, B, C, and G streptococci. Since Maxted found the reverse to be true after treatment of the same organisms with *Streptomyces albus* filtrate (12), it was of interest to determine whether extraction with a mixture of lysozyme and *Streptomyces albus* filtrate would permit the identification of all of the Lancefield serogroups.

Our studies demonstrated that the use of lysozyme plus *S. albus* filtrate provided a rapid and accurate method for extraction of group-specific material from all groupable streptococci. This has been confirmed by a prospective comparison of this method with that of Rantz and Randall (17) for the routine serogrouping of clinical isolates of streptococci.

MATERIALS AND METHODS

Lysozyme. No. L-6876, grade 1, three times crystallized, dialyzed, and lyophilized lysozyme from egg white was obtained from the Sigma Chemical Co.

S. albus enzyme. No. 3134-56 freeze-dried *S. albus* enzyme was obtained from Difco Laboratories. This reagent is a crude filtrate of a culture of *S. albus* and probably contains more than one enzyme.

When used separately, the lysozyme was dissolved in normal saline in a concentration of 5 mg/ml. The *S. albus* filtrate was reconstituted with distilled water according to the manufacturer's instructions. The enzyme mixture was prepared by adding a solution of lysozyme in distilled water instead of saline to the *S. albus* material. Combining the enzymes resulted in the formation of a heavy precipitate, which was removed by centrifugation at 3,000 rpm for 0.5 h.

The enzyme preparations were distributed in 0.5-ml quantities in cork-stoppered tubes (10 by 75 mm) and stored at -70 C. When refrigerated, the tubes containing the *S. albus* filtrate alone or in combination with lysozyme developed an opalescence that cleared on warming and did not appear to interfere with the effectiveness of the preparation. However, because of this phenomenon, it was important to avoid refrigeration of the extracts immediately before their use in the precipitin reaction.

The test organisms used for the initial studies consisted of clinical isolates as well as certain reference strains that were not encountered clinically but were obtained from the Center for Disease Control, Atlanta, Ga. All of these organisms had been serogrouped previously by the precipitin test using extracts prepared by the method of Rantz and Randall (17) and in some instances by the method of Lancefield (8) as well. The reliability of the reactions of the combined enzymatic extracts was then evaluated in a prospective study of 761 strains isolated from various clinical specimens in the Massachusetts General Hospital diagnostic bacteriology laboratory. The precipitin tests on the latter extracts were performed the day before the tests on the Rantz-Randall and Lancefield extracts.

The organisms were propagated on brucella agar (Albimi Laboratories) with 5% horse blood in an atmosphere of 5% CO_2 in air. The growth from the plate cultures was removed with sterile cotton swabs and transferred to tubes of the thawed enzyme solutions. In the studies on the previously grouped specimens, the turbidity of the cell suspensions was adjusted to approximate that of a 1:4 to 1:8 dilution of skim milk. Depending on the size of the colonies, this sometimes required the use of more than one heavily inoculated plate. After squeezing the swab dry against the wall of the tube, the suspensions were incubated in a water bath at 45 to 50 C for 1.5 h and centrifuged at 3,000 rpm for 15 to 20 min. The supernatants were then used in microprecipitin tests with antisera to serogroups A through H and K through T as described by Swift et al. (19). The grouping antisera were purchased from Burroughs, Wellcome and Co. with the exception of group D antiserum, which was prepared in this laboratory according to the method of Lancefield (9), and the group T antiserum, which was obtained from Difco Laboratories.

Subsequent modifications in these procedures are discussed in the following sections.

RESULTS

Initial studies with enzymatic extracts. Table 1 shows the effectiveness of lysozyme, *S. albus* filtrate, and a mixture of the two in extracting the group-specific substances of streptococci encountered commonly, less commonly, and either rarely or not at all in clinical specimens.

TABLE 1. *Results of precipitin tests performed on enzymatic extracts of previously grouped streptococci*

Serogroup	No. of strains	No. correctly identified after extraction with:		
		Lysozyme	*S. albus* filtrate	Both
Common				
A	14	2	14	14
B	14	10	14	14
C	12	8	12	12
D	19	19	10	19
F	15	11	14	15
G	17	9	16	17
Nongroupable	9	9	9	9
Less common				
H	20	0	7	12
K	2	2	2	2
L	18	1	17	17
M	15	2	14	14
O	7	1	0	0
Q	2	1	1	2
Rare[a]				
E	1	1	1	1
N	3	2	3	3
P	2	2	2	2
R	2	2	2	2
S	2	2	2	2
T	2	2	2	2

[a] Reference strains.

Treatment of the common serogroups with lysozyme was most effective for group D organisms. It was less effective for groups B, C, and F and poor for strains of groups A and G. By contrast, treatment with *S. albus* filtrate was excellent for groups A, B, and C, good with groups G and F, but unsatisfactory for group D strains. When the two enzymes were combined, the correlation with the results obtained by the extraction method of Rantz and Randall was 100% for each of these serogroups. The precipitin reactions observed with the mixed enzyme extracts, moreover, were usually as strong as or stronger than those of extracts made with either one of the two enzymes alone or those prepared by the Rantz and Randall method.

With exception of the two group K strains, the less common serogroups showed varying degrees of resistance to *S. albus* enzymes and especially to lysozyme. Although treatment with a mixture of both enzymes increased the number of specific reactions with organisms of serogroups H and Q, the results obtained with the other serogroups remained essentially similar to those obtained with the *S. albus* extracts.

The rare serogroups were equally susceptible to treatment with either one or both enzyme preparations except for one group N strain, which failed to react after treatment with lysozyme.

The occasional cross-reactions observed with some of the extracts were usually weaker and appeared later than the specific reactions. The cross-reactions occurred most frequently with antisera to the less common and rare serogroups.

Modifications in procedures to enhance identification of difficult organisms. Further tests on cultures that were not readily groupable after extraction with the enzymes indicated that some strains required the use of larger cell volumes and/or longer periods of treatment to produce reactive extracts. This consideration led to a change in the protocol for the prospective evaluation of the effectiveness of the mixture of enzymes for the preparation of grouping extracts. The strains that could not be grouped when harvested from the surface of the culture plates (immediate method) were subcultured overnight in 40 ml of Todd-Hewitt broth containing 1% dextrose (13). The sedimented cells were resuspended by adding the contents of one of the tubes which contained the combined enzymes. After vigorous agitation the mixture was returned to the original tube. The cell

suspension was then incubated at 45 to 50 C for 4 h (delayed method) and the centrifuged extract was used in precipitin tests as before. Cultures that grew poorly were enriched by the addition of 4 ml of horse serum to the Todd-Hewitt broth to ensure an adequate cell crop.

The total number of groupable strains identified in tests with antigens made by the immediate and delayed enzymatic extraction was larger than those identified in tests with the Rantz-Randall extracts (Table 2). In fact, of the 611 groupable isolates there were only four strains, or less than 1%, incorrectly identified as nongroupable after treatment with enzymes. Two of these strains were found to belong to group D and two to group H by the Rantz and Randall method. The number that could be grouped after extraction with enzymes but not with the standard method of Rantz and Randall, on the other hand, totaled 31, or 5%. They included single strains of groups B, C, and G, four group L, six group F, six group N, and 12 group D strains. That these results were not spurious was indicated by the fact that the differences in the reactions of the enzymatic and Rantz and Randall extracts were usually reconciled by doubling the cell volume used for Rantz and Randall treatment of the more refractory strains.

TABLE 2. *Prospective grouping of 761 clinical isolates of streptococci*

Serogroup	No. of strains tested	No. identified by Rantz-Randall method	No. identified by combined enzymatic method		
			Immediate	Delayed	Total
A	61	61	61		61
B	173	172	173		173
C	48	47	44	4	48
D	142	130	57	83	140
F	46	40	31	15	46
G	94	93	90	4	94
H	16	16	4	10	14
K	5	5	2	3	5
L	9	5	3	6	9
M	8	8	3	5	8
N	7	1	2	5	7
O	1	1		1	1
Q	1	1		1	1
Nongroupable	150	181[a]			154[b]
Total	761				

[a] Including 31 strains identified as nongroupable by the Rantz and Randall method but grouped by the enzymatic method.

[b] Including four strains identified as nongroupable by the enzymatic method but grouped by the Rantz and Randall method.

The immediate method using the mixture of enzymes identified 456 of 564 (81%) of the strains of the commonly occurring serogroups A, B, C, D, F, and G. With the exception of the two group D strains discussed above, the balance of these strains was identified by the delayed method. Of the remaining 197 isolates, 14 (7%) reacted with antisera to the less common serogroups after extraction by the immediate method and an additional 31 (16%) reacted after extraction by the delayed method; 152 (77%) proved to be nongroupable by either of these methods. The fact that a smaller proportion of strains was identified by the immediate method in the prospective study than in the original evaluation of the method may have been because the prospective study was carried out with routinely plated cultures in which the amount of growth was sometimes insufficient to prepare cell suspensions of the required turbidity.

Current protocol and use of ancillary data to limit the number of sera to be tested. Most of the strains with readily extractable antigens were beta-hemolytic and belonged to the commonly occurring serogroups A, B, C, D, F, and G. For routine testing, therefore, we now use only these six antisera in the immediate method. Most of the remaining strains were nonhemolytic (alpha- or gamma-reacting), and the majority of these were nongroupable. These strains as well as the beta strains that could not be grouped by the immediate method were processed by the delayed method. Streptococci belonging to serogroups E, P, R, S, and T have been encountered so rarely in our laboratory that we have abandoned the routine use of antisera to these groups. We have also discontinued routine use of antiserum for group Q since this antiserum has been shown to be type specific for certain strains of enterococci (4, 15). Thus, we have reduced the number of grouping antisera used in tests by the delayed method from 18 to 12. Because the group D streptococci require further speciation physiologically and biochemically in order to differentiate enterococci from non-enterococci, serogrouping of these organisms has been limited to strains that are not readily recognized visually or that show discrepancies in their biological reactions and antibiotic susceptibility patterns. Enterococci, for example, are usually resistant to methicillin and lincomycin, whereas non-enterococci are susceptible to both agents.

With experience, it is possible to use certain ancillary features such as colonial morphology, the character of the extract, and the source of the clinical isolate to predict the probable serogroup of a large number of the beta-

hemolytic streptococci. The extracts from such organisms can then be tested against only one or two antisera, with a considerable saving in effort and expense. If they fail to react, the procedure noted above can be followed (i.e., they are tested against the antisera of the balance of the six most likely groups).

The use of these modified procedures has made it possible for a single individual to serogroup up to 50 strains a day. A particularly attractive feature of the method has been the possibility of serogrouping streptococci harvested directly from primary isolation media when sufficient colonies were present. Occasional attempts to serogroup streptococci from differentially centrifuged positive blood cultures have also been successful.

DISCUSSION

Despite the advent of antibiotic chemotherapy, streptococci remain a significant cause of human disease (11). Indeed, in terms of numbers of isolates, streptococci rank third (after the enterobacteriaceae and staphylococci) among organisms cultured from clinical specimens submitted to the diagnostic bacteriology laboratory of the Massachusetts General Hospital. The clinical significance of group A streptococci is well known because of their propensity to cause serious disease if untreated and because of the potentially dangerous immunological complications that can follow infections with these organisms (10). Recently a number of investigators have also stressed the importance of non-group A streptococci as causes of human disease (1, 5, 7, 18). These studies demonstrate the importance of serogrouping all streptococci because knowledge of the specific serogroup has important diagnostic and therapeutic implications.

Most of the classical methods of serogrouping (6, 8, 17) are too time consuming or technically difficult to enable diagnostic laboratories to engage in the routine grouping of clinical isolates. In lieu of serogrouping, susceptibility to bacitracin has thus been used to distinguish group A and non-group A beta-streptococci in many laboratories. This method has the advantage of simplicity, but its limitations (including false-positive and false-negative results) are well known (14, 16). Immunofluorescent antibody techniques have also been used, but their effective application to streptococci, especially non-group A streptococci, requires frequent testing and standardization of the antisera and a detailed knowledge of the patterns of cross-reactions that occur (20).

Several rapid methods of preparing group-specific streptococcal antigens for serogrouping have also been described. Maxted utilized *S. albus* extract to prepare antigens (12). Although this method is simple, it is applicable only to certain groups of streptococci and is not effective for many strains of alpha-reacting streptococci and especially for group D streptococci. Ederer et al. (2) have recently described the use of Pronase B for the enzymatic extraction of group-specific antigens from beta-hemolytic streptococci. This method appears to be effective, but its reported use has been restricted to beta-reacting strains. Moreover, it requires the use of chemically clean glassware and double-distilled water, since heavy metal ions inactivate the enzyme use in extraction.

Our previous studies showed that lysozyme could be used to prepare extracts of group-specific antigen from group D streptococci for serogrouping (Watson, Moellering, and Kunz, unpublished data). Unfortunately, lysozyme was not as effective for preparation of antigens from group A, B, C, and G streptococci. Since Maxted had found *S. albus* extract effective in preparing group-specific antigen from group A, B, C, and G streptococci but not from alpha and group D and K streptococci, we experimented with a combination of lysozyme and *S. albus* extract for preparation of group-specific streptococcal antigens. The resulting method has proven useful for extracting group-specific antigen from virtually all clinically important strains of streptococci. Moreover, it provides a rapid and uncomplicated method that can be utilized in the diagnostic bacteriology laboratory. Reagents are easy to prepare and are stable under laboratory conditions. Chemically clean glassware is not necessary. It is important to emphasize, however, that reliable grouping antisera must be used in conjunction with the enzymatic extraction method. Commercial antisera have proven adequate in our hands for all of the commonly occurring groups except group D streptococci. Commercial group D antisera gave appropriate reactions for most strains of *Streptococcus faecalis* tested but tended to produce weak or negative reactions for certain strains of *S. faecium* and *S. bovis*. Because of this, we have prepared our own group D antiserum and found that it reacts with enzymatically prepared extracts of all species of group D streptococci and not with heterologous serogroups. A prospective evaluation of the combined enzymatic extraction method using clinical isolates of streptococci has demonstrated that it is reliable when used for routine serogrouping. Its rapidity and simplicity and the relatively low cost of the reagents involved make it practical for routine use even in small diagnostic laboratories.

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ADDENDUM

Since submission of this manuscript for publication, El Kholy et al. (3) have described a rapid extraction procedure for beta-hemolytic streptococci utilizing nitrous acid.

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