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Microtechnique for Serum Opacity Factor Characterization of Group A Streptococci Adaptable to the Use of Human Sera

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We have developed a microtechnique for detection of streptococcal serum opacity factor (OF) and for typing of group A streptococci by inhibition of OF. This technique, which involves the use of single wells of standard 96-well tissue culture plates, offers several advantages over previous methods: no advance test preparation is required, allowing tests to be quickly and easily performed; only small quantities of reagents are required; results can be determined visually (qualitative) or by using a photometric enzyme-linked immunosorbent assay plate reader (quantitative); and human serum samples may be quickly and easily screened for OF-inhibitory antibody and subsequently used in place of difficult-to-produce and expensive hyperimmune animal sera for OF characterization of group A streptococci. Fifty-eight samples of normal adult human serum were tested by this new microtechnique for anti-OF antibodies, and 49 (84%) were found to have antibody against 1 or more of the 27 recognized OF-positive serotypes. OF antibodies to M-4, M-2, M-75, and M-48 were most common in these individuals. These 58 human serum samples collectively contained antibody to 25 of the 27 different OF-producing serotypes. Serum samples from four individuals were tested for persistence of OF antibody. OF antibodies to eight different serotypes present in the serum samples collected 7 to 12 years previously were present in the freshly collected sera, indicating that OF antibody persists in human antisera for many years. This new technique has distinct advantages and makes it possible for many laboratories to use this technique to characterize group A streptococci.

Although the efficacy of using streptococcal serum opacity factor (OF) techniques for serotype characterization of group A streptococci is well established (12, 16), OF typing has not been widely used. Previously available techniques are labor intensive, the results may be difficult to interpret, and antisera for OF typing have been difficult and expensive to produce in laboratory animals. To eliminate these disadvantages, we have developed a microtechnique which is quick and simple to perform, is easy to interpret, conserves materials (especially antisera), and permits use of readily available and inexpensive human serum as a source of anti-OF antibodies. This useful technique can now be made available to more laboratories because of this simplicity.

MATERIALS AND METHODS

Group A streptococcal reference strains in our collection representing the 27 currently recognized OF-positive M types (M-2, M-4, M-8, M-9, M-11, M-13, M-22, M-25, M-28, M-48, M-49, M-58 to M-64, M-66, M-68, M-73, M-75 to M-79, and M-81) (1, 17) were obtained originally from Rebecca Lancefield, the Rockefeller University, New York, N.Y.; Richard Facklam, The Centers for Disease Control, Atlanta, Ga.; Geoffrey Colman, Central Public Health Laboratory, London, England; and Jiri Rotta, Institute of Hygiene and Epidemiology, Prague, Czechoslovakia. (Note that M-64 has been described as identical to M-52 [1]. The strain sent to us in 1975 as provisional M-64 [Rabinowitz 932] was not M-52 and is included in these studies as M-64.) Wild-type strains of group A streptococci were obtained from previous and ongoing epidemiological studies in our laboratory. Long-term storage of stock strains was by lyophilization. Short-term storage of strains was in blood broth frozen at −20°C.

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Horse serum (GIBCO Diagnostics, Grand Island, N.Y.) was used as the "substrate" in the serum opacity reaction after multiple lots from several suppliers were screened for suitability. The horse serum was stored at −20°C. Single 100-ml bottles were thawed as needed and heated at 56°C for 30 min to inactivate endogenous enzymes. The pH of the horse serum was not adjusted for use in these studies. Storage for daily use was at 4°C after addition of 0.2 mg of thimerosal per ml to inhibit contamination.

OF was obtained in two ways. Strains were grown for 16 to 18 h at 35°C in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with an additional 2% Neopeptone (Difco). The clear supernatant broth was examined for presence of OF as described below. In addition, standard Lancefield hot-HCl extracts (15) of the same strains were also tested for presence of OF. In the work described here, we generally used HCl-extracted OF.

The microwell method for detection of OF involved the use of standard 96-well tissue culture plates with lids (Costar, Cambridge, Mass.). Horse serum (100 μl) followed by a culture supernatant or HCl extract from the strain to be tested (10 μl) was added to each well. Each test included controls consisting of known OF-positive and OF-negative supernatants or HCl extracts. The plate was sealed with the low-evaporation lid that was provided; it was then inserted into a small plastic bag (to protect the optical surface of the plate). The protected plate was then enclosed in a larger plastic bag containing a moistened gauze sponge and incubated overnight at 35°C. To ensure mixing of reactants, the plate was rotated during the first 15 min of incubation on a Macro-Vue Test Card Rotator (Hynson, Westcott & Dunning, Baltimore, Md.). Before the test results were examined, 100 μl of normal saline was added to each well. Individual wells were visually examined for opacity by using a Microtiter test reading mirror (Dynatech Laboratories,

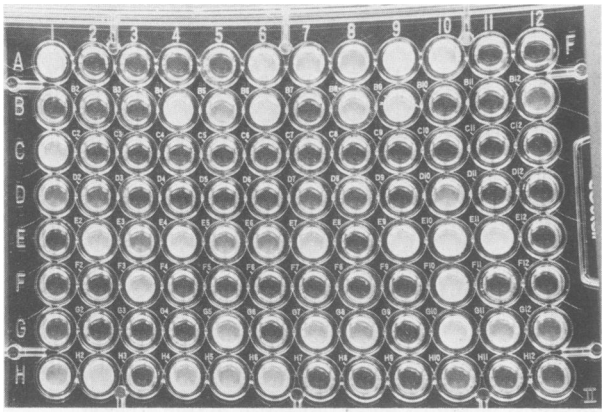

FIG. 1. Detection of OF by using the microwell plate technique (see text).

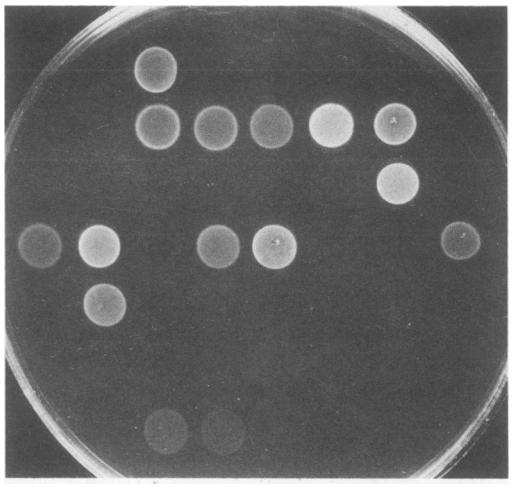

FIG. 2. Detection of OF by using the solid-agar technique (see text).

Inc., Alexandria, Va.). The plate was illuminated by a lamp placed above and behind the stand. A black surface was held above the microwell plate, which was then read by viewing into the mirror through the bottom of the plate, which appeared illuminated against the dark background. By this technique, wells with positive OF reactions appeared white (opaque), whereas wells with negative OF reactions appeared black (clear).

Detection of the presence of OF by the traditional solid-agar technique was done by the method of Maxted et al. (12).

Inhibition of OF by type-specific antibody (OF typing) was performed by the following microwell technique. Culture supernatant or HCl extract, ranging in volume from 2.5 to 10 μl (weak OF producers require the larger volume), from each streptococcal strain to be typed was combined with 10 μl of type-specific human or guinea pig antiserum in a microplate well. Plates were incubated for 1 h at 35°C to enhance antigen-antibody binding before 100 μl of inactivated horse serum was added; the plates were then reincubated at 35°C in a moist environment. After overnight incubation, 100 μl of normal saline was added to each well and the plates were examined visually. All tests incorporated appropriate controls including antibody-negative (or heterologous) sera and OF-negative extracts, supernatants, or saline. Quantitation of results was obtained when desired by measuring the A_{450} of the plates in a photometric enzyme-linked immunosorbent assay-type plate reader (Flow Laboratories, Inc., McLean, Va.). Each microplate contained wells with appropriate blank solutions. The A_{450}s of these blanks were subtracted from the A_{450}s of the corresponding test wells to give the most accurate quantitation of serum opacity. During the early phases of this study, concomitant determinations for OF inhibition were compared with the solid-agar method of Maxted et al. (12).

Antisera for OF typing were either raised in guinea pigs by the procedure of Fraser (2) or obtained from a bank of reference human serum samples collected from laboratory personnel. The earliest human serum samples tested were collected in 1975. All samples had been stored at −20°C; small aliquots were preserved with 0.02% thimerosal and held at 4°C for routine use during these studies.

RESULTS

Detection of OF production. The microwell method for detection of OF production is shown in Fig. 1. HCl extracts from 96 different strains of group A streptococci were added to the individual wells containing horse serum. Clearly visible are the strongly positive serum opacity reactions (opaque or white) produced by such strains as the M-4 OF-positive control in well A-1 and definite negatives (clear or dark) such as that in well A-2. All gradations can be seen, as in the less-evident but clearly positive reaction in well D-2. Complete preparation of this microwell plate for detection of OF production in 96 strains took only approximately 20 min.

In the macrotechnique (Fig. 2), the agar contains horse serum with HCl extract spotted on the agar surface. The opacity produced by the OF-positive strains, including the same M-4 positive control (top row, left), is visible. Various gradations of opacity are also evident with this technique. Preparation of this agar plate, which has only 40 tests, also took approximately 20 min, but this does not include time required for preparation, autoclaving or drying of the plates. This is considerably longer than the time required by the microtechnique.

OF typing. Inhibition of opacity by specific antibody (OF typing) with the microwell technique is illustrated in Fig. 3.

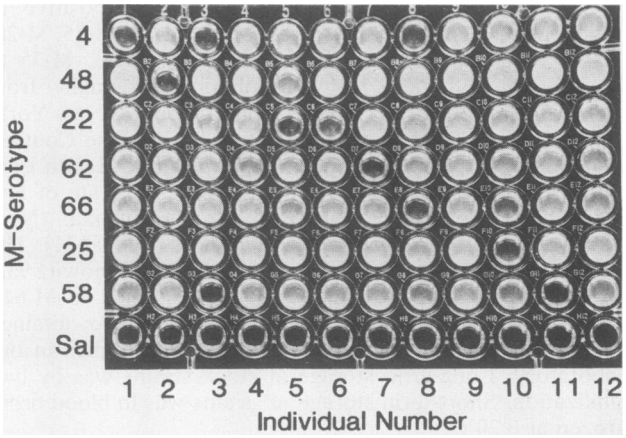

FIG. 3. Inhibition of the serum opacity reaction by specific antibody with the microwell technique (see text).

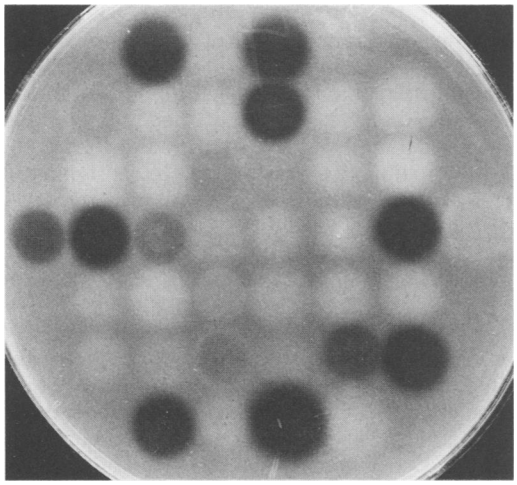

FIG. 4. Inhibition of the serum opacity reaction by specific antibody with the solid-agar technique (see text).

The wells of each vertical column contain 10 μl of a different anti-OF antiserum (in this case, human). The wells of each horizontal row contain 2.5 μl of OF (HCl extract) from a different reference group A streptococcal serotype. Strains which occasionally or commonly share T-agglutination typing patterns are grouped on the plate (e.g., M-4 and M-48 commonly share T-4 antigen; M-22, M-62, and M-66 may share the T-12 antigen; M-25 and M-58 are usually associated with the 8/25/Imp.19 T-agglutination pattern). In the wells of the bottom row, saline has been substituted for OF as a control (or blank) to detect unrelated antiserum turbidity (lipemic serum) which might result in a false impression of opacity. All wells also contain 0.1 ml of undiluted and inactivated horse serum.

Inhibition of the serum opacity reaction by type-specific antibody results in a clear (black) well. For example, in Fig. 3, M-4 OF is inhibited by serum from individuals 1, 3, and 8, and M-48 is inhibited by serum from individual 2 and less strongly by serum from individual 5. Other wells showing inhibition are also clearly visible. Microwell OF inhibition typing of unknown strains can be accomplished by this same technique. The total time involved in preparation of a 96-well plate for OF inhibition testing is only approximately 25 min.

For comparison, Fig. 4 shows OF inhibition by the agar method. The agar contains horse serum and OF from the same reference M-4 strain as in Fig. 3. The same 12 human serum samples shown in Fig. 2A, as well as 28 additional normal human serum samples, are spotted on the agar surface. Inhibition of opacity appears as a clear (dark) spot against the opaque (white) background. The three sera showing inhibition of M-4 OF correlate exactly by the microwell plate and the solid-agar method. Additional sera with anti-OF-4 activity are also evident. The agar plate took approximately 25 min to prepare, not including the time required for autoclaving and drying. If multiple strains were to be tested as in the microwell plate shown in Fig. 3, multiple agar plates, and hence a significant increase in preparation time, would be required.

OF antibodies in human sera. Fifty-eight recently collected normal adult human serum samples were screened for anti-OF antibody by the microwell technique with extracts from all 27 recognized reference OF-positive serotypes of group A streptococci. The plates were examined visually, and the results were also quantitated by the photometric microplate reader. The absorbance values for each well containing human serum plus extract were corrected by subtracting the value of the human serum-saline blank. The resulting absorbance more accurately represents the amount of opacity due to OF in the presence of that human serum sample. The sera were evaluated as having either strong inhibition (complete blocking of opacity), partial inhibition (partial blocking of opacity with an absorbance greater than two standard deviations below the mean for all antibody-negative human sera tested with extracts of that strain), or none (no visual inhibition and no significant decrease in absorbance). For example, in Fig. 3 there appeared to be inhibition of M-48 by serum from individual 5, although this inhibition was not as complete as that shown by serum from individual 2. The mean A_{450} for serum samples from all individuals (except individuals 2 and 5) with M-48 was 416 with a standard deviation of 37. Serum from individuals 2 and 5 gave A_{450}s of 1 and 113, respectively. Therefore, serum from individual 5 fulfilled the previously stated requirement for partial inhibition, and serum from individual 2 fulfilled the requirement for strong inhibition of the M-48 OF.

TABLE 1. Prevalence of antibody to group A streptococcal serotypes in 58 normal human serum samples

No. of OF antibodies/ sample	No. (%) of individual samples with OF inhibition
1	18 (31)
2	8 (14)
3	12 (21)
4	5 (9)
≥5	6 (10)

Of the 58 human serum samples tested, 49 (84%) contained detectable antibody to the OF of one or more of the established streptococcal serotypes (Table 1). Of these, nearly two-thirds (31 of 49) had antibodies to at least two different serotypes. One serum sample contained antibodies to nine different types. With the more stringent requirement of strong (total) inhibition, nearly two-thirds of individuals tested (37 of 58) were antibody positive, and of these, nearly 60% (22 of 37) had antibodies to more than one type.

In serum samples from these 58 individuals (adults living in Minnesota), OF-inhibitory activity was most frequently observed with M-4 (34% of individuals tested), M-2 (29%), M-75 (22%), and M-48 (19%) (Table 2). These 58 human serum samples had antibody to 25 of 27 (93%) recognized OF-positive reference strains of group A streptococci.

Persistence of OF antibody. Serum samples from several of the 58 individuals studied had been collected and stored in previous years. Samples from four of these persons were reexamined to determine the persistence of serum anti-OF antibody. Table 3 shows the inhibition reactions of the paired serum samples from these individuals; nine different streptococcal serotypes were used. OF antibodies to eight different serotypes present in the previously collected frozen serum samples were also present in the recently collected samples. Individual 2 had antibodies to types 2 and 28 in 1979 that were still present 8 years later. Individual 4 had antibodies to types 4, 48, 59, 75, and 78, all of which persisted for 7 years. Individuals 1 and 3 showed persistence of OF antibodies for 12 years. Loss of OF antibody over periods as long as 12 years was never detected.

Development of OF antibody in human sera was also observed. Individual 1 acquired anti-OF-4 following docu-

TABLE 2. Prevalence of OF antibodies to 27 reference serotypes in 58 normal human serum samples

M serotypes	No. (%) of individuals with OF inhibition
4	20 (34)
2	17 (29)
75	13 (22)
48	11 (19)
9, 22, 59	7 (12)
78	6 (10)
62	5 (9)
28	4 (7)
49, 58, 76	3 (5)
11, 25, 60, 63, 66, 77	2 (3)
61, 64, 68, 73, 79, 81	1 (2)
8, 13	0 (0)

mented pharyngitis with this serotype in his children, although he was not clinically ill and did not have streptococci of this type isolated during this period. Individual 3 developed OF-58 antibody following documented pharyngitis due to M-58 streptococci.

DISCUSSION

Identification of group A streptococci based on specific M-protein type is an essential component in studies of the pathogenesis, epidemiology, and therapy of streptococcal infections. However, this system is limited by the fact that many M serotypes are poorly immunogenic in rabbits. These M antisera are difficult to prepare (17), their availability is limited, and they are expensive. Many of these difficult serotypes produce OF, an enzymelike substance which has the ability to produce opacity when combined with various mammalian sera (9, 13, 14, 18). This OF production is consistently and exclusively associated with specific M-serotype streptococcal strains (3, 16, 17, 19). Demonstration of OF production not only allows the classification of that strain into OF-positive and OF-negative categories but also facilitates the typing process by limiting initial typing efforts to M types that correspond to the OF production status of that strain (OF^- or OF^+). This results in a significant saving in time and material, especially in M typing antisera. Equally important, characterization of group A streptococci by OF typing can be used instead of M typing for the 27 currently recognized OF-positive strains (6, 11, 12, 17). This greatly enhances the typing capability of laboratories involved in group A streptococcal serotype identification.

TABLE 3. Persistence and development of antibody to OF in human serum samples collected 7 to 12 years apart

Serotype	Presence of antibody in[a]:							
	Individual 1		Individual 2		Individual 3		Individual 4	
	3/75	3/87	6/79	3/87	2/75	2/87	12/79	2/87
2	−	−	+	+	+	+	−	−
4	−	+	−	−	+	+	+	+
28	−	−	+	+	−	−	−	−
48	−	−	−	−	−	−	+	+
58	−	−	−	−	−	+	−	−
59	−	−	−	−	−	−	+	+
62	+	+	−	−	−	−	−	−
75	−	−	−	−	+	+	+	+
78	−	−	−	−	−	−	+	+

[a] Symbols: +, antibody positive; −, antibody negative. Dates are given as month/year.

Turbidimetric methods (so-called tube tests) for detection of OF production have been used since the initial discovery of streptococcal OF by Ward and Rudd in 1938 (18). Photometric quantitation of OF as applied to OF typing of streptococci in a macrotube test was described by Top and Wannamaker (16), and quantitative macrophotometric methods continue to be used by those desiring quantitation of test results (4, 5, 11, 19). The reliability of photometric technology as applied to OF testing is well established. However, the most significant problem with the original photometric techniques has been the requirement for large quantities of sera and reagents. The solid-agar method reduced significantly the volume of one reagent (the reagent spotted on the agar surface) but increased the preparation time for the actual test. Other inherent limitations of the solid-agar methodology were the difficulties in typing weakly OF-producing strains and in visually demonstrating weakly positive inhibition reactions from true-negative reactions.

The microwell method described here essentially eliminates all of these problems. The test may be set up very quickly, since it requires no advance preparation of plates. This economy of labor is one of the most important benefits of the technique. Only small quantities of material are required, generally 10 μl or less, making the test economical in this regard as well. Weak OF producers may often be satisfactorily tested simply by increasing the amount of OF added to the well in the microtechnique. The ability to obtain rapid quantitation of results by the use of an enzyme-linked immunosorbent assay reader is another important advantage of this technique. An apparent weak-positive reaction can easily be compared with a large number of true-negative reactions to allow an objective, statistically based decision about the significance of the result. Also, the opacity caused by turbid antisera (often resulting in a visually unreadable test) can be eliminated by using a plate reader and subtracting appropriate blanks. The sensitivity of the microwell method for detecting OF production was higher than that of the agar method when read photometrically and slightly but insignificantly lower when read visually.

The technical aspects of the micro-OF technique are not complex. The effect of pH on detection of serum opacity was examined by using doubling dilutions of OF-positive extracts with horse serum adjusted to pH 6.0 versus unadjusted horse serum. The lower-pH extracts showed approximately a one-dilution increase in sensitivity in detecting opacity. However, we believe that the advantage of simplicity in using unadjusted horse serum outweighed this slight increase in sensitivity obtained by pH adjustment. In limited studies examining the effect of pH on OF typing in microwells, a pH in the range of 7.2 to 7.5 was found to give optimal inhibition of opacity. Since this was the normal pH of our horse serum, no pH adjustment was used in the routine procedures described. Since pH adjustment may be required with different lots of horse serum, researchers in individual laboratories may wish to evaluate this variable.

The traditional wavelength of 475 nm (4, 5, 7, 8, 11, 12, 16) for reading serum opacity reaction absorbance in the tube test was not available on our enzyme-linked immunosorbent assay plate reader. Parallel experiments performed at 410, 450, and 492 nm all gave satisfactory results. The final selection of 450 nm was largely arbitrary. There was excellent correlation between visual semiquantitative determination of opacity (negative to 4+) and the quantitative A_{450}

readings obtained from the plate reader. Our results were usually read by two individuals, and agreement was consistent.

Culture supernatants and HCl extracts of equal OF content performed comparably in these systems. Previous parallel OF testing by the solid-agar method of supernatants and HCl extracts from 244 OF-positive strains showed that in 232 (95%), the HCl extract was equal in strength to or stronger than the culture supernatant. Therefore, HCl extracts were used in the majority of these experiments. Strains whose HCl extracts were negative for OF and which were also M nontypable were routinely tested for OF production by using culture supernatants. To date, no additional OF-positive strains have been detected by this duplicate testing. Also, strains whose HCl extract OF levels were so low as to make inhibition typing difficult were retested with culture supernatants. For a very small percentage of strains, a higher-strength supernatant facilitated OF typing.

These studies verify that OF inhibition typing of group A streptococci may be performed with either hyperimmune animal or human sera. Parallel studies, not reported here, in which both human sera and guinea pig sera (serotypes 11, 25, 60, 77, 78, and 81) were used gave comparable results. Although clearly an advantage over production in animals, the use of human sera does have potential disadvantages. We (Table 1) and others (7, 8) have found OF antibodies to multiple serotypes in individual human serum samples. Therefore, it is possible that the use of such sera for OF typing will lead to incorrect interpretation unless special care is taken. It is important that all human serum samples to be used as antisera in the microtest be screened with all 27 currently recognized OF positive M types, and with OF-positive provisional types, so that monospecific samples may be selected if possible. If this is not possible, serum samples which react only with M serotypes not sharing a common T-pattern (e.g., M-2 and M-4) should be selected. To eliminate typing errors due to anti-OF antibodies directed against as-yet undescribed serotypes, we also require OF type-specific inhibition of a given unknown strain by serum samples from two or more individuals. These precautions have resulted in an accurate and reliable typing system when human sera are used. We have not recognized any errors when using these criteria.

We think there are important advantages to the use of human sera in OF inhibition typing by the microtechnique. Large quantities can be collected. Usable sera may be obtained immediately; it is not necessary to wait weeks or months for an adequate antibody response in rabbits or guinea pigs. Furthermore, it is not difficult to build a serum bank. By screening a relatively small number of individuals, we obtained antisera suitable for OF inhibition typing for more than 90% of the currently recognized OF-positive serotypes (Table 2). If these percentages are consistent and applicable in other geographical areas, use of human sera would allow a laboratory beginning an OF typing program to become operational very quickly. The microtechnique system is accurate even without the enzyme-linked immunosorbent assay reader, making the system practical for laboratories which cannot afford to purchase the equipment.

Determination of OF-antibody prevalence may be useful in evaluating previous population exposure to specific OF-positive serotypes, information which may not be projected with certainty from bacteriologic serotype prevalence data. Parallelism of OF antibody and protective anti-M bactericidal antibody has been demonstrated (11), and it is likely, as proposed by Iontova and Totolian (7), that this test can also provide a means for measuring population immunity to specific streptococcal serotypes.

Lancefield (10) demonstrated the very long persistence of anti-M bactericidal antibodies in human serum. Ours is the first study to document prolonged persistence of OF antibodies in human serum (Table 3). Of eight different OF type-specific antibodies found in serum samples collected and stored in past years, all were present in serum samples collected for this study after a time interval of 7 to 12 years. In fact, not once did we document loss of antibody activity or significant reduction in strength of antibody. The possibility that this was not true persistence but rather was due to an immunological boost from undocumented exposure or infection cannot be ruled out. However, the relative rarity of several of these strains, combined with the absence of acquisition of antibody to these types (except following documented exposure as noted in the text), would argue against that possibility.

The experienced streptococcal typing laboratory, as well as the newly established or less experienced laboratory, can benefit from the inclusion of OF technology in its streptococcal identification procedures. The microtechnique described here eliminates many of the drawbacks of traditional OF methodologies: it requires no significant preparation time, it requires small quantities of reagents, and it gives results which may be quantitated if desired. With proper precautions, human sera can be easily evaluated for OF antibodies and used to serotype OF-producing streptococci, thus eliminating (or at least postponing) the significant time and expense required for production of OF-specific antisera in animals.

We believe that the new microtechnique for detecting OF and for OF typing now allows the addition of a useful, specific, and economic method of characterization of group A streptococci.

ACKNOWLEDGMENTS

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