Indirect Enzyme-Linked Immunosorbent Assay for Detection of Antibody to a 110,000-Molecular-Weight Hemolysin of Actinobacillus pleuropneumoniae

JIANNENG MA AND THOMAS J. INZANA*
Department of Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Received 1 November 1989/Accepted 13 March 1990

Swine pleuropneumonia is caused by Actinobacillus (Hemophilus) pleuropneumoniae and is characterized by fibrinous pleuritis, with hemorrhagic and necrotic lesions. A peracute or acute outbreak in nonimmune herds may lead to high mortality, whereas chronic or subclinical infections may persist in carrier animals, which serve as a reservoir for new infections (27). Definitive diagnosis of pleuropneumonia is based on clinical symptoms, typical lung lesions, and isolation of the organism. Determination of past infection, chronic infection, or carrier animals requires serologic testing.

Although a variety of serologic methods have been developed to detect antibodies to the 12 serotypes of A. pleuropneumoniae (12, 23, 27), most utilize whole cells or crude extracts as the antigen. Complement fixation (CF) (12) is the standard assay used for detection of antibodies to A. pleuropneumoniae, and this test is used for national testing of sera at the Iowa State University Diagnostic Laboratory, Ames. However, CF may give false-positive results due to the use of whole cells as antigen, lacks sensitivity, and cannot be used with anticomplementary serum (24, 26). An enzyme-linked immunosorbent assay (ELISA) (16) and a radioimmunoassay (15) have been described that specifically detect antibody to the capsule of A. pleuropneumoniae, but both assays require the use of purified capsule and are serotype specific. Capsule purification and radioisotope usage are beyond the scope of most clinical laboratories, and the serotype specificities of the assays compel their use for serological confirmation, rather than for screening.

A. pleuropneumoniae produces one or more hemolysins that may be important in virulence (1, 4, 8–10, 17, 21) and are common to many of the serotypes (5, 11, 17, 25). Antibody to hemolysin can be quantitatively measured by neutralization of hemolytic activity (17, 21, 25). However, crude materials have been used in the neutralization assays, and since more than one hemolysin may be produced by this organism (11), it is difficult to ascertain what factor is being measured. Furthermore, neutralization assays are time-consuming, laborious, and require that the hemolysin retain biologic activity. Heat-labile 104,000- to 110,000-molecular-weight hemolysins (104K to 110K hemolysins) from serotypes 1 and 5 have been identified and purified, and at least some of the genes coding for this hemolysin have been cloned and sequenced (3, 4, 7, 8). To facilitate detection of antibody to this hemolysin (characterized as 110K in our laboratory and by others [3, 7]), an indirect ELISA was developed that did not require that the toxin retain biologic activity and detected antibodies to a 110K protein from all serotypes tested.

(This work was presented, in part, at the 70th Annual Meeting of the Conference for Research Workers in Animal Disease, Chicago, Ill., November 1989.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of A. pleuropneumoniae used have been previously described (14, 16), except for a nonhemolytic mutant which was recently isolated by chemical mutagenesis (J. Ma, J. Todd, and T. Inzana, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, B180, p. 60). For most assays, Actinobacillus isolates were grown in 500 ml of an ultraltrate of Casamino Acids-yeast extract medium (molecular weight, less than 10,000) contain-

* Corresponding author.
ing 5 μg of β-NAD per ml and 1 mM CaCl₂, as previously described (9, 14).

To ascertain the specificity of the capture antibody for the 110K A. pleuropneumoniae hemolysin, clinical isolates of Pasteurella haemolytica, alpha-hemolysin-producing Escherichia coli 1691 (obtained from the Virginia-Maryland Teaching Hospital Clinical Bacteriology Laboratory) and alpha-hemolysin-producing E. coli SH1 (33) (generously provided by Richard Hull, Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Tex.), and Bordetella bronchiseptica (obtained from Hugo Veit, Virginia-Maryland Regional College of Veterinary Medicine) were included in some assays. P. haemolytica and alpha-hemolysin-producing E. coli were examined because the toxins of these bacteria and A. pleuropneumoniae are antigenically and genetically conserved (3, 5). B. bronchiseptica was examined because it is a common swine pathogen. The presence of alpha-hemolysin in both E. coli strains was confirmed by hemolytic activity in culture supernatant, and the reactivity of an approximately 110K protein from concentrated culture supernatant (CCS) with monoclonal and polyclonal antibodies to the alpha-hemolysin (kindly provided by Sucharit Bhakdi, Institute of Medical Microbiology, University of Giessen, Federal Republic of Germany) was determined by immunoblotting. Bacteria other than A. pleuropneumoniae were grown in brain heart infusion broth.

For dot blot ELISA, A. pleuropneumoniae was grown overnight on brain heart infusion agar plates containing NAD, and other bacterial species were grown on Columbia agar containing 5% sheep blood. The bacteria were swabbed from the plates and suspended at 10⁷ CFU/ml in 0.1 M phosphate-buffered saline (PBS), pH 7.4.

Preparation of hemolysin. Bacterial cultures at mid-log phase were centrifuged at 10,000 × g, and the supernatant was concentrated by ultrafiltration with a YM-30 membrane (Amicon Corp., Lexington, Mass.) at 4°C. The CCS (3.5 ml in sample buffer) was electrophoresed in a single lane (13 cm) by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a 10% polyacrylamide separating gel (19), and stained with Coomassie brilliant blue R-250. Purified hemolysin was obtained by cutting the 110K band from the gel, which was then lyophilized and stored at 4°C. This protein was identified as the hemolysin because it was the major protein absent from a nonhemolytic mutant, because antisera to this protein neutralized hemolytic activity, and because production of the protein was enhanced by calcium (9).

Preparation of antiserum and IgG to hemolysin. The lyophilized gel and toxin were ground to a powder and suspended in 3 ml of PBS. Antiserum to purified hemolysin was prepared by subcutaneous immunization of a New Zealand White rabbit with 0.3 ml of the suspended band, mixed with an equal volume of Freund complete adjuvant, in each of four separate sites. The rabbit was reimmunized 2 weeks later with the same dose of antigen in Freund incomplete adjuvant, followed by three more immunizations without adjuvant at 2-week intervals. Two weeks after the last immunization, the titer of antiserum to hemolysin was 1:3,200, as determined by immunoblotting, and the rabbit was exsanguinated. The serum was found to have a neutralizing antibody titer of 1:64 to the hemolytic activity in CCS (a 1:4 dilution of CCS with a hemolytic titer of 1:64 from strain J45) (21). The neutralizing antibody titer of preimmune serum from this rabbit was less than 1:4. The immunoglobulin G (IgG) fraction of antiserum to the hemolysin was purified by affinity chromatography on protein A-Sepharose CL-4B by modification of the procedure of Ey et al. (6). Briefly, the serum was mixed with an equal volume of 0.14 M sodium phosphate buffer, pH 8.0, and applied to the affinity column equilibrated in the same buffer. The column was washed with several volumes of phosphate buffer until the absorbance of the eluent returned to baseline. The bound IgG was then eluted by reversing the flow of the column and washing with 0.1 M citrate buffer, pH 4.0. Fractions were collected into an equal volume of phosphate buffer (pH 8.0) to neutralize the acidity. Fractions containing protein were dialyzed against PBS and concentrated by ultrafiltration to 28.8 mg/ml (20).

Production of MAb to the 110K hemolysin. Mice were immunized with SDS-PAGE-purified hemolysin as described above. Hybridomas were produced by fusing the spleen cells from the primed mice with SP2/0 plasmacytoma cells as described elsewhere (30). Hybridomas secreting antibodies to the hemolysin were screened by indirect ELISA and confirmed by immunoblotting. The isotype of each monoclonal antibody (MAb) was determined with isotype-specific goat anti-mouse IgG conjugated to horseradish peroxidase (The Binding Site, Ltd., Birmingham, United Kingdom) by the indirect ELISA. MAbs IgG3 and IgG1 were purified from ascites fluids by euoglobulin precipitation (22) and by a MAb purification kit (Beckman Instruments, Inc., Fullerton, Calif.).

Swine sera. Preimmune and postimmune sera were obtained from pigs vaccinated intramuscularly with strain J45, noncapsulated or nonhemolytic mutants derived from this strain, or SDS-PAGE-purified hemolysin from strain J45. Antisera against A. pleuropneumoniae serotypes 1 to 5 and 7 were provided by Martha Mulks, Michigan State University, East Lansing, and Richard F. Ross, College of Veterinary Medicine, Iowa State University. Negative control sera from pigs about 6 weeks old from closed herds with no incidence of pleuropneumonia and which were negative for antibodies to pleuropneumonia by CF (titers, <1:8) and/or an ELISA for lipopolysaccharide (titers, <1:100) were also provided by Richard Ross and by Carlos Pijano, College of Veterinary Medicine, University of Minnesota, St. Paul. Swine sera positive for antibodies to A. pleuropneumoniae by CF and/or radioimmunoassay (15) were obtained from the Virginia Polytechnic Institute Swine Center herd, which is a clinically symptomatic herd from North Carolina, provided by Lorraine Hoffman, Iowa State University Diagnostic Laboratory. CF titers were determined at the Iowa State University Diagnostic Laboratory.

Indirect ELISA. Appropriate concentrations of each of the reagents used in the ELISA were first determined by checkerboard titration. Polystyrene microtiter plates (Immulon IV; Dynatech Laboratories, Inc., Chantilly, Va.) were coated with 100 μl of affinity-purified rabbit IgG (50 μg/ml) or MAb (1 μg/ml) in 0.1 M sodium carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were washed three times in PBS containing 0.05% Tween 20, and nonspecific binding was blocked with PBS containing 2% bovine serum albumin (PBS-BSA) for 1 h at 25°C. The blocking buffer was removed, 100 μl of CCS protein in PBS-BSA (50 μg/ml) was added to each well, and the plates were reincubated at 25°C for 1 h. The percentage of hemolysin protein in the CCS was 76% (determined by SDS-PAGE and densitometry). The plates were washed as described above, serial two-fold dilutions of test sera in PBS-BSA were added in triplicate, and the plates were reincubated at 25°C for 1 h. The washes were repeated, 100 μl of a 1:2,000 dilution of rabbit anti-swine IgG conjugated to horseradish peroxidase
(heavy and light chains; Organon Teknika, West Chester, Pa.) in PBS-BSA was added to each well, and the plates were incubated at 25°C for 1 h. The plates were washed and incubated with 100 μl of substrate (5-aminosalicylic acid in 0.005% H₂O₂) for 30 min at 25°C, and the reaction was stopped by the addition of 50 μl of 1 N NaOH. The A₄₅₀ was measured with a TiterTek Multiskan ELISA reader (Flow Laboratories, Inc., McLean, Va.).

Preliminary studies showed that the titers of positive and negative control sera did not change when the amount of CSS protein added to the wells varied from 30 to 65 μg/ml. Therefore, we could maintain reproducibility of the assay between batches of culture supernatant by determining the percent hemolysin in the preparation and adjusting the concentration added to the wells as necessary. Control wells were included in triplicate on each day of the test and contained known negative and positive sera (immune serum to serotype 5; titer, 1:10,240 in the ELISA), precoatslow serum, and buffer in place of capture IgG or toxin with positive control serum. The endpoint titer was considered the highest dilution of antiserum whose absorbance was greater than that of the positive control serum lacking capture IgG plus 3 standard deviations. Background absorbances of controls lacking toxin or enzyme-conjugated antibody or containing precoatslow serum were negligible (less than 0.010).

Dot blot ELISA was done as previously described (16), except that in place of antisera purified rabbit IgG to hemolysin was used (50 μg/ml).

Immunoblotting. Immunoblotting was performed by modification of the procedure of Towbin et al. (29). Briefly, 15, 30, 75, or 150 μg of culture supernatant protein was separated by SDS-PAGE (10% polyacrylamide gels) and transferred onto nitrocellulose paper. Nonspecific binding was blocked for 1 h with Tris-buffered saline (pH 7.5) containing 2% BSA (TBS-BSA) at 25°C. The paper was incubated with 3 μg of protein per ml of purified rabbit IgG or 25 μg of MAb IgG1 to hemolysin per ml in TBS-BSA for 1 h at 25°C. The paper was washed with TBS containing 0.05% Tween 20 and shaken with a 1:2,000 dilution of goat anti-rabbit IgG (heavy and light chains; Organon Teknika) or goat anti-mouse IgG antibody conjugated to horseradish peroxidase in TBS-BSA at 25°C. After being washed in TBS only and rinsed in distilled water, the paper was incubated for up to 15 min with horseradish peroxidase color development reagent (4-chloro-1-naphthol in 0.02% H₂O₂; Bio-Rad Laboratories, Richmond, Calif.) in TBS. The reaction was stopped by washing the paper in distilled water, and the paper was dried in the dark. Protein molecular weights of the toxin bands were determined by calculation of the Rf value in comparison with standards (Bio-Rad) by SDS-PAGE on 10 and 7% polyacrylamide separating gels.

Statistics. The percent sensitivity of the indirect ELISA in comparison with CF was calculated as [true-positives/true-positives + false-negatives] × 100. The comparative percent specificity was calculated as [true-negatives/true negatives + false positives] × 100.

RESULTS

Immune sera to each available serotype of A. pleuropneumoniae, sera from clinically normal pigs at the Virginia Polytechnic Institute Swine Center, sera from a herd clinically positive for pleuropneumonia, and sera from pigs from closed research herds free of pleuropneumonia were tested by indirect ELISA and compared with CF titers (Table 1).

<table>
<thead>
<tr>
<th>Serotype or source of</th>
<th>Indirect ELISA titer</th>
<th>CF titer *</th>
</tr>
</thead>
<tbody>
<tr>
<td>swine serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. pleuropneumonia b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>serotype:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>≥2,560</td>
<td>64</td>
</tr>
<tr>
<td>2</td>
<td>640</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>1,280</td>
<td>64</td>
</tr>
<tr>
<td>4</td>
<td>≥2,560</td>
<td>128</td>
</tr>
<tr>
<td>5</td>
<td>320</td>
<td>64</td>
</tr>
<tr>
<td>6</td>
<td>≥2,560</td>
<td></td>
</tr>
<tr>
<td>Virginia Polytechnic</td>
<td>320-1,280</td>
<td>8-128</td>
</tr>
<tr>
<td>Swine Center</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinically positive</td>
<td>80-10</td>
<td>Negative-256</td>
</tr>
<tr>
<td>herd d</td>
<td></td>
<td>10-80</td>
</tr>
<tr>
<td>Closed herds free of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pleuropneumonia c</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The CF assay was done at the Iowa State Diagnostic Laboratory.
* Immune sera provided by R. F. Ross, Iowa State University.
* Serum samples from nine animals were tested. The numbers of samples and respective titers are as follows: (by ELISA) 1, 320; 3, 640; 3, 1,280; (by CF) 1, anticomplementary; 1, 8; 1, 32; 4, 64; 2, ≥128.
* Serum samples from 38 swine were tested. The numbers of samples and respective titers are as follows: (by ELISA) 1, 80 (negative); 2, 320; 8, 640; 7, 1,280; 20, ≥2,560; (by CF) 1, negative; 1, 4 (negative); 7, 16; 8, 32; 11, 64; 8, 128; 6, 256.
* Sera from closed herds were obtained from Iowa State University (12 samples) and the University of Minnesota (4 samples).

On the basis of a wide sampling of CF-negative sera, an ELISA titer of <1:320 was considered negative. Immune serum to each serotype tested was positive by ELISA, indicating that different serotypes produced antigenically cross-reactive 110K proteins. In addition, serum specimens from all nine pigs at the Virginia Polytechnic Institute Swine Center and 37 of 38 serum specimens from the clinically positive herd were also positive for antibodies to the toxin (titers ranged from 1:320 to ≥1:2,560). Titers were 8- to 64-fold higher by indirect ELISA than the paired samples were by CF. All serum samples from 16 pigs from CF-negative sera, <1:8) closed herds were also negative by indirect ELISA (titers, <1:10 to 1:80). Of 69 serum samples tested, ELISA and CF results agreed for 50 positive samples and 16 negative samples. One sample from the clinically symptomatic herd positive by CF was negative by ELISA (sensitivity, 98.1%), and two samples from this herd negative by CF were positive by ELISA (specificity, 90%). The two samples that were negative by CF but positive by ELISA were also positive for antibodies to serotype 5 capsule by ELISA (16). The sample that was positive by CF but negative by ELISA (titer, 1:80) was CF positive only for serotype 7 (1:32), which was confirmed by capsule ELISA. Use of an IgG3 or IgG1 MAb to the hemolysin as the capture antibody did not change the sensitivity or specificity of the samples tested (data not shown). However, in order to obtain the same specificity, the MAb needed to be used at 1 μg of protein per ml and the CCS protein needed to be used at 0.4 μg/ml.

ELISA titers were also compared for immune sera from local commercial swine or from the University of Michigan and sera from the same pigs following intramuscular immunization (Table 2). Most of the immune sera from these pigs had titers of less than 1:160, except for two of the local pigs, which had titers of 1:320. Immunization or challenge of all the animals with serotype 1, 5 (encapsulated or noncapsulated mutant), or 7 boost the hemolysin titers of each pig from 4- to 128-fold. However, the antibody

---

Downloaded from http://jcm.asm.org/ on May 9, 2021 by guest
response to the 110K protein of serotype 7 was weaker and took longer to become positive than that to serotype 1 or 5. Immunization of pigs with purified hemolysin boosted each of their ELISA titers 8- to 64-fold. Immunization of some pigs with a nonhemolytic mutant paradoxically reduced the reactivities of sera from these pigs in the ELISA (data not shown).

Dot blot ELISA of whole cells of *A. pleuropneumoniae* serotypes 1 to 7, alpha-hemolysin-producing *E. coli*, *P. haemolytica*, and *B. bronchiseptica* with the affinity-purified rabbit IgG was used to screen whole cells for the presence of the 110K hemolysin or a related protein. Cells of *A. pleuropneumoniae* serotypes 1, 2, 4, 5, and 7 strongly reacted with IgG to the hemolysin. Serotypes 3 and 6 and *P. haemolytica* weakly reacted with the rabbit IgG; there was no detectable reactivity with *E. coli* or *B. bronchiseptica* (data not shown).

Immunoblotting confirmed the specific reactivity of the rabbit IgG for the 110K hemolysin (Fig. 1). A single, about 110K protein band from culture supernatants of serotypes 1 to 5 and 7 and a faint band from serotype 6 (not visible in Fig. 1) were recognized with the affinity-purified IgG. The molecular weight calculation of the band was based on SDS-PAGE analysis of CCS from each serotype on 7 and 10% polyacrylamide gels in comparison with molecular weight standards. However, the amount of protein loaded on the gel had to be increased 5-fold for serotype 7 and 10-fold for serotypes 3 and 6 to detect the bands. Culture supernatants of the nonhemolytic *A. pleuropneumoniae* mutant and alpha-hemolysin-producing *E. coli* were not reactive with the rabbit IgG when the amount of CCS protein used was 150 μg. In addition, approximately 105,000-molecular-weight doublet proteins, which presumably are the leukotoxin (2), from *P. haemolytica* were also reactive with the IgG (Fig. 1, lane 10). Of interest was the finding that reactivity of the IgG with the 110K protein of a lysate of strain J45 whole cells was weakly detectable but was not detectable with lysates of *P. haemolytica* whole cells (Fig. 1, lanes 11 and 12, respectively). Furthermore, none of the whole-cell suspensions that were more than 2 days old reacted with the IgG by dot blot ELISA. When immunoblotting was done with CCS employing the MAb to the 110K hemolysin, results were identical to those obtained with rabbit IgG (data not shown).

To determine whether the weak ELISA reactivities of the sera from CF-negative animals were specific for the 110K hemolysin, an immunoblot was done with a 1:20 dilution of sera from these animals against CCS of strain J45 (Fig. 2). Swine sera from the University of Minnesota reacted strongly with the hemolysin band (Fig. 2, lanes 1 to 3), whereas swine sera from Iowa State University were only weakly reactive with this protein (Fig. 2, lanes 4 to 8). Sera from normal pigs from a local commercial herd were also strongly reactive with the 110K hemolysin, as well as with several other proteins present in culture supernatant (Fig. 2, lanes 9 to 11). These results correlate well with ELISA titers of the individual sera (Fig. 2, legend).

**DISCUSSION**

A diagnostic assay for antibodies to the hemolysin of *A. pleuropneumoniae* would be valuable because the hemolysin is a common component of all serotypes, and presumably one set of reagents could be used to identify infection due to any serotype. Furthermore, the hemolysin is currently the subject of intense research as a factor in the pathogenesis of pleuropneumonia (4, 5, 8–11, 17, 21, 25), and an assay to measure the immune response to this protein would be useful to researchers. Although CF is the serological assay most often used for diagnosis of pleuropneumonia, the sensitivity and specificity of CF have not been confirmed. Serotype-specific assays, however, are too complicated for

<table>
<thead>
<tr>
<th><strong>Immunogen</strong></th>
<th><strong>ELISA titer</strong></th>
<th><strong>Preimmune sera</strong></th>
<th><strong>Postimmune sera (wk)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotype 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20–320</td>
<td>1,280–5,120 (4)</td>
<td></td>
</tr>
<tr>
<td>Serotype 1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>80</td>
<td>5,120 (3)</td>
<td></td>
</tr>
<tr>
<td>Serotype 5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;10</td>
<td>5,120 (3)</td>
<td></td>
</tr>
<tr>
<td>Serotype 7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>40</td>
<td>160 (5)</td>
<td></td>
</tr>
<tr>
<td>Purified hemolysin&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt;10–320</td>
<td>2,560 (4)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of weeks after final immunization.

<sup>b</sup> Serum samples from eight local pigs immunized with strain J45 or its noncapsulated mutant.

<sup>c</sup> Serum samples from pigs experimentally challenged at the University of Michigan.

<sup>d</sup> Serum samples from three local pigs immunized with hemolysin that was purified by SDS-PAGE.

FIG. 1. Immunoblot of culture supernatant or whole cells of *A. pleuropneumoniae* serotypes 1 to 7, a nonhemolytic mutant of serotype 5, and clinical isolates of alpha-hemolysin-producing *E. coli* and *P. haemolytica*. Reactive antigens were identified with 3 μg of affinity-purified rabbit IgG to the 110K antigen of serotype 5 per ml, followed by goat antibody to rabbit IgG conjugated to horseradish peroxidase and substrate. The strains (and amounts of protein in culture supernatant or numbers of whole cells) were as follows: lane 1, serotype 1 strain 4045 (15 μg); lane 2, serotype 2 ATCC strain 27089 (15 μg); lane 3, serotype 3 ATCC strain 27090 (150 μg); lane 4, serotype 4 ATCC strain 33378 (15 μg); lane 5; serotype 5 nonhemolytic mutant (150 μg); lane 6, serotype 5 strain J45 (15 μg); lane 7, serotype 6 ATCC strain 33590 (150 μg); lane 8, serotype 7 strain 53 (75 μg); lane 9, *E. coli* 822 (150 μg); lane 10, *P. haemolytica* P1148 (30 μg); lane 11, whole cells of strain J45 (10<sup>5</sup> CFU); lane 12, whole cells of *P. haemolytica* P1148 (2 × 10<sup>6</sup> CFU).
1360 MA AND INZANA

routine testing. Since the hemolysin itself is difficult to purify, we used affinity-purified polyclonal or monoclonal IgG to the toxin as a capture antibody, followed by CCS containing about 76% 110K hemolysin protein. IgG to the hemolysin of serotype 5 was capable of detecting antibody in immune sera to each of the serotypes available for testing. Each of these sera also had high CF titers. However, although high CF titers usually correlated with high ELISA titers, one immune serum to serotype 5 had a low positive ELISA titer of 1:320 but a relatively high CF titer of 1:64. The reason for this discrepancy is not clear, but this serum sample also had low ELISA and radioimmunoassay titers for antibody to serotype 5 capsule (15, 16). Sera from swine at the Virginia Polytechnic Institute Swine Center were positive for antibodies to hemolysin by ELISA and for antibodies to A. pleuropneumoniae by CF. Although these pigs were clinically normal, they were also strongly reactive in a radioimmunoassay for antibody to serotype 5 or 1 capsule (15). Sera from this herd are routinely assayed for CF titers, and most samples continue to be positive. It is therefore possible that this herd has had subclinical infections, which are common (27).

The sensitivity of the indirect ELISA compared with that of CF was 98.1%, while the specificity was 90%. The lower specificity was due to two CF-negative serum specimens, which were positive by indirect ELISA, from a clinically symptomatic herd. Both of these CF-negative serum specimens were positive for antibodies to serotype 5 capsule by ELISA (16). Since the titers by indirect ELISA were 8- to 64-fold higher than by CF, it is quite possible that the CF-negative samples were also positive. Furthermore, the one sample that was positive by CF but negative by indirect ELISA (titer, 1:80) was positive only for serotype 7 (determined by CF and capsule ELISA). The indirect ELISA results for serotype 7 may have been negative because the capture antibody was specific for the 110K hemolysin, and serotype 7 produced less of this protein or produced a less reactive protein, as evidenced by the fact that five times more CCS protein from serotype 7 than from serotype 1 or 5 was required to demonstrate reactivity with the 110K protein by immunoblotting. The weaker anti-hemolysin response to serotype 7 (compared with the response to serotype 1 or 5) was confirmed by experimental challenge of a nonimmune pig with another strain of serotype 7 (Table 2). However, inherent differences in the ability of individual animals to respond, or differences in the antigen or strain used, could also account for a weaker response because serum to serotype 7 provided by another laboratory had high titers to the hemolysin (Table 1).

On the basis of results with swine sera that were known to be negative by CF and that were from clinically normal pigs or from closed research herds, we established an antibody titer of 1:320 or greater as positive for pleuropneumonia. Although two samples from local preimmune pigs had antibody titers of 1:320 (Table 2), we had no CF titer for these pigs or their clinical histories. Therefore, we cannot rule out possible previous exposure to A. pleuropneumoniae or to an organism producing a cross-reacting protein. Immunization of all pigs with live, whole cells or with purified hemolysin boosted titers to the hemolysin 4- to 128-fold. Therefore, the accuracy of the ELISA would be best when used with acute- and convalescent-phase sera.

Immunoblotting using serotype-specific antisera to A. pleuropneumoniae confirmed that the reactivities of these sera in the indirect ELISA were due to antibody to the 110K hemolysin, thus confirming that the assay can detect infection due to any serotype of A. pleuropneumoniae. However, greater quantities of culture supernatant protein from serotypes 3, 6, and 7 had to be used in the blots to obtain reactivity with the capture antibody, indicating that these serotypes produce less of the 110K protein or that the protein is less reactive.

Infections of pigs with A. pleuropneumoniae are widespread in this country (27), and therefore exposure to this organism and its hemolysin may be common. Furthermore, a 104K hemolysin of A. pleuropneumoniae has been found to cross-react with P. haemolytica leukotoxin, a similar-molecular-weight protein from A. pleuropneumoniae taxon "minor groups," Actinobacillus suis, and E. coli alpha-hemolysin (5). Since the P. haemolytica leukotoxin has regions genetically homologous with hemolysins from A. pleuropneumoniae, E. coli, Actinobacillus actinomycetemcomitans, and possibly others (3, 18, 28), it is likely that some antigenic determinants of the 110K hemolysin are conserved in proteins produced by a variety of genera. Sera from pigs free of pleuropneumonia that reacted weakly in the sensitive ELISA may have antibodies produced to antigens of other bacteria that cross-react with the 110K hemolysin used in the assay. This assumption was supported by the reactivity of CF-negative sera with the 110K protein of A. pleuropneumoniae as determined by immunoblotting. Immunoblotting and dot blotting confirmed that our capture antibody reacted with a protein that matched the reported molecular weight of P. haemolytica leukotoxin (2) and with whole cells of P. haemolytica. However, the antibody did not react with cells...
or the alpha-hemolysin of *E. coli* or with *B. bronchiseptica* cells. The lack of reactivity with the alpha-hemolysin of *E. coli* differs from results reported by Devenish et al., who reported weak reactivity of antibody to *A. pleuropneumoniae* hemolysin with alpha-hemolysin (5). The discrepancy in these results may be due to a difference in the sensitivities of the antibodies used or the amount of alpha-hemolysin produced by the different *E. coli* strains.

The indirect ELISA described here has the advantages of high sensitivity and convenience. Hemolysin that had lost hemolytic activity was still active antigenically by ELISA and immunoblotting. Furthermore, the crude hemolysin used in the ELISA could be stored at −20°C for more than 6 months without a loss of antigenic activity in the ELISA (data not shown). The application of a MAb for capture in the ELISA did not appreciably improve the sensitivity or the specificity of the assay, although the amounts of reagents required when the MAb was used were considerably smaller.

ACKNOWLEDGMENTS

We thank Jean Todd, Carlos Pijano, Martha Mulks, Richard F. Ross, Lorraine Hoffman, and David Sprecher for providing serum samples for this study, and Gerhardt Schurig for critical review of the manuscript.

This work was supported, in part, by grant 88-34116-3641 from the U.S. Department of Agriculture/Cooperative State Research Service and by Hatch funds from the Virginia Agricultural Experiment Station.

LITERATURE CITED


