

Simplified Extraction Procedure for Serological Grouping of Beta-Hemolytic Streptococci

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An adaptation of the nitrous acid extraction of streptococci proved to be a reliable and practical method for the preparation of extracts for routine serological group identification. The extracts of all groups tested gave strong capillary precipitin reactions as well as reactions of double diffusion in gel. For routine grouping, extracts were prepared from the first one-half-plate subculture of the initial throat culture. The technique is simple and reliable, and it requires a minimum of technical skill, reagents, and equipment. Its use would facilitate epidemiological surveillance of group A streptococci and rapid diagnosis of streptococcal infections at a low cost.

The clinical diagnosis of streptococcal infections is strengthened by the identification of group A streptococci in the pharyngeal culture. Identification is commonly achieved by either serological means (3, 4, 6, 9, 10) or by the detection of sensitivity to bacitracin (6). Current serological procedures often require at least 72 h for completion after the initial throat culture is taken. Whereas results from the bacitracin test and from fluorescent microscopy can be obtained in less time, false identification may occur with the bacitracin test, particularly when the procedure is not carefully standardized. Identification by fluorescent microscopy requires special equipment and highly specific antisera (9, 10).

Swanson et al. (12) reported that a single overnight nitrous acid extraction of group A streptococcal cell walls released the bulk of the group-specific carbohydrate. Because of its technical simplicity, the preparation of extracts of beta-hemolytic streptococci by this procedure has been evaluated as an alternate method to prepare extracts for use in routine bacteriologic identification.

MATERIALS AND METHODS

Streptococcal strains. Streptococcal strains from the World Health Organization Reference Laboratory (Prague, Czechoslovakia) for typing streptococci were used as prototypes for each of the corresponding groups: A, B, C, D, E, F, G, H, L, M, N, O, P, and Q.

Field strains (210) of beta-hemolytic streptococci

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were obtained from throat culture surveys of a general population in the Qalyub area near Cairo, Egypt (1).

Grouping antisera. Streptococcal grouping antisera groups A, B, D, E, F, G, L, M, N, and O were obtained from R. C. Lancefield (The Rockefeller University, New York, N.Y.). Grouping antisera for groups C, P, and Q were obtained from the WHO Reference Center (Prague).

Additional rabbit grouping antisera were prepared for groups A, B, C, G, and D with the Lancefield technique of immunization (4) (WHO Laboratory reference strains) and were used for identifying field strains. Antisera and hydrochloric acid extracts of prototype strains were cross-checked for specificity and potency.

Extraction procedures. Three different procedures for the extraction of the group carbohydrates from beta-hemolytic strains were employed. These included: the hot hydrochloric acid (Lancefield) extraction procedure (4), the formamide (Fuller) extraction procedure (3), and the new, nitrous acid extraction procedure recently described by Swanson et al. (12).

The hot hydrochloric acid extraction was performed on the streptococci obtained from 50 ml of a Todd-Hewitt broth culture. The formamide extraction was performed on the streptococci obtained from 5 ml of broth.

The nitrous acid extraction procedure was carried out by suspending the cells of the 5-ml overnight broth culture in 1 drop of saline, 2 drops of 4 M sodium nitrite, and 1 drop of glacial acetic acid for 5, 15, 30, and 60 min, and 24 h. Pasteur pipettes were used to deliver the reagents. Extracts were made at 4 C, 40 C, and at room temperature (22 C). The supernatant fluids obtained after centrifugation at 2,000 rpm for 10 min were adjusted to pH 7.5 with 0.5 N sodium hydroxide.

The same nitrous acid procedure was also used on the streptococci collected, by scraping, from an 18-h

culture on one-half of a sheep blood agar plate. Broth (Todd-Hewitt) and sheep blood agar (Difco base, Difco Laboratories, Detroit, Mich.) were extracted by the nitrous acid procedure to determine whether media components were rendered reactive with the grouping antisera. These extracts gave no reaction with the grouping sera.

Streptococci after carbohydrate extraction with nitrous acid were extracted by the hot hydrochloric acid procedure (13). The extracts were reexamined for group carbohydrate, the serum opacity factor (8), and M protein (13).

Group- and type-specific antigen-antibody reactions were performed by the capillary precipitin technique described by Swift et al. (13), and by the immunodiffusion technique reported by Rotta et al. (11) for M typing.

RESULTS

Nitrous acid, formamide, and hot hydrochloric acid extracts on the prototype strains of each serological group of streptococci were tested with the panel of grouping antisera. In all instances, the extracts in the capillary precipitin reactions and the double-diffusion reaction in agar gave specific reactions with homologous antisera. In general, the precipitates with the nitrous acid extract appeared more flocculent than with the other two extracts, a property that enhanced the visibility of the reaction in the capillary tube. The group D and the group O substances were serologically active after the nitrous acid extraction, a consideration that is important since these group antigens are destroyed by the formamide procedure.

The effects of variation in time and temperature on the nitrous acid extraction of group A carbohydrate are given in Table 1. Extractions were carried out for 5, 15, 30, and 60 min, and 24 h, at 4 C, 22 C, and 40 C. Whereas some carbohydrate was obviously released after 5 min, the 15-, 30-, and 60-min extracts gave the strongest precipitin reactions. The 24-h extracts appeared to be somewhat less reactive than the 60-min extracts, a possible indication of carbohydrate destruction after prolonged treatment. Data similar to those presented in Table 1 were also obtained for groups B, C, D, E, F, G, L, M, N, O, P, and Q. These results suggest that satisfactory extracts for grouping by the capillary test can be obtained in 15 min at 22 C.

In the experiments described thus far, the nitrous acid extracts were prepared from the streptococci grown in 5 ml of Todd-Hewitt broth. However, it would be of considerable practical advantage for the routine diagnostic laboratory if the streptococci which grew on one-half of a blood agar plate were sufficient for

TABLE 1. *Effects of time and temperature on the nitrous acid extraction of group A carbohydrate as detected by the capillary precipitin tests^a*

Temp (C)	Time				
	5 min	15 min	30 min	60 min	24 h
4	++	++	++	+++	+++
22	++	+++	++++	++++	+++
40	+++	++++	++++	+++	+

^aPrecipitin reactions in the capillary tube were graded in the usual fashion; +, ++, +++, +++++.

the preparation of the extract. It is usual for streptococci to be isolated from the primary plate and subcultured onto one-half of a second plate which then serves as a source culture for further identification. The subsequent study shows that sufficient streptococcal growth occurs on such a one-half-plate culture for the preparation of the nitrous acid extract. The bulk of the streptococci which grew on one-half plate after 18 h of incubation was scraped from the surface. Two different streptococcal cultures were tested for each of the streptococcal groups. The streptococci were suspended in one drop of saline and extracted with nitrous acid for 15 min at 22 C. All the extracts gave precipitin reactions as strong as those seen with extracts prepared from the 5 ml of broth culture. The comparison was made with the broth culture extract which had been extracted with nitrous acid for 15 min at 22 C.

Preliminary data suggest that the group A carbohydrate in the nitrous acid extract is identical to that of the formamide extract. As shown in the double-diffusion analysis in agar gel (Fig. 1), the group A carbohydrate in the nitrous acid extract gave a precipitin line of identity with the group A carbohydrate in the formamide extract. The group precipitin reaction in the capillary tube obtained with either extract was inhibited when *N*-acetylglucosamine was added to the group A antiserum at a concentration of 2%, which indicated that the terminal *N*-acetylglucosaminide residues had not been cleaved from the antigen to any significant extent by the nitrous acid treatment. Further work must be done on the carbohydrates isolated from the nitrous acid extract to determine whether it is chemically identical to that isolated from the formamide extracts.

Use of the nitrous acid method in a survey of field strains. Extracts were made from 210 field strains by the formamide procedure and the nitrous acid procedure. The formamide

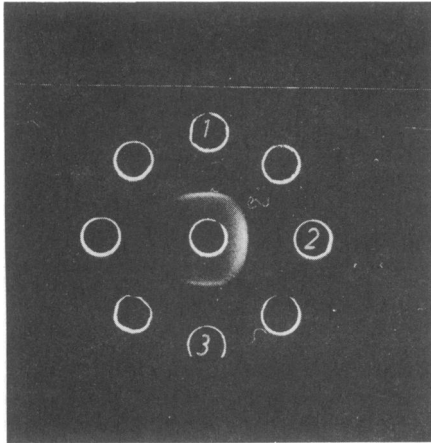


FIG. 1. Double diffusion reaction in agar gel with group A streptococcal extracts and group A antiserum. Center well: group A antiserum; peripheral wells: 1 and 3, formamide extract; 2, nitrous acid extract.

extracts were prepared from a 5-ml Todd-Hewitt broth culture, and the nitrous acid extracts were prepared from the streptococci scraped from one-half of a sheep blood agar plate. The grouping results obtained from the two different extract preparations were in complete agreement. Sixty-three strains were identified as group A, 57 as group C, 68 as group G, and 22 as group B.

Effect of nitrous acid on M protein. In agreement with the findings of Swanson et al. (12), none of the nitrous acid extracts on the 63 group A streptococcal strains gave precipitin reactions when tested with a complete panel of M-type antisera. Thirty of these 63 group A strains were M-typable when hydrochloric acid extracts were employed. To eliminate the possibility that no reactions occurred because the nitrous acid extracts had been prepared from an insufficient growth of bacteria, 50-ml Todd-Hewitt cultures were prepared on 10 M-typable strains. The collected streptococci were extracted with the M-type antisera. In a similar way, nitrous acid extracts were prepared from 10 serum opacity reaction positive strains. These also failed to M-type when nitrous acid extracts were used.

It was assumed that the streptococcal residues after nitrous acid extraction still contained the M protein because: (i) none was detected in the nitrous acid extract, and (ii) Swanson et al. (12) had observed that nitrous acid-extracted streptococci still reacted with ferritin-tagged, M-type-specific antibody. Therefore, hot hydrochloric acid extractions (pH 2) were performed

on the nitrous acid residues of 20 M-typable strains. None of those hydrochloric acid extracts gave a precipitin reaction in capillary tube or in gel agar with the M-type antisera. They did, however, react with group A antisera, which indicated that not all group A carbohydrate had been extracted by the prior nitrous acid treatment.

DISCUSSION

The capillary precipitin test is widely employed in the serological identification of beta-hemolytic streptococci (13). Either hydrochloric acid (4) or formamide treatment (3) has been used to extract the group-specific antigens. However, there are several disadvantages in the use of these extracts for the group precipitin reaction. Considerable effort is required for their preparation, a matter of practical importance when a large number of strains must be identified for epidemiological studies. In addition, the isolation, extraction, and serological identification of the organism usually requires several days after the initial culture was taken. These disadvantages have been circumvented by application of nitrous acid extraction to streptococci recovered from the first blood agar subculture of the initial isolate. Nitrous acid extraction is an adaptation of a procedure described by Swanson et al. (12) which was employed to enhance the demonstration of ferritin-labeled M protein fimbriae by electron microscopy.

The nitrous acid method as described here has proved to be a specific and practical technique for rapid and dependable preparation of extracts for routine grouping of beta-hemolytic streptococci. The method was successful for all serogroups tested; groups A, B, C, D, E, F, G, L, M, N, O, P, and Q. The inhibition of the reaction between the group A nitrous acid extract and group A antiserum by *N*-acetylglucosamine confirms the group A nature of the carbohydrate extracted in this way (5).

For routine identification, extraction was performed for 15 min at 22 C on the streptococci collected from one-half of a blood agar plate, the first subculture after the initial isolation. Use of this subculture rather than a subsequent broth culture simplifies the procedure of grouping streptococci, and this also shortens the time required for identification. Whereas streptococci from a plate could also be used for the *Streptomyces albus* enzyme procedure and for the formamide procedure, the enzyme for the former is not readily available, and the formamide procedure is technically more difficult.

It is true that the nitrous acid extract is not useful for M-typing of streptococci; nevertheless, for many epidemiological surveillance studies recognition of the serological group is adequate, and indeed M-typing sera are unavailable in many areas of the world.

The group-specific carbohydrate of most serogroups of streptococci is a cell wall component. In the case of group D, the group antigen (a teichoic acid) is located elsewhere in the cell (2). Nevertheless, the group D antigen is extracted by the nitrous acid. Furthermore, the serological reactivity is not destroyed, which is also the case of groups O and N. This is an important consideration, because groups N and O antigens are destroyed by formamide extraction. It has yet to be learned whether the procedure is useful for extraction of the type antigens of group B streptococci.

As was mentioned above, prior electron microscopic studies demonstrated that the M protein fimbriae are retained after nitrous acid extraction. It was surprising, therefore, that M protein could not be detected in the hydrochloric acid extract of the nitrous acid residue. It is possible that the nitrous acid treatment has altered the M protein sufficiently so that after subsequent extraction with hydrochloric acid it is no longer serologically reactive in the precipitin test. This matter is currently under investigation.

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