Purification and Serological Characterization of a Type-Specific Antigen of *Streptococcus equisimilis*

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A microtiter complement fixation (CF) procedure was developed for use in detection of antibodies in sera of swine and rabbits vaccinated with *Streptococcus equisimilis*. Crude ultrasonic as well as acid-extracted preparations contained CF antigen, but the ultrasonic procedure resulted in a higher yield of active antigen. Evidence of serotype specificity with varying degrees of cross-reactivity was detected with the CF procedure when representative strains of four different serotypes of *S. equisimilis* were compared by using their respective unadsorbed antisera. Fractionation of crude sonic extract or acid extract by centrifugation, precipitation with ammonium sulfate, and chromatography on carboxymethyl cellulose yielded a purified, type-specific antigen that reacted only with the homologous antiserum in the CF test and formed a single band by immunodiffusion. Complement-fixing antibodies in immune swine sera were predominately immunoglobulin G.

Beta-hemolytic streptococci, especially Lancefield group C strains identified as *Streptococcus equisimilis*, are frequent causes of septemia and polyarthritis in neonatal pigs (24). The same organisms are common in tonsils of normal swine of various ages and in vaginal secretions and mammary glands of normal sows and sows with reproductive disorders and agalactia. The significance of the infections in sows is not known, except that they undoubtedly are the principal source of streptococci causing disease in baby pigs.

In studies of the immune response of man to group A streptococci, particular emphasis has been focused on M protein, a factor associated with virulence and protective immunity (17). Immunity to these organisms in man has been shown to be directly related to the presence of homologous type-specific anti-M antibodies. M protein-like antigens were originally described in group C streptococci by Lancefield (16) and Griffith (14). Subsequently, type-specific protein antigens have been described in strains of *S. dysgalactiae* (7), *S. zooepidemicus* (20), *S. equisimilis* (15), and *S. equi* (3, 4). Immunity against *S. equi* in horses has been shown to be directly related to the presence of antibodies against a single proteinaceous substance found in the capsule (27). Evidence obtained by Baskhi and Singh (1, 2) indicated that immunity to group C organisms causing mastitis in cattle was associated with an antigen found in *S. dysgalactiae* and that it was capable of cross-protection against *S. equisimilis*.

The purpose of the work presented here was to further characterize the serological activity of a type-specific antigen from *S. equisimilis* with the ultimate goal of applying techniques developed for study of the immune response of swine to the organism.

**MATERIALS AND METHODS**

**Streptococcal cultures.** Group C streptococci used in this work consisted of *S. equisimilis* strain 78-A (serotype I), R-8 (serotype II), F-49 (serotype III), and 78-B (serotype IV). The strains were originally isolated from arthritic joints and vaginal secretions of swine (15). A *S. zooepidemicus* strain of unknown origin was obtained for R. A. Packer (Iowa State University, Ames, Iowa), and a group L strain of unknown origin was obtained from the American Type Culture Collection (Rockwell, Md.). Cultures were stored lyophilized at 4 C. All work was done with cultures that were no more than five passages from original isolation.

Strain 78-A was passaged three times in young pigs by intravenous inoculation in an attempt to enhance its virulence and immunogenicity. This strain, designated 78-A3X, had an arthritic dose 50 in swine of 2 × 10⁴ colony-forming units (CFU), whereas the parent strain 78-A had an arthritic dose 50 of 7.2 × 10⁵ CFU. The term "arthritic dose" was devised to denote the number of *S. equisimilis* cells required to produce arthritis in 50% of inoculated swine. The swine-passage strain had a mean lethal dose for Swiss Webster mice of 5 × 10⁴ CFU, whereas the parent strain had a mean lethal dose of 4 × 10⁶ CFU

**Antigen preparations.** Streptococcal cells used for preparation of antigens were grown in Todd-Hewitt broth for 16 to 18 h at 37 C and harvested by
centrifugation at 5,000 × g for 20 min in a refrigerated centrifuge. Cells used for production of ultrasonically disrupted (UD) antigen were resuspended in 0.1 M potassium phosphate-buffered saline (PBS) pH 7.55, and the suspensions were adjusted to contain 10⁶ CFU/ml. A curve prepared by plotting the number of CFU in a series of culture dilutions of known density at 550 nm was used for standardization of cell suspensions. Cells were disrupted by use of a Biosonic II ultrasonic disintegrator (Bromwell Scientific Co., Rochester, N.Y.) at 95% capacity for 20 min and centrifuged at 3,000, 30,000, or 100,000 × g for 30 min at 4 C.

Hot acid extracts were prepared by the procedure developed by Lancefield and Perlmann (18) with slight modifications. Sedimented cells were mixed with an equal volume of 0.2 N HCl, heated in a boiling water bath for 10 min, cooled, and centrifuged. The supernatant was neutralized by adding 0.2 N NaOH in PBS. The extracts were stored frozen at −20 C until used.

Alkaline extracts were prepared from whole cells by the procedure originally developed by Fox and Wittner (12). A 25% (vol/vol) suspension of whole cells in PBS was adjusted to pH 10.0 with 1.0 M NaOH, heated in a boiling water bath for 10 min, cooled, and centrifuged. The supernatant was neutralized by adding 0.1 M HCl. This material was centrifuged, and the supernatant fluids were held at −20 C until used.

Antigens used for indirect hemagglutination (IHA) consisted of supernatant of UD cells (UD 3000) that had been centrifuged at 3,000 × g, whereas those used for complement fixation (CF) consisted of supernatant of UD cells (UD 30000) that had been centrifuged at 30,000 × g. Both preparations were stored at −70 C.

Partially purified, type-specific S. equisimilis antigen was prepared by the procedure used by Fox (9) for preparation of type-specific antigen from group A streptococci. Cell walls were obtained by passing a slurry of cells in PBS (0.15 M NaCl, 0.1 M K₂PO₄, pH 7.55) through a French press at 18,000 lb/in² or from ultrasonically disrupted cell suspensions. After disruption, the suspensions were washed three times in PBS, and the walls were separated by centrifugation at 5,000 × g for 30 min at 4 C. The walls were resuspended in 4 volumes of PBS, treated with ribonuclease (10 μg/ml) (Worthington Biochemical Corporation, Freehold, N.J.) for 4 h at 37 C, washed twice with PBS 5,000 × g, and resuspended at a concentration of 25% in PBS. The cell walls were extracted with acid by usual procedures, centrifuged twice, neutralized, and pooled.

Fractionation of the pooled supernatant fluids was done with 33 to 60% ammonium sulfate. Initially, 17 g of ammonium sulfate was slowly dissolved in each 100 ml of supernatant fluid, and the mixture was allowed to stand at room temperature overnight. The resulting precipitate was removed by centrifugation at 17,000 × g for 30 min at 4 C and discarded. The supernatant fluid was adjusted to pH 8.0 with 1 N NaOH and brought to 60% saturation by adding 13.9 g of ammonium sulfate per 100 ml. This solution was mechanically stirred for 1.5 h at 4 C and then centrifuged at 17,000 × g for 30 min at 4 C. The 33 to 60% pellet was solubilized in distilled water and dialyzed against PBS for 18 h at 4 C. After dialysis, 2 ml, equivalent to 50 mg (dry weight) of the 33 to 60% pellet, was layered onto a carboxymethyl cellulose (CMC) column (1.5 by 24 cm) equilibrated with 0.003 M sodium acetate, pH 4.0. Fractions (5 ml) were collected with a fraction collector (Instrumentation Specialties Co., Lincoln, Neb.), and the protein peaks were monitored with an ISCO model UA-5 ultraviolet analyzer at 280 nm with a 3-mm optical path. The column flow rate was regulated to 0.5 to 1.0 ml per min by means of a peristaltic pump. The pH and ultraviolet absorption of each sample was monitored and recorded. Elution was begun with a 0.1 M sodium acetate buffer at pH 5.5. When the effluent reached pH 5.5, a three-chambered gradient elution was initiated by using 75 ml of 0.1 M potassium phosphate buffer at pH 6.0, 75 ml at pH 6.5, and 200 ml of pH 7.0. The gradient was continued until the effluent reached pH 7.0. After fractionation, each peak was lyophilized, reconstituted in a small amount of PBS, and dialyzed against PBS for 18 h at 4 C. After dialysis, the fractions were stored at −20 C.

Type-specific antigen was separated from crude sonic extract by a modification of the method used with acid extracts of cell walls. Prior to chromatography on CMC, the crude sonic extract was dialyzed for 2 days at 4 C against four changes of PBS prepared with sodium instead of the usual potassium salts. This saline was designated PBS-Na.

Antiser production. Swine were given a series of six intravenous injections of merthiolate-killed whole cells (1, 2, 2, 4, 4, and 10 billion) at 3-day intervals. Epinephrine was given to prevent a non-specific shock that sometimes developed in swine following intravenous injection of protein-containing material. Live streptococcal cells (3.35 × 10⁶ and 1.85 × 10⁶ CFU) were given intravenously to one group of swine at 7 and 14 days, respectively, after the last killed antigen had been inoculated. All swine were bled 7 days after the final injection of live or killed cells. Rabbits were inoculated intravenously at 3-day intervals with six increasing doses of cells (1, 2, 2, 4, 4, and 10 billion). Ten days later, an additional 10 billion cells were given, and the rabbits were bled 7 to 10 days later. Sera were stored at −20 C and heated at 56 C for 30 min prior to use.

Type-specific antisera were prepared by adsorbing rabbit or swine sera with at least two heterologous strains as described previously (15). Sera samples (1 ml) were adsorbed with 0.2 ml of wet, packed, whole cells at 37 C for 2 h and then centrifuged to remove particulate material.

Serological methods. Indirect hemagglutination was done with UD 3,000 antigen by the microtiter procedure developed by Zimmerman et al. (28) with slight modifications. Sheep erythrocytes, which had been collected and stored in Alsevers solution for 3 to 5 days at 4 C, were washed in PBS and treated with 1:10,000 tannic acid. The tanned cells were washed, suspended with an optimum antigen-sensitized solution, rewashed, and suspended in PBS at a concentration of 0.5% (vol/vol). The sensitized cells were
added to diluted antisera and held at 4°C overnight. The results were recorded the following morning. Optimum antigen dilutions were determined by block titration against standard streptococcal antisera.

The Laboratory Branch CF procedure as modified for microtiter (13) was used with UD 30,000 antigen. Complement was reconstituted with normal swine sera by the method of Slavik and Switzer (25). Optimum antigen dilutions were determined by block titration against standard streptococcal antisera.

Immunodiffusion analysis of streptococcal antigens was done as described previously (15). Chromatography and immunoelectrophoresis of swine sera were conducted by methods previously described (22).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was conducted by the method of Razin and Rottem (21) with a slight modification. Approximately 140 μl of antigen preparation containing approximately 150 μg of protein was applied to each tube. Electrophoresis was carried out in 7.5% gel with a cationic system at room temperature for 3 h at a constant current of 3.5 mA per gel.

RESULTS

Comparison of nonadsorbed and adsorbed antisera prepared in rabbits against four different serotypes of *S. equisimilis* with the CF procedure revealed marked evidence of serotype specificity (Table 1). Similarly, specificity also was detected when antigen preparations from the four different serotypes were compared in the CF and IHA tests with hyperimmune sera prepared in swine against strain 78-A. Similar complete specificity was detected with the sera used in Table 1 when they were tested against their respective antigens in immunodiffusion and IHA tests.

Comparison of various *S. equisimilis* preparations indicated that supernatant from ultrasonically disrupted cells (UD 30,000) was the most potent CF antigen and that it resulted in minimal nonspecific hemolysis and anticomplementary activity. The UD 30,000 procedure resulted in a CF antigen yield approximately 180 times greater, on a per cell basis, than that obtained by extraction with HCl (Table 2). In addition, the optimally diluted UD 30,000 preparation reacted at a fourfold higher dilution of a standard swine antisera than did HCl extract.

Immunodiffusion (ID) analysis revealed that UD 30,000 and UD 3,000 preparations of strain 78-A3X were essentially the same and contained at least three separate precipitating antigens when evaluated against a homologous, nonadsorbed rabbit antisera (Fig. 1). Acid extracts derived from *S. equisimilis* strain R-8 (serotype II) and *S. zooepidemicus* contained one precipitating antigen common to strain 78-A3X that was probably the group C carbohydrate. Other antigens demonstrated by ID in the HCl and UD preparations of *S. equisimilis* strain 78-A3X were a nontype-specific antigen and *S. equisimilis* serotype I antigen. Precipitating antigens cross-reactive with *S. equisimilis* serotype I antisera were not detected in an HCl extract of a group L strain. Adsorbed type-specific sera formed a single precipitin band.

### Table 1. Comparison of four serotypes of *S. equisimilis* by CF

<table>
<thead>
<tr>
<th>Antigen prepn</th>
<th>Serotype I (78-A)</th>
<th>Serotype II (R-8)</th>
<th>Serotype III (78-B)</th>
<th>Serotype IV (F-49)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NA*</td>
<td>TS*</td>
<td>NA</td>
<td>TS</td>
</tr>
<tr>
<td><em>S. equisimilis</em> 78-A HCl</td>
<td>320&lt;sup&gt;4&lt;/sup&gt;</td>
<td>320</td>
<td>160</td>
<td>&lt;10</td>
</tr>
<tr>
<td><em>S. equisimilis</em> 78-A UD</td>
<td>640</td>
<td>320</td>
<td>80</td>
<td>&lt;10</td>
</tr>
<tr>
<td><em>S. equisimilis</em> R-8 HCl</td>
<td>40</td>
<td>&lt;10</td>
<td>2,560</td>
<td>1,280</td>
</tr>
<tr>
<td><em>S. equisimilis</em> R-8 UD</td>
<td>40</td>
<td>&lt;10</td>
<td>1,280</td>
<td>640</td>
</tr>
<tr>
<td><em>S. equisimilis</em> 78-B HCl</td>
<td>20</td>
<td>&lt;10</td>
<td>40</td>
<td>&lt;10</td>
</tr>
<tr>
<td><em>S. equisimilis</em> 78-B UD</td>
<td>20</td>
<td>&lt;10</td>
<td>160</td>
<td>&lt;10</td>
</tr>
<tr>
<td><em>S. equisimilis</em> F-49 HCl</td>
<td>20</td>
<td>&lt;10</td>
<td>160</td>
<td>&lt;10</td>
</tr>
<tr>
<td><em>S. equisimilis</em> F-49 UD</td>
<td>20</td>
<td>&lt;10</td>
<td>80</td>
<td>&lt;10</td>
</tr>
<tr>
<td><em>S. zooepidemicus</em> HCl</td>
<td>10</td>
<td>&lt;10</td>
<td>40</td>
<td>&lt;10</td>
</tr>
<tr>
<td><em>S. zooepidemicus</em> UD</td>
<td>10</td>
<td>&lt;10</td>
<td>80</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Gr L 9932 HCl</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Gr L 9932 UD</td>
<td>&lt;10</td>
<td>20</td>
<td>40</td>
<td>80</td>
</tr>
</tbody>
</table>

* NA, nonadsorbed antisera.
* TS, Type-specific antisera prepared by whole cell adsorption.
* HCl extract.
* Reciprocal titer determined by duplicate block titration.
* Ultrasonic-derived antigen centrifuged at 30,000 × g.
Table 2. Potency of complement-fixing antigen in various preparations of S. equisimilis 78A

<table>
<thead>
<tr>
<th>Prepn</th>
<th>Optimal dilution for maximal sensitivity</th>
<th>Reciprocal serum titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Working dilution</td>
<td>Actual dilution</td>
</tr>
<tr>
<td>Acid-extracted whole cells</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>Acid-extracted walls</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Alkaline-extracted whole cells</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>UD 3,000&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8</td>
<td>1,240</td>
</tr>
<tr>
<td>UD 30,000</td>
<td>32</td>
<td>5,760</td>
</tr>
<tr>
<td>UD 100,000</td>
<td>32</td>
<td>5,760</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reciprocal of dilution of each preparation which gave the highest titer in a block titration with a standard antiserum.

<sup>b</sup> Relative potency of each preparation, determined by combining the working dilution factor with the dilution resulting during preparation of the extract from packed cells.

<sup>c</sup> Nonabsorbed, homologous swine sera.

<sup>d</sup> Ultrasonically disrupted and centrifuged at 3,000 × g, 30,000 × g, or 100,000 × g.

with homologous antigen and failed to react with antigens prepared from strain 78-B.

Attempts to prepare purified, type-specific CF antigens from sonic extracts and acid or alkaline extracts by starch block electrophoresis and molecular sieving chromatography failed. Immunodiffusion analysis of fractions from these procedures indicated that all fractions containing type-specific antigen were contaminated with the group antigen. Fractionation of acid extract by differential centrifugation, precipitation with ammonium sulfate, and chromatography on CMC or fractionation of crude sonic extract by precipitation with ammonium sulfate and chromatography in CMC yielded a preparation that contained one antigen detectable by immunodiffusion (Fig. 2 and 3). Two bands were demonstrated by electrophoresis of the acid extract-derived CMC preparation in polyacrylamide gel. The elution profile of hot-acid extract chromatographed on CMC is presented in Fig. 4. The elution profile for the sonic extract was nearly identical to that of the hot-acid extract. The purified antigen (peak 3 of Fig. 4) reacted with nonadsorbed or adsorbed antiserum against the homologous serotype in the CF and immunodiffusion procedures; however, it did not react with nonadsorbed or adsorbed antiserum against heterologous serotypes, suggesting that it was type specific (Table 3 and Fig. 3).

Postimmunization sera of six swine exhibited a sequential rise in CF and IHA antibodies to S. equisimilis strain 78-A when compared to the preimmunization sera. The geometric mean titers of CF antibodies were <10 on day 0, 40 on day 14 (3 injections), and 267 on day 35 (8 injections). The geometric mean titers of IHA antibodies in the same sera were <4 on day 0, 12 on day 14, and 48 on day 35. None of sera collected from four swine that received placebo vaccine had detectable CF or IHA antibodies against S. equisimilis. Fractions of serum from a vaccinated pig obtained by chromatography on Sephadex G-200 were shown by immunoelctrophoresis to contain predominately immunoglobulin M in peak 1 and immunoglobulin G in peak 2. Most of the IHA activity (reciprocal titers; peak 1 = 128 and peak 2 = 16) was present in the immunoglobulin M fraction, whereas the immunoglobulin G fraction contained most of the CF

![Fig. 1. Immunodiffusion analysis of ultrasonic-derived antigens. Well AS contained nonabsorbed rabbit antiserum against S. equisimilis 78-A (serotype I), well 1 contained a crude HCl extract of S. equisimilis 78-A, well 2 contained a UD 30,000 extract of S. equisimilis 78-A, well 3 contained a UD 3,000 extract of S. equisimilis 78-A, well 4 contained a crude HCl extract of S. equisimilis R-8 (serotype II), well 5 contained a crude HCl extract of S. zoonepedemicus, and well 6 contained a crude HCl extract of group L strain 9932.](http://iai.asm.org/Downloadedfrom)
activity (reciprocal titers; peak 1 = 20 and peak 2 = 160).

DISCUSSION

Immunity to group A streptococcal infection in man has been shown to be at least partially type specific (17). The in vitro bactericidal test has been one of the most reliable methods for

serological assessment of this immunity (23). Results obtained with bactericidal immunity tests in our work with S. equisimilis and swine sera were inconclusive; thus, other test methods were devised for evaluation of the humoral antibody response of swine. Bone et al. (6) used crude acid extracts to detect CF antibodies in serum from glomerulonephritic patients that also had type-specific bacteriostatic activity. Recently,

FIG. 2. Stages in the purification of S. equisimilis serotype I antigen. Well AS contained nonabsorbed rabbit antiserum against S. equisimilis 78-A3X, well 1 contained a crude HCl extract of S. equisimilis 78-A3X, well 2 contained an HCl extract of S. equisimilis 78-A3X after ammonium sulfate precipitation, and well 3 contained purified type-specific antigen from S. equisimilis 78-A3X.

FIG. 3. Specificity of purified serotype I antigen. Well CMC contained purified serotype I antigen, well 1 contained rabbit antiserum against S. equisimilis 78-A3X, well 2 contained rabbit antiserum against S. equisimilis R-8 (serotype II), well 3 contained rabbit antiserum against S. equisimilis 78-B (serotype III) and well 4 contained rabbit antiserum against S. equisimilis F-49 (serotype IV).

FIG. 4. Elution pattern of S. equisimilis serotype I antigen from a CMC column (1.5 by 24 cm). The protein content is recorded by the solid line; pH is recorded by the broken line.
Wittner and Fox (26) used a purified acid extract in a micro CF test for detection of type-specific antibody in serum of patients with group A infection. They presented evidence that it could be substituted for the bactericidal test as a measure of protective immunity. In our initial work, crude acid extracts and ultrasonically derived whole cell antigens were used in CF and IHA procedures to demonstrate a serological response in vaccinated swine. Because of cross-reactions with other serotypes, it was necessary to purify the antigens. Further purification of the preparations suggested that the principal antigens in the crude extracts involved in the CF test were identical to the type-specific antigen. The IHA activity of the purified antigen was not evaluated.

Evidence has been presented that ultrasonic disruption of streptococcal cells results in liberation of antigen that is in a more natural form than that obtained by acid extraction (5). The native protein is apparently hydrolyzed during the acid-extraction procedure, resulting in alteration of its physicochemical form and antigenicity. Extraction under alkaline conditions has also resulted in less alteration than occurs with the acid-extraction procedure (12). Use of these various extraction procedures has resulted in comparable yields of type-specific antigen from group A streptococci (5). In contrast, ultrasonic treatment of S. equisimilis resulted in a much higher yield of antigen active in the CF test than did acid extraction. Possibly the type-specific antigen of S. equisimilis is partially destroyed under acidic conditions.

Purification of type-specific antigen from crude sonic extract with CMC required a variation in dialysis before addition to the column. Antigen that had been dialyzed against PBS containing potassium salts bound irreversibly to the CMC. Dialysis of the antigen against PBS-Na produced an antigen that behaved similarly to the HCl extract on the CMC column, although there still appeared to be some retention of antigen.

Analysis of the UD 30,000 and UD 3,000 preparations by immunodiffusion revealed essentially no difference; both preparations contained the group and type-specific components. Evidence obtained by starch block electrophoresis and molecular sieving chromatography indicated that the type-specific component was not of a single species, but rather a composite with different densities and molecular weights, all of which appeared to react similarly with the type-specific antisera. The antigen may have a multiple molecular structure similar to the type-specific antigen found in group A (11).

The need for multiple injections of streptococcal cells or their components to produce high-titer antibodies in man and animals has been demonstrated previously. Bazeley and Battley (3, 4) and Engelbrecht (8) found that three doses of S. equi vaccine were necessary before complete immunity developed in horses. Similar results have also been obtained during vaccination attempts in humans with purified M protein extracts (10, 19). The sequential rise in swine CF and IHA antibodies suggest this may also be required in swine. The serological activity of chromatographed swine streptococcal antibodies in CF and IHA tests was consistent with previous reports concerning immunoglobulin G and M antibodies.

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**LITERATURE CITED**


