Inhibition Enzyme-Linked Immunosorbent Assay for Serotyping of Group B Streptococcal Isolates

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Group B Streptococcus (GBS) is one of the most common organisms causing neonatal sepsis as well as serious infections in adults. Serotyping the organism is important in studying the epidemiology of the disease as well as deciding a course of treatment. There are several methods available for serotyping. Most of them need high-titered sera and are not quantitative. We are reporting a new inhibition enzyme-linked immunosorbent assay (ELISA) for serotyping which is sensitive and specific compared to the conventional methods but does not need high-titered serotype-specific antisera, as the specificity is controlled by the polysaccharide coating on the ELISA plates. The method can also be quantitative, and we have measured polysaccharide elaborated by different serotype V strains. Thus, the inhibition ELISA method will be useful in serotyping for epidemiological studies, assessing virulence, and performing strain selection for vaccine production.

Materials and Methods

Strains and typing sera. The GBS strains used in our studies were provided by one of the authors (P.E.), Vincent Fischetti (Rockefeller University, New York, N.Y.), and Rick Schumann (Virion Corporation, Rockville, Md.). The IGIV was obtained by immunizing volunteers with a GBS vaccine containing purified polysaccharides from types Ia, Ib, II, and III and then obtaining plasma during a 2- to 3-month period after immunization. The plasma was pooled into two lots and processed by the Swiss Red Cross into a preparation suitable for intravenous administration (7). This preparation in a lyophilized form was a gift from Gerald Fisher (Uniformed University for Health Sciences, Bethesda, Md.). As the IGIV did not contain antibodies to type V GBS polysaccharide, the inhibition ELISA for serotyping GBS type V strains was performed with rabbit anti-type V serum, which was a gift from Rick Schumann. GBS strains were grown on sheep blood agar plates (Remel, Columbia, Md.) at 37°C overnight, and colonies were transferred to 5 ml of Todd-Hewitt liquid medium (Difco Laboratories, Detroit, Mich.). Frozen cultures were also directly transferred to Todd-Hewitt liquid medium and grown for 20 to 24 h at 37°C. The liquid cultures were then heat killed for 45 min in a 56°C water bath. The cell suspensions were then neutralized by adding 1 M sodium hydroxide, with 10 μl of phenol red used as an indicator. These culture suspensions were used in the inhibition ELISA for serotyping. Immununon 4 plates (VWR Scientific) were coated with purified GBS polysaccharides of types Ia, Ib, II, III, and V (North American Vaccine Inc., Beltville, Md.). The coating solution consisted of 50 μl of polysaccharide solution (1 mg/ml) and 5 μl of methylated human serum albumin (1 mg/ml) in 10 μl of 1× phosphate-buffered saline made in tissue culture-grade, pyrogen-free sterile water (Bio fluids Inc., Rockville, Md.) containing 0.02% sodium azide (3). A 100-μl aliquot of this solution was added to coat each well of the microtiter plate. Plates were sealed and left overnight at room temperature.

Inhibition ELISA. The steps in the inhibition ELISA procedure are depicted in Fig. 1. Briefly, 160 μl of a broth culture from each isolate (heat killed and neutralized) was dispensed in duplicate into a 96-well Nunc microtiter plate. A 160-μl aliquot of a 1/1,000 dilution of IGIV (lot 006) in serum conjugate buffer (1× phosphate-buffered saline containing 0.5% newborn calf serum, 0.1% Brij 35, 0.05% sodium azide) was added to each sample well and incubated for 40 min at room temperature (3). Immunon 4 plates, previously coated with GBS polysaccharides Ia, Ib, and III, were washed; 100 μl of serum conjugate buffer was added to each well and incubated for 1 h at room temperature (3). The solutions were then removed and plates were washed. To the wells of the microtiter plate, 160 μl of GBS type V antigen was added and incubated for 1 h at room temperature. Next, plates were washed and 150 μl of a 1/1,000 dilution of rabbit antibody to type V GBS polysaccharide was added and incubated for 30 min at room temperature. The plates were then washed, and 150 μl of a 1/2,000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories, Hercules, Calif.) was added and incubated for 30 min at room temperature. To the plates, 150 μl of a 1/5 dilution of 3,3′,5,5′-tetramethylbenzidine hydrochloride was added, and the reaction was stopped by adding 150 μl of 1 N sulfuric acid. The absorbance at 450 nm was then read, and the results were compared with the absorbance of negative control wells (3).

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Grow strains in 5 ml of liquid Todd-Hewitt medium for 20-24 h.

Heat inactivate for 45 minutes at 56°C in a water bath.

Add 10 μl of 0.5% phenol red solution and about 100 μl of 1 M NaOH to neutralize the cultures.

Vortex the suspensions and pipette 160 μl of each strain in duplicate into to a 96 well microtiter plate and add 160 μl of diluted GBS polysaccharides and incubate for 1 h at room temperature.

Wash plates and add a suitable dilution of anti human IgG alkaline phosphatase conjugate, incubate plates at 37°C for 1 h.

Wash plates and develop color with p-nitrophenyl phosphate.

FIG. 1. Serotyping by inhibition ELISA.

RESULTS

To determine the sensitivity of the inhibition ELISA, serial dilutions of purified polysaccharides of GBS serotypes Ia, Ib, II, and III from 0.08 to 2.50 μg/ml were incubated with IGIV (1/1,000 dilution) for 40 min and then transferred to Immulon 4 plates coated with type Ia, Ib, II, or III GBS polysaccharide. Percent homologous inhibitions with type Ia GBS polysaccharides and heterologous inhibitions with type II and III GBS polysaccharides are shown in Fig. 2A, and percent homologous inhibitions with type Ia, II, and III GBS polysaccharides are shown in Fig. 2B. As can be seen in Fig. 2A, incubation of IGIV with GBS polysaccharide Ia inhibits the binding of IGIV to GBS type Ia polysaccharide-coated plates by >90%, while the heterologous polysaccharide types II and III do not inhibit the binding of IGIV to GBS type Ia polysaccharide-coated plates. Figure 2 depicts the concentration of GBS polysaccharides and percent inhibition of IGIV binding as measured by the inhibition ELISA. Types Ib and V gave results comparable to those shown in Fig. 2B. Taking 50% inhibition as the minimum essential for determining a serotype, the amount of homologous polysaccharide that can be detected at 50% inhibition is about 0.1 μg/ml.

Validation of the results obtained by the inhibition ELISA method was done by testing 98 isolates (from the collection of P.F.) under code. Eighty-six were invasive isolates, and of these 75 were from a prospective study of early-onset disease (12, 13). Four additional GBS isolates from the Rockefeller University collection were also tested blindly. The isolates were first typed at the University of Minnesota (UM) reference laboratory by immunodiffusion in agarose using Lancefield hot-HCl cell extracts and reference antisera to GBS polysaccharides Ia to VIII (12, 13). These strains were then typed by inhibition ELISA. Table 1 shows the results of typing by the two methods. There was complete agreement between the two methods.

FIG. 2. (A) Inhibition of binding of IGIV to GBS polysaccharide type Ia by homologous and heterologous GBS polysaccharides. IGIV (1/1,000 dilution) was incubated with GBS polysaccharide types Ia, II, and III at concentrations of 2.50 to 0.08 μg/ml. For homologous inhibition, 100 μl from the preincubated mixture of type Ia GBS and IGIV was transferred to GBS type Ia polysaccharide-coated Immulon 4 plates. For heterologous inhibition, 100 μl from the preincubated mixture of IGIV with GBS polysaccharide type II or III was transferred to GBS type Ia-coated Immulon 4 plates. (B) Homologous inhibition of binding of IGIV to GBS polysaccharide type Ia, II, and III coated plates, respectively. Inhibition ELISA was performed as described in the text.
methods for the results obtained for serotypes Ia, Ib, II, and III. With regard to type V, there was a discrepancy with two strains. One strain, AP101692, was typed as type V by inhibition ELISA and as type IV with a weak cross-reaction with type V by immunodiffusion. Another strain, MM97002132, was typed as type V by ELISA and as NT by immunodiffusion. Eight other strains were not typeable by ELISA into any of the five types examined. These NT strains were typed as types IV, VI, VII, and VIII by immunodiffusion using sera specific for types not examined by ELISA.

We also received 12 strains with assigned serotypes by capillary precipitation from the Rockefeller University. The results for 10 strains were concordant with those from the Rockefeller Laboratories. The two discordant strains as well as two other discordant strains were retyped blindly at the UM. The results matched those of the inhibition ELISA for all four of the strains. Two strains of GBS type V were provided by Rick Schumann and were typed by the inhibition ELISA. Both strains were correctly identified by inhibition ELISA as type V.

The inhibition ELISA method can also be used to quantitate the amount of polysaccharide present in a culture. This can be very useful in comparing strains from patients and carriers or in selection of strains for vaccine production. Five strains of type V exhibiting different degrees of inhibition by inhibition ELISA serotyping were selected, and 24-h broth cultures were adjusted to the same initial cell density. Inhibition ELISA was performed with serial dilutions of the cultures, and the results are shown in Fig. 3. Three strains have similar inhibition curves, whereas two others showed varying degrees of inhibition, suggesting the production of different amounts of polysaccharide.

### DISCUSSION

Serotyping methods that have been used in the last 30 years for typing GBS include immunodiffusion, capillary tube precipitation, and CIE (4, 10, 14). Pulsed-field gel electrophoresis is a new addition and was found to be a reliable method for typing the most common streptococci which cause bovine mastitis, but it requires specialized equipment (2). The relative sensitivities of capillary precipitation (tube and ring), slide agglutination, immunodiffusion, and fluorescent antibody techniques are similar, as are the specificities of all these methods. They all require high-titered type-specific antisera which are not commercially available. CIE is more sensitive and less time-consuming than these other serotyping methods. CIE has an additional advantage of using the patient specimen directly, without requiring overnight growth of cultures or an extraction procedure (10, 14). CIE detected 14 μg of antigen per ml when a commercial antiserum from Difco was used and 0.7 μg of GBS type III polysaccharide per ml when a high-titered burro antiserum to type III GBS polysaccharide (serum not commercially available) was used (14). None of these methods are more than semiquantitative.

ELISA methods have been used for the measurement of innumerable antigens and antibodies (11). We report here an inhibition ELISA for serotyping GBS strains. Initially, high-titered type-specific antibodies were not available in the laboratory, but a GBS hyperimmune IGIV was available. However, it is important to note that the inhibition ELISA can also be performed with rabbit antiserum raised against individual serotypes, as we have shown with rabbit antiserum to type V.

Purified type-specific GBS polysaccharides and not whole bacteria are used for coating the plates which determine the specificity of the assay and not the type of antiserum that is used. In the inhibition ELISA using purified GBS polysaccharides from different serotypes, the amount of homologous polysaccharide detected at 50% inhibition was 0.1 μg/ml, which could have been determined using inhibition ELISA.

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<th>Typing method</th>
<th>No. of strains determined to belong to GBS serotype:</th>
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<tr>
<td></td>
<td>Ia</td>
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<tr>
<td>Inhibition ELISA</td>
<td>32</td>
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<tr>
<td>Immunodiffusion</td>
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* Nine strains NT by inhibition ELISA were serotyped as IV (n = 5), VI (n = 2), VII (n = 1), and VIII (n = 1) in the UM reference laboratory.
be quantitated with inhibition curves. With regards to specificity, the inhibition ELISA is as specific as the conventional methods. Comparison of serotyping of samples that were earlier analyzed by immunodiffusion and tube precipitation methods revealed that the results obtained by the inhibition ELISA agreed with the analysis of the five serotypes. In the case of two strains whose inhibition ELISA results were type V and whose immunodiffusion results showed one strain to be nontypeable and the other to be type IV with a slight cross-reaction with type V, it was possibly an issue of sensitivity of the methods, as inhibition ELISA may be more sensitive. For the other strain, one cannot exclude the possibility that it is truly type IV with a cross-reaction to type V antiserum.

Forty samples in duplicate can be assayed at one time in a microtiter plate. The process can also be automated for washing plates when a large number of plates are being handled at one time. We have serotyped over 1,600 strains of GBS by this method. This procedure is suitable for large-scale serotyping either with IGIV or with antiserum that has been raised to different serotypes of GBS.

An interesting outcome of this procedure is that the amount of polysaccharide made by a given strain can be quantitated by the inhibition ELISA by using dilution curves with pure polysaccharides as references, similar to the method reported by Holm and Hakansson (11). As shown in Fig. 2, different type V strains cause inhibition to different extents, depending on the amount of capsular polysaccharide present. This can be useful for studying strains isolated from patients with invasive disease and healthy carriers to see if there are inherent differences among them. It is well known that capsular polysaccharide plays a role in virulence although it is not the only factor. Hakansson et al. (9) isolated high- and low-density subpopulations of GBS and observed that the low-density variants had enhanced virulence. Among the strains that have been serotyped by inhibition ELISA, there are strains with varying inhibitions among all the serotypes that have been tested, and further studies are planned to pursue these differences.

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REFERENCES


