Serologic Detection of *Actinobacillus pleuropneumoniae* in Swine by Capsular Polysaccharide-Biotin-Streptavidin Enzyme-Linked Immunosorbent Assay

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Serologic detection of *Actinobacillus pleuropneumoniae* infections in swine have been problematic due to antigenic cross-reactivity of Apx toxins, lipopolysaccharide, and outer membrane proteins between *A. pleuropneumoniae* serotypes and other bacterial species. To maximize serologic specificity and sensitivity, we developed an assay that uses highly purified *A. pleuropneumoniae* capsular polysaccharide (CP) conjugated to biotin, which is then bound to streptavidin-coated enzyme-linked immunosorbent assay (CP-BS-ELISA) plates. This assay was used to test a panel of 240 serum samples from pigs prior to challenge, after challenge with bacterial species other than *A. pleuropneumoniae*, or after challenge with *A. pleuropneumoniae* serotype 1, 5, or 7. Overall assay results for the individual sera tested were reproducible on the same day and on separate days. The sensitivity of the assay was 100% by ELISAs with biotin-CPs of serotypes 1 and 7 and 87.5% by ELISAs with biotin-CP of serotype 5. Specificity was 100% by ELISAs with biotin-CPs of serotypes 1 and 5 and 94.5% by ELISAs with biotin-CP of serotype 7. The biotin-CPs of at least three *A. pleuropneumoniae* serotypes could be combined for use in a screening assay to detect antibodies to CPs from strains of different serotypes. In conclusion, the CP-BS-ELISA proved to be a serotype-specific and species-specific assay with high sensitivity for the identification of pigs exposed to *A. pleuropneumoniae*.

Actinobacillus pleuropneumoniae is the etiologic agent of swine pleuropneumonia, which is a major cause of economic loss to the swine industry throughout the world. There are 14 recognized serotypes of NAD-dependent *A. pleuropneumoniae* that vary in predominance depending on geographic location. One method used to control pleuropneumonia in a herd is adjustment of management practices (4). Therefore, knowledge of the immune status of the herd and of individual animals within the herd (for the presence of antibodies to *A. pleuropneumoniae*) is essential. To address this need a wide variety of serologic tests have been developed to detect antibodies to *A. pleuropneumoniae* in swine. The complement fixation test was initially used to detect antibodies to distinct components of *A. pleuropneumoniae* (11), but a variety of other tests have been developed that are more sensitive and specific, particularly enzyme-linked immunosorbent assays (ELISAs) (4). Nonetheless, cross-reactions between strains of the various *A. pleuropneumoniae* serotypes and other bacterial species have remained problematic. Most of these cross-reactions are due to common epitopes in outer membrane proteins (12). The O side chain of the lipopolysaccharide (LPS) has been proposed for use as a serotype-specific antigen in serologic assays (5, 9, 17). However, cross-reactions between serotypes 1, 9, and 11, 4 and 7, and 3, 6, and 8 have been attributed, at least in part, to the O-side-chain antigen (15, 19, 20). Recently, Lebrun et al.

(10) have shown that monoclonal antibodies to the O side chain of *A. pleuropneumoniae* serotype 7 also cross-react with *Actinobacillus lignieresii*.

On the basis of structural and immunologic analyses, the capsular polysaccharide (CP) has been shown to be the appropriate serotype-specific antigen for use in serologic tests for *A. pleuropneumoniae* (9, 17). Direct ELISAs have been developed with the CP as well as the LPS O side chain as antigen (2, 5, 9, 21). However, CP and O-side-chain antigens are hydrophilic and do not bind as well as hydrophobic antigens to most ELISA plates. A radioimmunoassay with purified CP was developed to obtain high sensitivity and serotype specificity (8). However, the complexity of the radioimmunoassay and the need for use of radioisotopes limit the routine use of this test in clinical laboratories. To maximize sensitivity and specificity with an ELISA format, biotin-long chain-hydrazide was covalently conjugated to purified CP of serotype 1, 5, or 7, and the combination was bound to streptavidin-coated microtiter plates. These serotypes account for the majority of clinical cases of swine pleuropneumonia in the United States and Canada (13, 18). The CP-biotin-streptavidin ELISA (CP-BS-ELISA) proved to be highly reproducible, sensitive, and specific when tested with 240 serum samples from pigs before and after experimental challenge with *A. pleuropneumoniae* serotype 1, 5, or 7 or challenge with other pathogenic bacterial species of swine.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Reference strains of each *A. pleuropneumoniae* serotype were obtained from the American Type Culture Collection (Manassas, Va.) or from J. Nicolet (Institute of Veterinary Bacteriology, University of Berne, Berne, Switzerland). Clinical isolates of *A. pleuropneumoniae*

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and other swine pathogens were isolated and identified at the Kansas State University Diagnostic Laboratory from swine with spontaneously occurring cases of pleuropneumonia by standard techniques (3). Bacterial isolates were recovered in pure culture from the respiratory tract. Clinical isolates consisted of *A. pleuropneumoniae* serotype 1, 5, or 7, *Actinobacillus suis*, *Escherichia coli*, *Pasteurella multocida*, and *Haemophilus parasuis*. Each species was grown in RPMI medium supplemented with 5% fetal bovine serum and 10 µg of NAD per ml, if needed.

Pigs and challenge procedures. Pigs were obtained from a genetic multiplier herd of a large swine genetics company that provides replacement pigs to pork production herds nationwide. The health status of the pigs from the source farm is by design often better than that of pigs from production-level farms, but the pigs are not specific pathogen free. These pigs are the most appropriate target population because control of *A. pleuropneumoniae* transmission is most effective when the introduction of *A. pleuropneumoniae* carrier pigs is prevented through serologic testing of pigs prior to their introduction into a herd. The most common source of introduced commercial farm pigs is herds of the type from which these pigs were obtained (a genetic multiplier herd).

The challenge dose for each strain was determined from preliminary experiments and was based on the dose required to establish a sublethal, clinical infection. Pigs were exposed via nasal inoculation with between 10^5 and 10^8 CFU of an isolate in 3 ml of RPMI medium. The clinical response, the time course of the disease, and the pathologic findings for pigs challenged experimentally were identical to those for pigs that develop pleuropneumonia through natural exposure.

Collection of sera. Sera were collected from the anterior vena cava of pigs before challenge and at the following times after challenge with *A. pleuropneumoniae*. Sera from pigs infected with serotype 1 were obtained at 14 and 120 days postchallenge, sera from pigs infected with serotype 5 were obtained at 22 and 110 days postchallenge, and sera from pigs infected with serotype 7 were obtained at 20 and 108 days postchallenge. Sera were collected from pigs challenged with all heterologous species at 28 and 49 days postchallenge; however, serum from pigs challenged with *P. multocida* was collected between 43 and 70 days postchallenge.

Purification and biotinylation of CP and ELISA. Highly purified CP was prepared as described previously (6). CPs from strains of serotypes 1 and 7 were conjugated to biotin following oxidation with sodium *meta*-periodate to generate aldehyde groups (16) and reacted with biotin-LC-hydrazide according to the manufacturer's instructions (Pierce Chemical Co., Rockford, Ill.). Biotin-LC-hydrazide was coupled directly to the carboxyl groups of 2-keto-3-deoxy-D-manno-2-octulosonic acid in the serotype 5 CP with ethyl-dimethylaminopropylcarbodiimide (Bio-Rad Laboratories, Richmond, Calif.) according to the manufacturer's instructions (Pierce Chemical Co.).

Optimization of the reagents used, serum dilutions, and other variables that influence the sensitivity and specificity of the ELISA are described in the Results section. The optimized ELISA procedure is described here. Biotin-CP at 10 µg/ml in 100 µl of phosphate-buffered saline (PBS; pH 7.4) containing 20 mM $MgCl_2$ (PBS-M) or PBS-M only was added to streptavidin-coated ELISA plates (LabSystems, Helsinki, Finland), and the plates were incubated covered for 1 h at 37°C. For the polyclonal CP ELISA, 100 µl of a mixture of serotypes 1, 5, and 7 at 30 µg/ml each was added to the wells. The samples were discarded, blocking buffer (5% nonfat dry milk in PBS-0.05% Tween 20 containing 10% normal goat serum) was added, and the plates were incubated for 1 h at 37°C. The wells were washed three times with PBS-0.05% Tween 20, and test or control swine sera diluted 1:200 in blocking buffer was added in duplicate to wells coated with CP or buffer only. After 1 h of incubation at 37°C, the wells were washed five times with PBS-0.05% Tween 20, and goat anti-swine immunoglobulin G conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, Pa.) diluted 1:3,000 in blocking buffer was added. The plates were incubated for 1 h at 37°C and washed five times with PBS-0.05% Tween 20, and 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added. After 15 min of incubation at room temperature, the absorbance was determined at 405 nm (A_{405}) in a Molecular Devices (Sunnyvale, Calif.) ELISA reader. The net absorbance of each test sample was determined by subtracting the absorbance of the negative control well (coating buffer without biotin-CP) from the absorbance of the well containing biotin-CP. A serum sample was considered weakly positive (+), moderately positive (++), or strongly positive (+++) for the presence of antibodies to a given serotype if the net A_{405} was ≥ 0.20 to 0.50, 0.51 to 0.80, or ≥ 0.81 , respectively.

Statistical analysis. The mean A_{405} values and standard deviations (SDs) were calculated by the *t* test with InStat computer software (GraphPad, Inc., San Diego, Calif.). The percent specificity of the CP-BS-ELISA was determined from the following formula: [number of true-negative samples/(number of true-nega-

tive samples + number of false-positive samples)] $\times 100$. The percent sensitivity of the CP-BS-ELISA was determined from the following formula: [number of true-positive samples/(number of true-positive samples + number of false-negative samples)] $\times 100$. The positive and negative predictive values were calculated from the following equations: [number of true-positive samples/(number of true-positive samples + number of false-positive samples)] $\times 100$ and [number of true-negative samples/(number of true-negative samples + number of false-negative samples)] $\times 100$, respectively (14).

RESULTS AND DISCUSSION

Preliminary analyses showed that conjugation of biotin to CP, under the conditions described above, did not reduce the antigenic activity of the CP in a latex agglutination test (7). Furthermore, the use of biotin-CP in conjunction with streptavidin-coated ELISA plates increased the A_{405} s for the test wells by 0.3 to 0.8 unit (depending on the CP preparation and the dilution of serum) compared to those for an ELISA with nonconjugated CP and non-streptavidin-coated plates (9). About 10 random serum samples from pigs before and after exposure to one of the three *A. pleuropneumoniae* serotypes were serially titrated from 1:50 to 1:6,400 in triplicate in order to select a single serum dilution and cutoff A_{405} that would yield the highest sensitivity and the highest specificity. These preliminary experiments resulted in the selection of a 1:200 dilution of test serum and a cutoff A_{405} of 0.20 after subtraction of the A_{405} for the control wells containing test serum but lacking antigen. A variety of blocking buffers were initially tested, such as 5% bovine serum albumin, 3% gelatin, and 5% nonfat dry milk with and without 10% goat serum (all diluted in PBS-0.05% Tween 20). The 5% nonfat dry milk containing 10% goat serum yielded superior results in regard to sensitivity and specificity, particularly when it was used with serotype 7 biotin-CP.

The reproducibility of the assay was tested with 15 serum samples reactive with *A. pleuropneumoniae* serotype 1, 5, or 7 (five samples of each serotype). Each of the serum samples was tested in triplicate on the same day and on 3 different days with different plates. The mean and SD A_{405} for sera from five pigs challenged with each serotype, after subtraction of the A_{405} for the no-antigen control, are shown in Table 1. The SD of the A_{405} s ranged from a maximum of 0.310 (mean, 1.331 for sample 1846, serotype 1) to a minimum of 0.010 (mean, 1.720 for sample 10110, serotype 7) for a single sample on any given day. The SDs of the A_{405} s of all assays done for each serum sample ranged from 0.283 (mean, 1.385 for sample 228, serotype 1) to 0.067 (mean, 1.268 for sample 6525, serotype 7). Therefore, assay results did not vary significantly when samples were tested on different days or on different plates. In all cases the SD of the absorbance was less than 24% of the mean absorbance, and variation of the SD did not change any of the positive results to a negative result. In most cases, the A_{405} for sera from pigs prior to challenge or after challenge with heterologous bacteria was less than 0 after subtraction of the A_{405} for the no-antigen control well. For five random samples for which the A_{405} was greater than 0, the mean $\pm$ SD A_{405} was 0.022 ± 0.007 (Table 1).

The specificity of the assay with sera from prechallenged swine or sera from pigs challenged with swine pathogens other than *A. pleuropneumoniae* was 100% with serotype 1 biotin-CP and serotype 5 biotin-CP. The specificity of the assay with

TABLE 1. Mean and SD A_{405} s for swine sera from pigs challenged with *A. pleuropneumoniae* serotype 1, 5, or 7 as determined by ELISA

Serum sample no. (serotype)	Mean $\pm$ SD A_{405} /test day ^a	Mean $\pm$ SD A_{405} for all tests ^b
228 (1)	1.154 $\pm$ 0.272, 1.654 $\pm$ 0.134, 1.348 $\pm$ 0.194	1.385 $\pm$ 0.283
1846 (1)	1.077 $\pm$ 0.202, 1.331 $\pm$ 0.310, 1.254 $\pm$ 0.083	1.221 $\pm$ 0.221
1903 (1)	1.857 $\pm$ 0.109, 1.514 $\pm$ 0.198, 1.372 $\pm$ 0.267	1.581 $\pm$ 0.278
2401 (1)	1.745 $\pm$ 0.099, 1.380 $\pm$ 0.236, 1.321 $\pm$ 0.060	1.482 $\pm$ 0.238
6503 (1)	1.114 $\pm$ 0.084, 1.365 $\pm$ 0.120, 1.416 $\pm$ 0.124	1.298 $\pm$ 0.170
6510 (5)	1.087 $\pm$ 0.213, 1.456 $\pm$ 0.119, 1.350 $\pm$ 0.246	1.298 $\pm$ 0.239
13584 (5)	0.988 $\pm$ 0.061, 0.993 $\pm$ 0.065, 1.136 $\pm$ 0.040	1.039 $\pm$ 0.088
13954 (5)	0.700 $\pm$ 0.124, 0.478 $\pm$ 0.061, 0.585 $\pm$ 0.108	0.588 $\pm$ 0.130
8352 (5)	1.097 $\pm$ 0.169, 0.996 $\pm$ 0.097, 1.059 $\pm$ 0.176	1.051 $\pm$ 0.138
3573 (5)	1.118 $\pm$ 0.074, 1.035 $\pm$ 0.106, 1.202 $\pm$ 0.220	1.118 $\pm$ 0.147
3437 (7)	1.405 $\pm$ 0.096, 1.563 $\pm$ 0.047, 1.541 $\pm$ 0.042	1.503 $\pm$ 0.094
6525 (7)	1.192 $\pm$ 0.013, 1.334 $\pm$ 0.041, 1.276 $\pm$ 0.031	1.268 $\pm$ 0.067
10110 (7)	1.720 $\pm$ 0.010, 1.848 $\pm$ 0.042, 1.870 $\pm$ 0.042	1.813 $\pm$ 0.074
12921 (7)	1.904 $\pm$ 0.053, 2.072 $\pm$ 0.014, 1.986 $\pm$ 0.039	1.987 $\pm$ 0.080
15769 (7)	1.444 $\pm$ 0.121, 1.744 $\pm$ 0.032, 1.689 $\pm$ 0.023	1.626 $\pm$ 0.152
Various ^c	0.022 $\pm$ 0.007, ND, ^d ND	ND

^a Sera from 15 different pigs reactive with serotype 1, 5, or 7 were tested in triplicate on 3 different days, and the mean $\pm$ SD was calculated for each day with InStat software (GraphPad, Inc.).

^b The mean $\pm$ SD was calculated for all nine assays with each serum sample tested on the same day and on different days.

^c Sera from various pigs were randomly chosen and tested prior to challenge or after challenge with non-*A. pleuropneumoniae* species. The A_{405} for most sera from pigs prior to challenge or after challenge with heterologous species was less than 0 after subtracting the A_{405} for the no-antigen control well.

^d ND, not determined.

antisera to *A. suis* or *E. coli* and serotype 7 biotin-CP was 94.1%, and the specificity of the assay with antisera to *H. parasuis* and *P. multocida* and with preimmune sera was 100% (Table 2). Serum samples obtained from one pig challenged with *A. suis* and one pig challenged with *E. coli* at 49 days postchallenge, but not at 28 days postchallenge, were weakly positive by the serotype 7 biotin-CP-ELISA. It is possible that these two pigs were exposed to *A. pleuropneumoniae* serotype 7 or to an organism with cross-reacting antigens sometime after the 28-day postchallenge serum collection. The overall sensitivity of the CP-BS-ELISA was 100% with postchallenge sera to *A. pleuropneumoniae* serotypes 1 and 7 and 87.5% with postchallenge sera to *A. pleuropneumoniae* serotype 5 (Table 3). Four serum samples from pigs challenged with serotype 5 were negative by ELISAs with serotype 5 biotin-CP at 110 days postchallenge but were positive at 22 days postchallenge. It is

TABLE 2. Specificity of the CP-BS-ELISA for *A. pleuropneumoniae* with prechallenge and heterologous swine antisera

Challenge species	No. of samples with reactivity with biotin-CP/no. of samples tested ^a		
	CP1	CP5	CP7
<i>A. suis</i>	0/16	0/16	1/16 (+) ^b
<i>E. coli</i>	0/10	0/10	1/10 (+) ^b
<i>H. parasuis</i>	0/16	0/16	0/16
<i>P. multocida</i>	0/18	0/18	0/18
Prechallenge	0/73	0/73	0/73

^a A positive reaction was considered a net A_{405} of ≥ 0.2 . CP1, CP5, and CP7, serotype 1, 5, and 7 CPs, respectively.

^b +, A_{405} between 0.20 and 0.50 above the A_{405} for the no-antigen control. Serum samples were collected from pigs at 49 days postchallenge. Serum samples from these pigs were nonreactive at 28 days postchallenge.

TABLE 3. Sensitivity of CP-BS-ELISA for detection of serotype-specific *A. pleuropneumoniae* antibodies in experimentally challenged swine

Challenge <i>A. pleuropneumoniae</i> serotype	No. of samples with the following reactivity with homologous purified biotin-CP ^a :			
	–	+	++	+++
Serotype 1	0	6	14	39
Serotype 5	4 ^b	5	6	13
Serotype 7	0	5	3	12

^a –, A_{405} of <0.2 unit above that for the no-antigen control; +, A_{405} of 0.2 to 0.5 unit above that for the no-antigen control; ++, A_{405} of 0.5 to 0.8 units above that for the no-antigen control; +++, A_{405} of >0.8 unit above that for the no-antigen control.

^b Serum samples were collected at 110 days postchallenge. Serum samples collected from these pigs at 22 days postchallenge were positive.

likely that the immune response to serotype 5 CP was weak in these four pigs and that antibody titers fell to below the cutoff value by 110 days postchallenge. The overall positive predictive value was 100% by ELISAs with *A. pleuropneumoniae* serotype 1 biotin-CP and *A. pleuropneumoniae* serotype 5 CP and 91% by ELISAs with *A. pleuropneumoniae* serotype 7 biotin-CP. The overall negative predictive value was 100% by ELISAs with *A. pleuropneumoniae* serotype 1 biotin-CP and *A. pleuropneumoniae* serotype 7 CP and 97% by ELISAs with *A. pleuropneumoniae* serotype 5 biotin-CP.

Due to the serotype specificity of any assay based on detection of antibodies to CP, a disadvantage of such a test is that multiple aliquots of one serum sample must be analyzed to screen for antibodies to multiple serotypes. Thus, we sought to determine if biotin-CP from serotypes 1, 5, and 7 could be combined in a polyvalent CP-BS-ELISA to act as a screening test for the detection of antibodies to the most prevalent serotypes. Eighteen serum samples with absorbance values that ranged from weakly positive (1+) to strongly positive (3+) for serotypes 1, 5, and 7 were tested (Table 4). Reactivity remained high or increased for serotypes 1 and 5 but diminished somewhat for serotype 7, with two samples converting from weakly positive to negative. Nevertheless, these results indicated that biotin-CPs of different serotypes could be combined to screen sera for reactivity to multiple serotypes. Our results were similar to those obtained by a mixed-antigen ELISA for the detection of antibodies to *A. pleuropneumoniae* serotypes 1, 5, and 7 in swine reported by Bossé et al. (1).

TABLE 4. Sensitivity of a polyvalent CP-BS-ELISA for detection of *A. pleuropneumoniae* antibodies in experimentally challenged swine

Challenge <i>A. pleuropneumoniae</i> serotype	No. of samples with the following reactivity in polyvalent CP-BS-ELISA ^a to biotin-CPs of serotypes 1, 5, and 7 ^b :			
	–	+	++	+++
Serotype 1	0	0	0	6
Serotype 5	0	0	1	5
Serotype 7	2	2	2	0

^a –, A_{405} of <0.2 unit above that for the no-antigen control; +, A_{405} of 0.2 to 0.5 unit above that for the no-antigen control; ++, A_{405} of 0.5 to 0.8 unit above that for the no-antigen control; +++, A_{405} of >0.8 unit above that for the no-antigen control.

^b A mix of strong and weakly positive reactors to each serotype was tested.

The CP-BS-ELISA that we have described here has the advantages of serotype specificity, enhanced sensitivity, and convenience. At one postchallenge time point or another, the assay was 100% sensitive and specific for the detection of serotype-specific antibodies to each CP in challenged pigs. However, by 7 weeks postchallenge two pigs challenged with heterologous species became positive for serotype 7 biotin-CP, and by 16 weeks postchallenge four pigs that were previously reactive and that were challenged with serotype 5 became nonreactive to serotype 5 biotin-CP. We determined that plates could be coated with biotin-CP, the nonspecific sites could be blocked, and the plates could be stored dry at -20°C for at least 6 months without a loss of reactivity (data not shown).

In conclusion, serotype-specific serologic tests should use highly purified CP in order to maximize specificity. Tests that use crude antigens, cell extracts, or LPS may result in reactions with antibodies to multiple serotypes or even heterologous species (10–12, 15, 19, 20). The CP-BS-ELISA described here uses optimum specificity, its sensitivity approaches the sensitivity of radioimmunoassay (8), and it has the convenience of an ELISA format suitable for most clinical laboratories.

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