Use of an Inhibition Enzyme-Linked Immunosorbent Assay for Quantification of Capsular Polysaccharide or Proteins in Vaccines

Thomas J. Inzana* and Anna Champion
Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

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An inhibition enzyme-linked immunosorbent assay (ELISA) is described for quantification of capsular polysaccharide or proteins in vaccines and other samples containing whole cells or extracts of Actinobacillus pleuropneumoniae. The assay can be used to quantify any antigen that can be purified and for which highly specific antibodies are not available. The assay can be carried out by any laboratory capable of performing an ELISA.

Actinobacillus pleuropneumoniae is the etiologic agent of swine pleuropneumonia and causes severe economic losses to the swine industry worldwide. Pigs with this disease present with hemorrhagic and necrotic lesions in the lungs and associated fibrinous pleuritis. There are 15 serotypes and two biotypes of A. pleuropneumoniae (3), and serotype specificity is determined by capsular polysaccharide (CP) composition (14, 25). However, respiratory lesions are predominately due to one or more of the extracellular cytotoxins (Apx toxins) produced by this bacterium (5, 6, 28). Attempts to control swine pleuropneumonia include management practices and vaccination, for which live attenuated, subunit, and killed-whole-cell vaccines are commercially available. Protective antibodies induced by bacterins are serotype specific and may be specific for CP and other somatic antigens (22, 23). Furthermore, each serotype produces one or two of the Apx toxins I, II, and III and Apx toxin production is serotype related, not strain related (5, 6, 18). Although Apx IV is produced by all serotypes in vivo, it is not expressed by bacteria in culture (28). Antibodies to the Apx toxins are cross-reactive (4, 5, 17, 26), and neutralizing antibodies provide optimum protection against the serotypes that produce those toxins (4, 5, 10, 15, 26). Therefore, vaccines should have measurable quantities of desirable antigens present, which could be documented for quality control purposes. An inhibition radioimmunooassay has been developed for highly sensitive quantification of CP (2, 11), but this assay requires the use of purified, radiolabeled CP and a scintillation counter. A latex agglutination assay has also been developed to detect CP (9), but it is semiquantitative. For antibody-based quantification of a particular antigen in a mixture, monoclonal antibodies are required because polyclonal antibodies are likely to cross-react with other antigens in the sample, resulting in either false detection of more antigen than is present or, due to inhibition, less antigen than actually present. However, if a purified antigen is available, the antigen can be used in an inhibition-style assay with polyclonal antibodies to accurately quantify a specific antigen. Therefore, we developed an inhibition enzyme-linked immunosorbent assay (I-ELISA) for sensitive quantification of CP, Apx toxins, or other antigens that can be purified and for which monospecific or monoclonal antibodies are not available. The assay can be carried out in most laboratories capable of doing ELISA.

A. pleuropneumoniae serotypes 2 (strain S 1536), 3 (strain S 1421), and 4 (strain M62) were obtained from the American Type Culture Collection (Manassas, VA). Serotype 1 strain 4045 and serotype 5 strain J45 were obtained from Bradley Fenwick (Virginia Tech, Blacksburg, VA). Serotype 7 strain 53 was obtained from Martha Mulks (Michigan State University, East Lansing, MI). Although different strains of A. pleuropneumoniae may be present in vaccines, particularly between manufacturers, the CP is the serotype-specific antigen (14), and polyclonal antibodies to the CP of one strain will react with the CP of the same serotype regardless of the strain. Bacteria were grown at 37°C in brain heart infusion broth supplemented with 5 μg/ml of NAD (BHI-N) with shaking to 10⁹ CFU/ml. The CPs of A. pleuropneumoniae serotypes 1 to 5 and 7 were purified from the supernatant of bacteria grown in BHI-N by Cetavlon precipitation, NaCl extraction, phenol extraction, and ultracentrifugation as previously described (8), except that enzyme digestion to remove nucleic acids and proteins (13) was used prior to phenol extraction in place of column chromatography. Our analysis has shown that if enzyme digestion is used prior to phenol extraction, the CP does not require further purification by column chromatography (unpublished data). Although this procedure was developed for isolation of CP from serotype 5, we have found that it works equally well for all A. pleuropneumoniae serotypes. CPs from serotypes 1, 2, 3, 4, and 7 were conjugated to biotin following oxidation with sodium metaperiodate to generate aldehyde groups (24), followed by reaction with biotin-LC-hydrazide according to the manufacturer’s instructions (Pierce Chemical Co., Rockford, IL). Biotin-LC-hydrazide was coupled directly to the carboxyl groups of 3-deoxy-d-manno-2-octulosonic acid in the serotype

* Corresponding author. Mailing address: College of Veterinary Medicine, 1800 Kraft Dr., Suite 200, CMMID, Virginia Tech, 0491, Blacksburg, VA 24061. Phone: (540) 231-4692. Fax: (540) 231-5553. E-mail: tinzana@vt.edu.

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5 CP with ethyl-dimethylaminopropyl-carbodiimide HCl (EDC; Bio-Rad Laboratories, Richmond, CA) as previously described (12). The biotin-CP ELISA was done as previously described (12).

Polyclonal antibodies to Apx I, II, and III are highly cross-reactive, and the toxins can only be distinguished by monoclonal antibodies (17). Therefore, we did not attempt to distinguish between the amounts of Apx I and II, which almost comigrate on polyacrylamide gels as 103- to 105-kDa peptides, or between the amounts of Apx II and III in preparations. Apx II and III were purified from serotype 2, and Apx I and II were purified from serotype 1 as follows. Following growth to mid-log phase in BHI-N, A. pleuropneumoniae cells were washed three times in phosphate-buffered saline (PBS [pH 7.4]) and incubated in RPMI medium containing 2.5% horse serum, 2 g/liter sodium bicarbonate, and 25 mg/liter NAD (Sigma-Aldrich, St. Louis, MO) with shaking overnight at 37°C. The bacterial cells were removed by centrifugation at 10,000 × g for 15 min at 4°C, and 55% ammonium sulfate was slowly added to the supernatant while stirring at 4°C, which was continued for 24 h. The semipurified Apx precipitate was pelleted by centrifugation at 10,000 × g for 30 min at 4°C, resuspended in 425 ml of 10 mM Tris-HCl (pH 7.5), and dialyzed in the same buffer overnight at 4°C. The amount of protein from serotype 1 (Apx I and II) was 1.261 μg/ml, and the amount of protein from serotype 2 (Apx II and III) was 431 μg/ml. Final purification of the Apx toxins was done by polyacrylamide gel electrophoresis, as previously described (19). The Apx toxins, as for other RTX toxins, are highly labile and lose biological activity soon after release from the bacterial cells (20, 21). Although biologically inactive toxin does not induce neutralizing antibodies, antigenic activity can still be measured by ELISA (19).

Antisera to A. pleuropneumoniae serotypes 1, 2, 5, and 7 were prepared previously (9). For serotypes 3 and 4, New Zealand White rabbits were immunized with formalin-killed cells by the protocol described previously (14). Briefly, the rabbits were immunized with killed whole cells of each serotype in Freund’s complete adjuvant followed by Freund’s incomplete adjuvant and then were given weekly intravenous immunizations without adjuvant until the antibody titer reached a plateau. Since antiserum to killed, washed A. pleuropneumoniae cells lacks antibodies to Apx toxins, antisera to the Apx toxins were made in New Zealand White rabbits immunized with the pooled, purified toxins following gel electrophoresis (19). The rabbits received one injection of toxin without adjuvant in four sites subcutaneously. The ELISA titer was ~1:6,000. The rabbits were then exsanguinated by cardiac puncture, and the serum was collected and stored in aliquots at −20°C. As mentioned above, antiserum was prepared to a collective pool of the Apx toxins because polyclonal antibodies to each Apx toxin cross-react with the other two. Therefore, without monoclonal antibodies specific to each Apx toxin, only the collective Apx toxin pool could be quantified. All vaccine samples were commercial-grade preparations provided by Boehringer-Ingelheim Vetmedica (Guadalajara, Mexico). The vaccine contained mixtures of serotypes 1 to 5 and 7. Some samples contained adjuvant (not disclosed by the company), while others did not, to evaluate the effect of adjuvant on reactivity with the antigens in the ELISA.

The direct CP ELISA was done using streptavidin-coated plates (Nunc AS, Roskilde, Denmark) and biotin-labeled CP as described previously (12). All incubations were at 37°C for 1 h, and all washes were done five times with PBS–0.5% Tween 20. Briefly, 100 μl of biotin-CP at 10 μg/ml in PBS–20 mM MgCl2 was added to microtiter wells, followed by incubation, washing, and blocking of nonspecific binding with blocking buffer (BB; PBS–0.5% Tween 20–10% normal goat serum). High-titer rabbit antiserum was serially diluted in 100 μl BB, the plates were incubated and washed, and goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (diluted 1:5,000 in BB; Jackson ImmunoResearch Laboratories, West Grove, PA) was added. After incubation and washing, O-phenylenediamine substrate (Sigma-Aldrich Co., St. Louis, MO) was added. The plates were incubated at room temperature for 10 min, and the optical density at 490 nm was determined in a Molecular Devices (Sunnyvale, CA) microplate reader. The net optical density of each test sample was determined by subtracting the optical density of the negative control wells (coating buffer without biotin-CP) from the optical density of the matching wells containing biotin-CP. The dilution of antiserum that resulted in an optical density in the middle of the logarithmic binding curve (1:25,000 for CP1) was used for the I-ELISA (Fig. 1). A similar direct ELISA was used to determine the optimal dilution of antiserum to pooled Apx toxins, except that Immulon IV microtiter plates (Thermo Electron, Milford, MA) were used and the Apx toxin pool was diluted in carbonate-bicarbonate coating buffer (not shown).

For the serotype 1 I-ELISA, log dilutions of purified CP1 from 1,000, 300, 100, 30, 10, 3, 1, and 0 ng/ml were made in 1.8 ml of BB in separate tubes. One hundred microliters of rabbit antiserum (to serotype 1) diluted 1:1,315 in BB was added to these CP standards, and the mixture was incubated overnight at 4°C to inhibit the binding of antibody to biotin-CP bound to streptavidin-coated microtiter wells. One hundred microliters of the preincubated, inhibited serum samples (1:25,000 final dilution) was added to the plates in triplicate and incubated, covered, for 1 h at 37°C. The plates were washed five times with PBS–0.5% Tween 20, and goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase diluted 1:5,000 in BB was added. Following washing and addition of substrate, the optical density at 490 nm (A490) was determined (12) and

**FIG. 1.** Dilution curve of antiserum to A. pleuropneumoniae serotype 1 (A.p. 1) with purified, biotinylated CP1. Serum diluted to about mid-optical density in the log range of the dilution curve was used for the I-ELISA. A similar curve was obtained and used for the CPs of other serotypes or pooled Apx toxins.
antiserum dilution curve with purified antigen. The determined to yield an optical density in the mid-log range of the using rabbit anti-pooled Apx toxin at a 1:300 dilution, which was corresponds to 56 ng of CP or 56 ng/ml of pooled Apx toxins was calculated to be in the vaccine (Fig. 2B). The absence of adjuvant from the vaccine did not significantly affect the amount of antigen detected (data not shown).

We sought an alternative assay as a control to determine the amount of antigen in the sample. However, the toxins in the commercial vaccine were not biologically active and could not be differentiated from other proteins in the sample by protein assay. The vaccine also contained several serotypes and was inadequate in volume to quantify each CP by purification. Therefore, we also tested the same dilution of vaccine in comparison to 10-fold dilutions of purified serotype 1 CP with latex particles sensitized with monospecific antibody to serotype 1 CP (9). Although latex agglutination is only semiquantitative at best, there was a clear difference in the degree of agglutination of the latex particles incubated with 10 ng/ml of CP and 100 ng/ml of CP. At 50 ng/ml of CP, the degrees of agglutination, which were about 3+ (9), were similar between the diluted vaccine sample and the I-ELISA (data not shown).

Quantification of most of the other CP serotypes by I-ELISA also worked well, and the results were similar to that for serotype 1 (not shown). It was calculated that the same vaccine sample contained 77.6 ± 4.10 μg/ml of serotype 2 CP, 21.4 ± 2.46 μg/ml of serotype 3 CP, 11.6 ± 6.70 μg/ml of serotype 5 CP, and 65 ± 4.04 μg/ml of serotype 7 CP. The amount of CP measured from each vaccine sample was consistent for each CP measured and for the pooled Apx toxins. Two separate rabbits did not make an immune response to serotype 4 CP, and the concentration of this CP was not calculated. However, it was later determined that immunized pigs did make antibodies to serotype 4 CP (data not shown). In addition, the rabbit antibody response to individual serotypes did vary. While very little response was made to serotype 4 CP, a strong response was made to serotypes 1 and 7 CP, with titers of 1:20,000 and 1:13,000, respectively, at an A_{490} of 1.0. A weaker response was made to serotype 2 and 5 CP (titers of 1:3,000 for both at an A_{490} of 1.0), and the weakest workable response was made to serotype 3 CP (titer of 1:640 at an A_{490} of 1.0). The exact reason for the variation in the antibody response of rabbits to polysaccharide antigens is not clear, but it may be related to whether the host recognizes the polysaccharide epitopes as foreign. Although polysaccharides presented to the host on bacterial cells may become T-dependent antigens, some polysaccharides remain nonimmunogenic following infection, such as the sialic acid capsule of Neisseria meningitidis type B (29). The A. pleuropneumoniae serotype 4 CP is a simple polymer of glucose, N-acetylgalactosamine, and phosphate, with no teichoic acid or acyl group modifications (1). Therefore, the immune response to polysaccharides may be highly variable and dependent on composition and host

an inhibition antigen curve was established (Fig. 2). Simultaneously, triplicate samples of vaccine sample were serially diluted from 1:10 to 1:10,000 in 1.8 ml of BB, 100 μl of each replicate of diluted (1:1,315) rabbit antiserum was added, the mixture was incubated overnight at 4°C, and the ELISA was carried out as described above. Each sample was tested in triplicate and on 3 separate days. The dilution of sample yielding an optical density in the mid-log region of the inhibition curve was used to determine the amount of CP (Fig. 2A) or Apx toxin (Fig. 2B) in the sample from the standard antigen inhibition curve using the respective plate-bound antigen. The slope of the Apx toxin curve was not as steep as the curve using the respective plate-bound antigen. The slope was 0.89 at a 1:100 dilution, which was extrapolated from the curve to an inhibition curve using the respective plate-bound antigen. The rabbit anti-serotype 1 primary antibody was used at a dilution of 1:25,000, which resulted in an optical density in the mid-log range I-ELISA from a dilution of vaccine preincubated with diluted antiserum. The amount of serotype 1 CP in the sample was extrapolated from the A_{490} chosen (0.4727), which corresponds to 56 ng of CP or 56 ng/ml of sample. (B) I-ELISA using rabbit anti-pooled Apx toxin at a 1:300 dilution, which was determined to yield an optical density in the mid-log range of the antiserum dilution curve with purified antigen. The A_{490} of the vaccine was 0.89 at a 1:100 dilution, which was extrapolated from the curve to give 16.2 μg/ml of toxin in the vaccine.

Delutions of a vaccine sample (containing adjuvant) preincubated with diluted antiserum to A. pleuropneumoniae serotype 1 followed by incubation with purified, biotin-labeled serotype 1 CP resulted in an inhibition curve similar to that of the antigen standard (not shown). An optical density in the mid-log region of the vaccine inhibition curve was selected that corresponded to an optical density in the mid-log region of the standard inhibition curve (0.4727). For this particular sample, it was determined that it contained 56 ng of CP (Fig. 1), corresponding to a concentration of 56 ± 2.54 μg/ml of CP when multiplied by the dilution factor (1,000) used for the assay (Fig. 2A). Following a similar procedure using the Apx toxins for inhibition, a concentration of 16.2 ± 1.40 μg/ml of pooled Apx toxins was calculated to be in the vaccine (Fig. 2B). The absence of adjuvant from the vaccine did not significantly affect the amount of antigen detected (data not shown).

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species. The rabbit immune response to the pooled Apx toxins was 1:400 at an \( A_{490} \) of 1.0, but this was in response to a single immunization without adjuvant, whereas the rabbits immunized with CP were immunized twice in adjuvant and then immunized intravenously weekly until a high-titer serum was obtained.

It should be emphasized that quantification of antigen by the I-ELISA is based on antigenic determination and is not necessarily a measure of vaccine efficacy. Protection against pleuropneumonia requires neutralizing antibodies to the Apx toxins (4, 16, 26), which are highly labile and lose biological activity shortly after secretion (20, 21, 27). Neutralizing antibodies are only made to native, active toxin, and not to denatured toxin that has lost cellulytic activity. Nonetheless, antibodies are made to denatured toxin that can be detected by ELISA (4, 17, 19). Immunization of pigs with bacteria also provides serotype-specific protection, and therefore antibodies to CP and other somatic antigens may also be protective. However, it is not known if there is a direct correlation between vaccine efficacy and the amount of CP or Apx toxin present in the vaccine.

Quantification of antigens in samples has been carried out by a wide variety of assays (7). If a monoclonal or highly specific antibody is available, an antibody capture assay can be used in which the sample is incubated in a microtiter plate and the amount of antigen bound is determined by the amount of antibody bound in comparison to a standard curve. However, the amount of antigen that binds to the plate may vary considerably between samples, or in the case of polysaccharides may not bind well at all, and therefore not be an accurate estimation of the total amount of antigen in the sample. Alternatively, a two-antibody sandwich assay can be used in which one antibody is bound to a solid phase, followed by the sample or a known standard and a second, labeled antibody. For this assay, it is important that the antibodies are directed to different epitopes on the antigen (which may be limited in polysaccharides) and that the affinities of each antibody for the antigen are similar. Therefore, monoclonal antibodies are preferred, or at least affinity-purified monospecific polyclonal antibodies. If the latter are used, reproducibility of the assay when a given batch of antibodies is depleted may be problematic. Antigen capture assays detect antigen by a competition-type assay. A highly specific antibody is bound to a plate, and then a purified, labeled antigen is mixed with an unknown sample, and the mixture is incubated with the bound antibody. However, if the antigen is not highly immunogenic, such as with many polysaccharides, a highly specific antibody to the purified antigen can be difficult to obtain.

The I-ELISA described here is a variation of the inhibition radioimmunoassay (2, 11), but does not require the use of radiolabeled antigen or the chemicals and equipment required for radioimmunoassays. The I-ELISA did require the use of purified antigen. However, in the case of CP, the procedure was simplified in that it was identical for each CP and the Apx toxins produced in defined medium culture supernatant could be further purified by a single electrophoresis step. By using an inhibition-style format, only a single antiserum was required (provided antibodies to all desired antigens are present), antibody monospecificity was not required, and preincubation of the sample or standard antigen with diluted antiserum im-

proved consistency because critical epitopes were never bound to a solid support and unavailable for binding. Biotinylation and use of streptavidin plates should only need to be done for polysaccharide antigens to enhance binding and reproducibility of results. In summary, the I-ELISA described here should be a relatively simple, reproducible assay for antigen quantification that can be carried out in most laboratories capable of some antigen purification and ELISA techniques.

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