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Comparison of Methods for Obtaining Serum Opacity Factor from Group A Streptococci

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Streptococcal serum opacity factor (OF) is an important type-specific strain marker produced by certain serotypes of group A streptococci (GAS). The accurate determination of OF production and subsequent serotyping of a strain require a reliable method for extracting OF. Traditional OF extraction methods have utilized either culture supernatants or Lancefield HCl extracts. However, these methods do not consistently yield adequate amounts of OF for accurate characterization of all strains. To improve the accuracy of OF detection and serological characterization, a 1% sodium dodecyl sulfate (SDS) OF extraction method was utilized and found to be reproducibly sensitive and suitable for routine laboratory use. A total of 3,014 GAS were examined for OF production by each of the three methods. The SDS extraction method accurately detected OF in all 1,302 OF-producing strains, whereas the HCl extraction method correctly identified 1,202 (92%) and the supernatant method identified only 1,141 (88%) of the isolates. When the 1,302 OF-producing strains were further evaluated to determine if sufficient OF had been extracted for reliable OF serotyping, 1,147 (88%) of the SDS extracts were found to be satisfactory compared with 1,081 (83%) of the HCl extracts and only 936 (72%) of the culture supernatants. The improved ability to characterize OF-producing strains by using SDS was statistically significant compared with the results obtained by the other two techniques. This technique offers distinct advantages, including enhanced detection of OF production and improved reliability of serotyping OF-producing GAS.

Streptococcal serum opacity factor (OF), an enzyme that reacts with and causes opacity in mammalian sera (9, 17, 18), is produced by group A streptococci (GAS) of certain M protein serotypes (3, 5, 14, 21). The differentiation of OF-producing (OF^+) from non-OF-producing (OF^-) strains not only provides a means of classifying GAS into two categories but also provides valuable information about a strain and its potential pathogenic association with suppurative and nonsuppurative sequelae. This information also defines and limits the possible M serotypes to which a strain might belong, thus reducing the time required for M typing and saving valuable M antisera (5). Antibody directed against OF inhibits the activity of the enzyme and is specific for the corresponding M serotype of the producing strain. This type-specific inhibition of OF activity is frequently used as an alternative method for determining the M serotypes of OF^+ GAS, strains which are typically the most difficult to M type. Currently, strains of at least 27 of the officially designated M serotypes and a large number of additional uncharacterized strains are known to produce OF (5, 7).

Thus, both detection of OF production and subsequent OF inhibition serotyping can be extremely useful laboratory techniques. However, it is not uncommon to encounter OF^+ strains that are difficult to characterize because of an inability to obtain sufficient quantities of OF. To address this problem, an OF extraction method using 1% sodium dodecyl sulfate (SDS) and suitable for routine use was developed on the basis of a procedure originally described by Hallas and Widdowson (4). GAS strains (n = 3,014) were randomly selected and extracted by the SDS method as well as the two traditional OF extraction methods, i.e., culture supernatants and Lancefield HCl extracts. The SDS method was found to significantly increase the amount of OF extracted from streptococcal isolates, resulting in the improved detection of OF production and an enhanced ability to characterize strains by OF inhibition serotyping tests.

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MATERIALS AND METHODS

Strains. GAS strains (n = 3,014) representing 38 GAS serotypes, including OF^- types 1, 3, 5, 6, 12, 14, 18, 24, 29, 33, 34, 41, 42, and 55 and OF^+ types 2, 4, 9, 11, 13, 22, 28, 44, 48, 49, 52, 58, 59, 60, 62, 66, 68, 73, 75, 76, 77, 78, 79, and 81, as well as M/OF-nontypeable strains, were evaluated for OF production. These strains included 2,979 clinical isolates randomly selected from strains received in this laboratory between mid-1991 and mid-1994 from the United States and 16 other countries, as well as 35 GAS serotype reference strains included as controls. The GAS clinical isolates were obtained from patients with uncomplicated pharyngitis as well as from patients with severe systemic infections. Additional analyses were performed with GAS M serotypes 8 (University of Minnesota 71682, Lancefield C265, and ATCC 12349), 27 (University of Minnesota 85350, Rotta 38/58), and 44 (University of Minnesota 71853; Colindale original identification number unknown).

Culture supernatants and Lancefield HCl extracts. Culture supernatants and Lancefield extracts were prepared as previously described (7, 20).

1% SDS extracts. The 1% SDS extraction technique used in this study is a modification of the method originally described by Hallas and Widdowson in 1982 (4). GAS strains were grown overnight at 36°C in 8 ml of Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) containing an additional 1% Neopeptone (Difco) and then were centrifuged at 700 (× g) for 30 min. The cell pellets were resuspended in 200 μl of 1% SDS (International Biotechnologies, Inc., New Haven, Conn.) prepared in a sterile 0.85% NaCl solution and transferred to sterile 1.5-ml microcentrifuge tubes. The streptococcus-SDS suspensions were rotated end over end for 1 h at 36°C and then centrifuged for 3 min at 14,926 × g. The clear SDS extracts were collected and then tested for OF activity. All culture supernatant, HCl, and SDS preparations were stored at 4°C until serotyping tests were completed.

Serum OF detection and OF inhibition serotyping tests. OF detection and OF inhibition serotyping tests were performed by the previously described microwell

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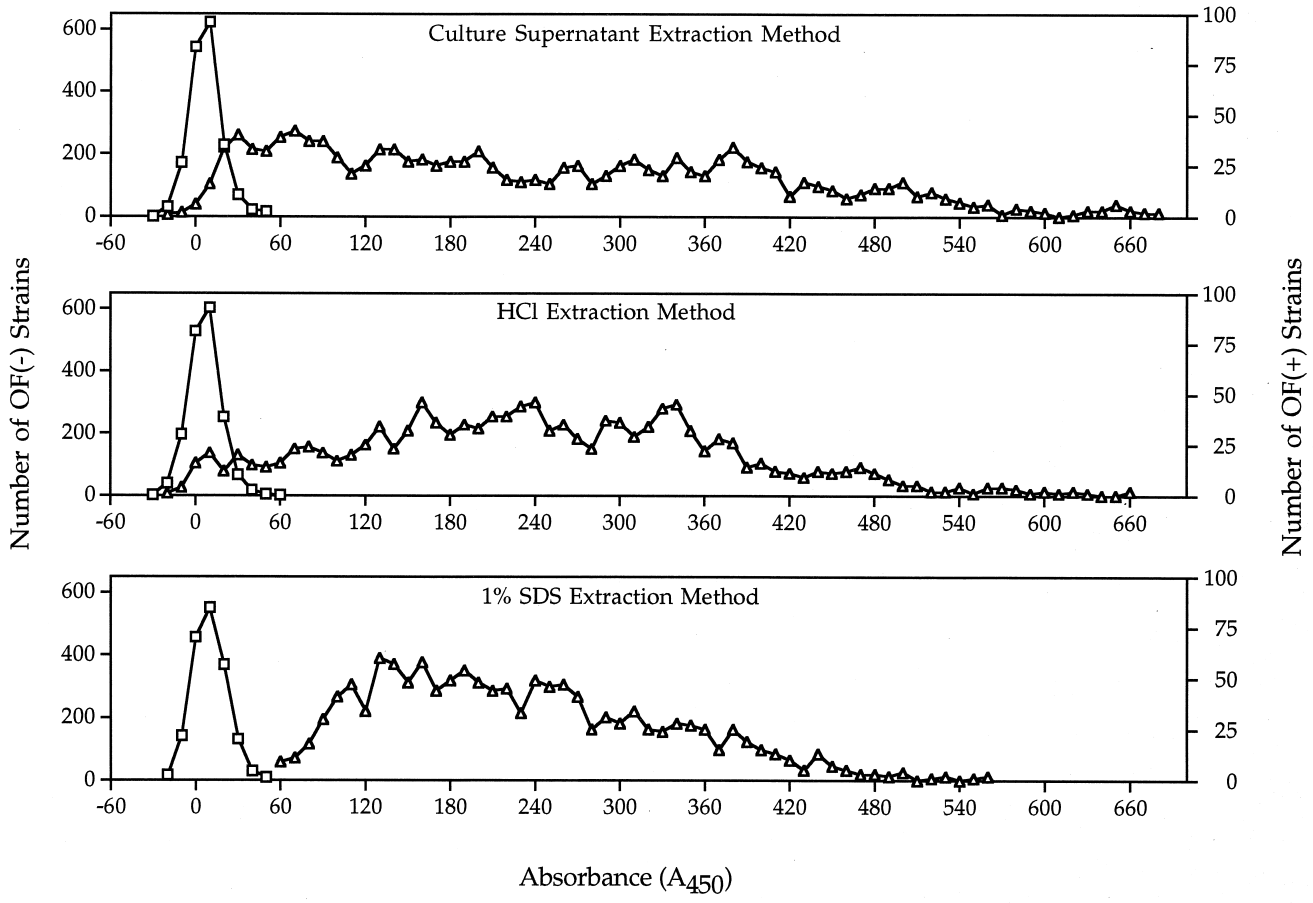

FIG. 1. A_{450} distributions for 3,014 GAS strains compared by OF extraction method. Squares, OF^- extracts; triangles, OF^+ extracts.

method with horse serum (Gibco Laboratories, Grand Island, N.Y.) as the substrate (7, 23). OF activity (opaqueness produced) was spectrophotometrically measured (A_{450}) with a Bio-Tek model EL 340 microplate reader (Bio-Tek Instruments, Winooski, Vt.). OF inhibition serotyping was performed with human anti-OF antisera by a technique previously described by members of this laboratory (7). Anti-OF antisera were available for the following OF^+ serotypes: M/OF types 2, 4, 9, 11, 22, 25, 27, 28, 44, 48, 49, 58, 59, 60, 62, 64, 66, 68, 73, 75, 76, 77, 78, and 81.

Serological characterization. Serological grouping, T protein agglutination, and M protein serotyping tests were performed by previously described methods (10, 11, 16).

Statistical analysis. Chi-square tests with Yate's correction were used for all statistical analyses and were performed with Instat statistical software (Intuitive Software for Science, San Diego, Calif.).

RESULTS

Culture supernatants, Lancefield HCl extracts, and SDS extracts from each of 3,014 GAS strains were evaluated for the ability to produce a serum opacity reaction (SOR). The SORs produced by these extracts were measured spectrophotometrically (A_{450}), thus allowing a quantitative comparison of the three methods (Fig. 1). More opaque (stronger) reactions produced higher absorbance values; less opaque (weaker) reactions produced lower absorbance values. When strains of M serotypes known to be OF^- were analyzed, all except one produced absorbance values of <0.060 U (see below). Therefore, for this study, 0.060 absorbance unit was considered to be the upper absorbance limit for OF^- strains: a strain was defined as OF^- if its SOR was <0.060 U by all three extraction methods and OF^+ if its SOR was ≥0.060 U by any one of the three methods. (It should be noted, however, that the specific absorbance value used in this study to discriminate OF^+ from OF^- reactions is influenced by several factors, especially lot-to-lot variation in the serum used as the OF substrate. Each laboratory should determine an appropriate value for its specific techniques and reagents.)

Virtually identical absorbance values were observed for culture supernatant, Lancefield HCl extracts and SDS extracts of the 1,712 strains defined as OF^- by the criterion described above. However, although the mean absorbance values obtained for the 1,302 OF^+ strains prepared by the different methods were very similar (Table 1), an examination of results for individual strains revealed that 12% (n = 161) of the culture supernatants and 8% (n = 100) of the HCl extracts produced SORs of less than 0.060 absorbance unit and therefore were indistinguishable from OF^- strains (Fig. 1). In con-

TABLE 1. Quantitative comparison of OF preparation methods for OF-producing GAS strains

Method	A_{450} obtained with OF^+ GAS strains (n = 1,302)		No. of strains[a]			
	Mean ± SD	Range	TN	TP	FN	FP
Supernatants	0.246 ± 0.160	−0.016 to 0.687	1,712	1,138	161	0
HCl extracts	0.248 ± 0.130	−0.011 to 0.665	1,711	1,199	100	1
SDS extracts	0.234 ± 0.100	0.061 to 0.568	1,712	1,302	0	0

[a] TN, true OF^- strains; TP, true OF^+ strains; FN, false OF^- strains; FP, false OF^+ strains.

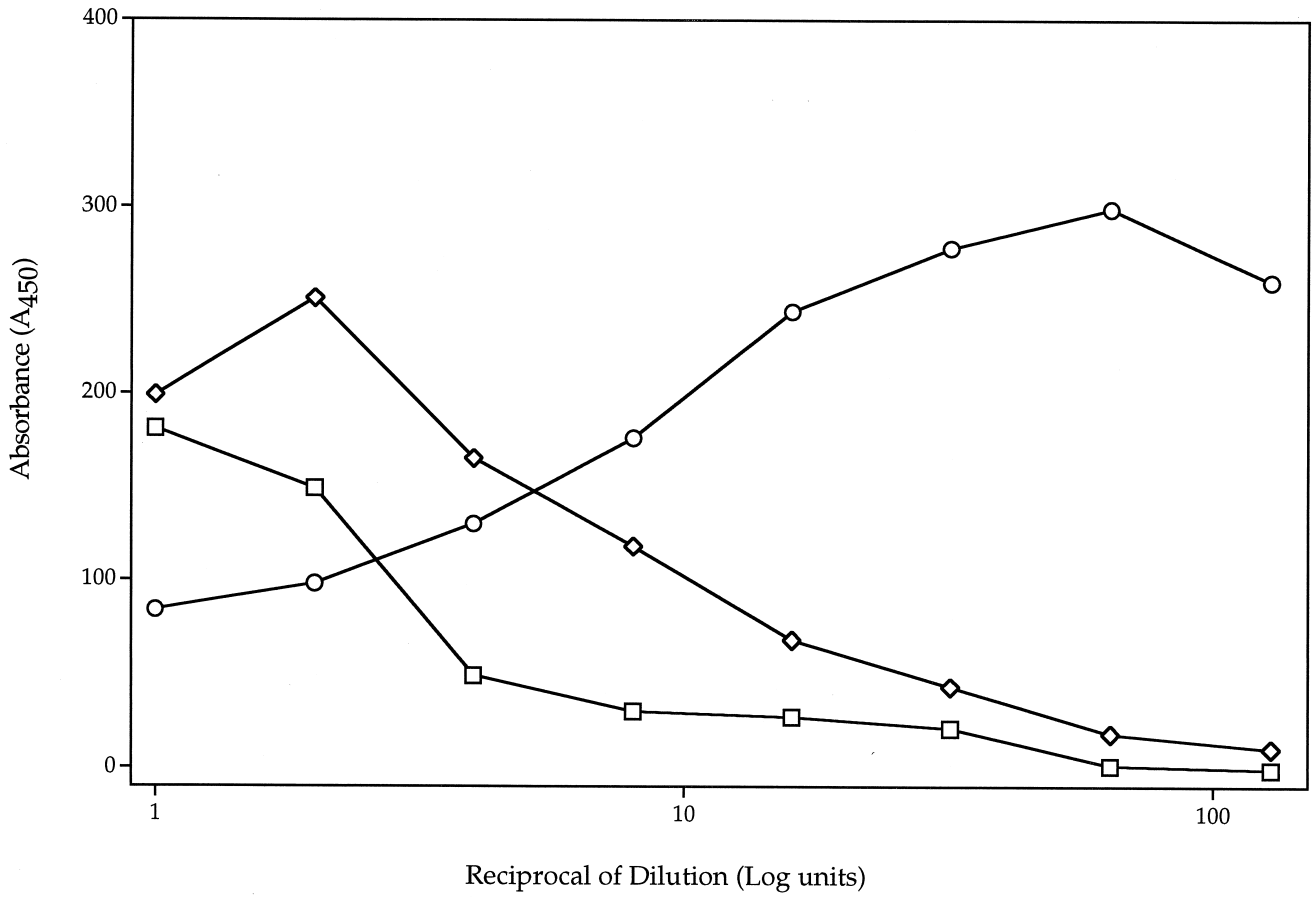

FIG. 2. A_{450} values obtained from twofold serial dilutions of OF prepared by three different extraction methods. The strain tested was an M-nontypeable GAS clinical isolate. Squares, dilutions of the culture supernatant; diamonds, dilutions of the HCl extract; circles, dilutions of the SDS extract.

trast, the SDS extraction method correctly identified all OF producers; no false-negative results were observed. All strains producing SORs of ≥0.060 U by the SDS method only were carefully examined. All either were found to be typeable by the OF inhibition method or, if nontypeable, were found to have their OF inhibited by at least one antiserum. Thus, none of these results were considered to be false positive. The increased number of strains identified as OF^+ by the SDS method was statistically significant compared with the results obtained by either the supernatant or HCl extract method ($P <$ 0.0001 by the chi-square test in both cases).

Serotyping of OF^+ GAS by inhibition of the SOR requires a higher concentration of OF than is necessary for simple detection of the antigen. This is because it is difficult to accurately assess inhibition of opacity if the intensity of the uninhibited SOR is very close to the threshold for detection. Therefore, the 1,302 OF^+ strains were reevaluated by using 0.120 absorbance unit as the minimum for satisfactory use in inhibition tests (twice the upper absorbance limit for OF^- strains). With this higher limit, 28% (n = 366) of the culture supernatants and 17% (n = 221) of the HCl extracts from these strains produced absorbance values of less than 0.120 U. In contrast, only 12% (n = 155) of the SDS extracts produced reactions that were below this value. The improvement in the results obtained with SDS compared with supernatants or HCl extracts was statistically significant ($P \leq 0.0003$ in all cases).

Superficially, this difference between SDS and HCl extracts appears to be minimal (12 versus 17% of the OF^+ isolates with results of <0.120 absorbance unit). However, when SDS extracts of 40 weakly OF^+ GAS strains (0.060 U $< A_{450} <$ 0.120 U) were serially diluted with 0.85% NaCl and retested, all but two of the diluted extracts produced SORs significantly greater than 0.120 absorbance unit (data not shown). In similar tests with culture supernatants and HCl extracts which produced weak opacity reactions, only an occasional HCl extract was found to have an increase in OF activity when diluted. Even then, none of the values increased to the extent observed with the SDS extracts. Therefore, the improvement in the results obtained with SDS extracts compared with supernatants or HCl extracts was probably much greater than is indicated by the percentages shown above.

To illustrate this increase in OF activity with dilution, extracts of an M-nontypeable clinical isolate (University of Minnesota 93037) were prepared by each of the three methods. These extracts were then serially diluted, and the SORs produced by each dilution were compared (Figure 2). Progressive dilution of the culture supernatant resulted in a steady decline in the intensity of the SOR. Dilution of the HCl extract resulted in an initial increase in the intensity of the SOR, with a peak at the 1:2 dilution, followed by a steady decline. In contrast, dilution of the SDS extract resulted in an increase in the intensity of the SOR with a peak occurring at a dilution of 1:64. Therefore, the intensity of the SOR of an undiluted extract may lead to a drastic underestimation of the true OF concentration.

The improved ability of the SDS extraction method to detect

OF was utilized to evaluate three GAS serotypes, M types 8, 27, and 44. These serotypes have frequently been omitted from published lists identifying OF^+ and OF^- M types, thus causing uncertainty regarding their OF production status. Both the M-27 and M-44 strains tested had essentially no OF activity in either undiluted or diluted supernatants or HCl extracts. In contrast, the SDS extracts of both strains produced OF activity in excess of 300 absorbance units, and these strains were clearly OF^+. The M-8 GAS reference strain tested was found to be OF^+ by all three methods. However, the SDS extract contained significantly more OF than did either the culture supernatant or the HCl extract (data not shown).

The antigenic specificity of OF produced by the three extraction methods was also examined and compared. Culture supernatant, Lancefield HCl extracts, and SDS extracts were prepared from an OF^+ M-75 GAS clinical isolate (University of Minnesota 93607), and OF inhibition serotyping was performed with the three extracts. Two human sera (DJ and TB) previously screened and shown to contain antibody to the M/OF-75 serotype, as well as two human sera (JMD and ELK) lacking antibody to M/OF-75 (included as negative controls), were used in the inhibition serotyping tests. Before the testing, each of the OF extracts was diluted to give a comparably intense SOR of approximately 0.200 to 0.300 absorbance unit, thereby standardizing the amount of OF present and requiring neutralization.

On the basis of dilution curves for this OF^+ M-75 isolate, OF inhibition serotyping was performed in duplicate with 10 μl of 1:2-diluted culture supernatant, 10 μl of undiluted Lancefield HCl extract, and 10 μl of 1:256-diluted SDS extract. The mean absorbance values of these duplicate OF extracts in the presence of the two antibody-negative control sera were 0.213 U for the supernatant, 0.241 U for the HCl extract, and 0.309 U for the SDS extract. In the presence of the OF-75 antibody, however, the ability of all three extracts to produce opacity was essentially eliminated. The HCl extracts produced a mean absorbance of only 0.020 U, and both the culture supernatants and the SDS extracts gave mean absorbance values of 0 U, demonstrating that the OF produced by all three methods is equally inhibited by serotype-specific OF antibody. Additional extensive serotyping has been performed for the 1,302 OF^+ isolates by using both SDS-extracted OF and traditionally prepared OF. Twenty-three different serotypes were identified, and, when adequate OF was present, identical results were obtained with all three OF extraction methods (data not shown).

DISCUSSION

The accurate characterization of GAS strains is crucial for a more complete understanding of the epidemiology and pathogenesis of GAS infections and their sequelae. The most widely used method of identification has been by the use of M protein, a cell surface antigen considered to be the primary virulence factor of GAS. However, identification based solely on M protein is complicated by the fact that there are nearly 80 recognized M serotypes as well as many additional uncharacterized types. Furthermore, the preparation of reliable M antisera, which is necessary for accurate strain identification, has proven to be difficult for many serotypes, especially those serotypes producing serum OF (23).

The determination of OF production, a stable characteristic of certain M serotypes (22, 23), not only allows classification of a strain into one of two categories (OF producer or OF nonproducer) but also limits the possible M serotypes to which the strain might belong, greatly reducing the laboratory reagents and effort necessary for serotyping (5). In addition, the OF produced by a strain of a given M serotype is antigenically distinct and specific for that M type (3, 21–23). Type-specific OF antisera, which frequently are easier to prepare than M antisera (2), can be used in OF inhibition serotyping tests as an alternative to M typing (14). Furthermore, if care is exercised, human antisera may be used in this procedure, significantly reducing the need for production of OF antisera (7, 8, 13). Thus, while not obviating the need for M typing, the use of OF techniques considerably enhances the capability to serologically characterize strains and makes at least limited serotyping readily available to more laboratories working with streptococci.

OF production appears to be related, although in ways that are not completely understood, to the pathogenic potential of GAS. Serotypes commonly associated with skin infections (impetigo) and their sequel poststreptococcal acute glomerulonephritis are frequently OF producers. In contrast, OF nonproducers often belong to serotypes considered "throat strains" and are more likely to be associated with acute rheumatic fever or with severe invasive infections such as necrotizing fasciitis and toxic shock-like syndrome (6, 12). Even the M protein of OF^+ strains appears to be basically different from the M protein of OF^- strains: so-called class II M proteins are more commonly associated with OF^+ serotypes, and class I M proteins are more commonly associated with OF^- serotypes (1). Although no specific role of OF in virulence has yet been demonstrated, recent findings suggest that the expression of OF is regulated by *virR*, the regulon which also controls the expression of other well-documented streptococcal virulence factors, including M protein (15).

Understanding the role of OF in infection, as well as successful application of OF techniques for strain characterization and identification, is largely dependent on the ability to reliably extract sufficient quantities of OF from strains. Unfortunately, traditional OF extraction methods (culture supernatants or Lancefield HCl extracts), frequently yield false-negative or very weakly positive SORs. This may be due to the fact that OF appears to be more tightly bound to the cell membrane of certain strains and is therefore more difficult to extract (4, 23, 24). The SDS extraction method significantly improved the ability to detect OF production and increased the number of strains yielding sufficient OF for accurate OF serotyping. Many strains producing SORs too weak for reliable OF serotyping by traditional methods could be serotyped by using SDS-extracted OF.

However, the large amounts of OF extracted by the SDS method, while beneficial, did create some unanticipated complications. High concentrations of OF can actually reduce the intensity of the SOR, giving the impression that the OF extract contains less OF than is actually present (19). As shown in Fig. 2, an SDS extract which initially appeared to possess less OF activity than either the supernatant or the HCl extract did, in fact, contain significantly more OF than either of the other preparations. For OF^+ GAS strains, diluting the SDS extract usually increased the intensity of the SOR to an absorbance value higher than that produced by the undiluted or diluted culture supernatant or HCl extract. It is important to be aware that the use of very high-titer OF preparations would almost certainly lead to errors in serotyping. An assessment of OF concentration by serial dilution and subsequent selection of a dilution giving an appropriate absorbance value falling on the downward slope of the absorbance-dilution curve (Fig. 2) should be performed if this problem is suspected. This will ensure that any inhibition of OF will result in a decrease in the intensity of the SOR.

The SDS streptococcal OF extraction method was found to be suitable for routine use in the streptococcal research laboratory. It significantly increased the yield of OF obtained from GAS and improved the ability to differentiate between OF producers and nonproducers. The use of the SDS method increased the number of strains yielding sufficient OF for accurate OF serotyping, and OF obtained by this method reacted identically to OF obtained by traditional methods in OF inhibition serotyping tests. The improved accuracy of GAS strain characterization obtainable through use of the SDS OF extraction method should prove valuable both to epidemiologists and to scientists involved in studies of this important human pathogen.

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