Serodiagnosis of Pleuropneumonia Using Enzyme-linked Immunosorbent Assay with Capsular Polysaccharide Antigens of *Actinobacillus pleuropneumoniae* Serotypes 1, 2, 5 and 7

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ABSTRACT

Capsular polysaccharide antigens of serotypes 1, 2, 5 and 7 of *Actinobacillus pleuropneumoniae* were used in enzyme-linked immunosorbent assays (ELISAs) to test sera from experimentally infected and field pigs. Specific reactions were found in sera of experimental pigs with antigens of serotypes 1, 5 and 7 whereas the serotype 2 antigen was cross-reactive. A 1:200 serum dilution was used for testing of 300 sera from 21 swine herds in southern Ontario. Cases of pleuropneumonia had occurred in 11 of these herds, but not in the others. The negative cut-off value was the mean optical density at 405 nm (OD_{405}) + three standard deviations (SD) for 16 negative reference sera. Sera from four pigs naturally infected with *Actinobacillus suis* were tested and found to react to varying degrees with each of the antigens. Therefore a second cut-off value was determined as the mean OD_{405} + 2 SD for the *A. suis* sera. Sera which, in the ELISA produced OD readings above the latter cut-off were considered positive for antibodies to *A. pleuropneumoniae*; those which were lower than the former cut-off were considered negative. Readings between the two cut-off values may have been due to low positive titers or cross-reactivity, possibly with *A. suis*, and could not be used to predict pleuropneumonia. Of the pleuropneumonia-free herds, none had positive reactors to serotypes 5 or 7, whereas one and two herds had positive reactors to serotypes 1 and 2, respectively. Of the pleuropneumonia positive herds, six had positive reactors to serotype 1, one to serotype 2, four to serotype 5, and eight to serotype 7.

RÉSUMÉ

Les antigènes polysaccharidiques capsulaires (APSC) provenant des sérotypes 1, 2, 5 et 7 d'*Actinobacillus Pleuropneumoniae* ont été utilisés en ELISA pour évaluer des sérums de porc. Des réactions spécifiques ont été notées pour les sérums obtenus de porcs infectés expérimentalement pour les sérotypes 1, 5 et 7 alors que des réactions croisées furent notées pour le sérotype 2. Trois cents sérums provenant de 21 élevages porcins du sud de l'Ontario ont été testés. Une dilution 1:200 a été utilisée pour tous les sérums. Des cas de pleuropneumonie ont été rapportés dans 11 de ces élevages. Le seuil de négativité a été établi comme étant la moyenne de la densité optique à 405, nm (DO_{405}) + trois déviations standards (DS) et ce pour 16 sérums de référence négatifs. Les sérums de quatre porcs infectés naturellement avec *Actinobacillus suis* ont réagi à des degrés divers avec chacun des antigènes. En conséquence, un deuxième seuil à été établi comme étant la moyenne de la DO_{405} + 2 DS obtenue avec les sérums anti-*A. suis*. Les sérums ayant des DO en ELISA au dessus de ce deuxième seuil ont été considérés comme positifs pour *A. pleuropneumonia*. Les valeurs compromises entre les deux seuils pourraient être le résultat de faibles titres d'anticorps ou de réactions croisées dues possiblement à *A. suis* et ne peuvent donc pas servir à évaluer la présence de pleuropneumonie. Des élevages exempts de pleuropneumonie, aucun sérum n'a réagi avec les APSC des sérotypes 5 et 7 alors qu'un élevage a réagi avec les APSC du serotype 1 et deux avec ceux du sérotype 2. Dans les élevages où la pleuropneumonie était présente, six élevages ont réagi avec le sérotype 1, un avec le sérotype 2, quatre avec le sérotype 5 et huit avec le sérotype 7. ***(Traduit par Dr. Daniel Dubreuil).***

INTRODUCTION

An epizootic of porcine pleuropneumonia in southwestern Ontario began in 1978 with a high prevalence of acute, often fatal disease (1). During the following decade the prevalence of acute disease decreased, whereas that of subclinical or chronic infections increased (2). Chronically infected pigs may show no clinical signs (3), and are thought to be the main source of spread of the disease when they are introduced into susceptible herds (4,5). Hence detection of *Actinobacillus pleuropneumoniae*-infected herds and pigs is important in control of the disease, and in maintenance of pleuropneumonia-free herds. The most practical method of detecting subclinical infections is by serodiagno-

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sis (6). Among the serological tests for diagnosis of *A. pleuropneumoniae*, the most widely used has been the complement fixation (CF) test (7-9). However, it is a cumbersome test and not always reliable due to the procomplementary and sometimes anticomplementary activities of swine serum (10,11). The tube agglutination test with pretreatment of sera with 2-mercaptoethanol (TAT-2ME) was shown to be more sensitive than the CF test for detecting early infections, but less sensitive for detection of chronic infections (11,12). Recently, there has been interest in developing an enzyme-linked immunosorbent assay (ELISA) for serodiagnosis of *A. pleuropneumoniae* infections. Nicolet *et al* (10) used an ethylenediaminetetraacetate (EDTA)-extracted antigen, which they suggested contained superficial polysaccharide and protein antigens, and found it to be serotype-specific. In limited field tests using a serotype 2 EDTA-extracted antigen, this ELISA proved to be more sensitive than the CF test (10). Willson *et al* (13) conducted a field survey using a serotype 5 EDTA-extracted antigen in ELISA, and reported similar results. Serotype-specific results were also obtained using a hot saline-extracted antigen in ELISA (14). This ELISA was found to be as specific and more sensitive than CF and TAT-2ME tests.

In applying these ELISA techniques to serotype-specific diagnosis of *A. pleuropneumoniae* infections in commercial swine, we encountered high levels of nonspecific reactivity not evident in sera from minimal disease swine. Such nonspecific reactions probably reflect exposure to antigenically related organisms. Cross-reactions between *A. pleuropneumoniae* and other *Actinobacillus* spp. have been observed in the CF test (6), and *A. suis* infections have become very prevalent in Ontario swine herds (15). Because serotype-specific antigens of *A. pleuropneumoniae* reside in the capsule (16,17), we evaluated capsular extracts of serotypes 1, 2, 5 and 7 in ELISA (18). When tested with sera raised in swine and rabbits, the more purified extracts of capsular polysaccharide (CPS) provided a high degree of serotype specificity in detection of antibodies to serotypes 1, 5 and 7 (18). Here we report evaluation of the ELISA using these antigens in tests of sera from commercial swine herds with known pleuropneumonia status. Sera from pigs infected with *A. suis* were also tested to determine the possible cross-reactivity of antibodies to this common organism with capsular antigens of *A. pleuropneumoniae* used in the ELISA.

MATERIALS AND METHODS

BACTERIAL STRAINS AND CULTURE CONDITIONS

Four strains of *A. pleuropneumoniae*, representing the main serotypes isolated in Canada, were chosen: Shope 4074 (serotype 1); VLS 400 (serotype 2); MG 131 (serotype 5); and WF 83 (serotype 7). The serotype 1 strain was obtained from Dr. R. Nielsen, Copenhagen, Denmark. The other strains were field isolates from southern Ontario (own collection). Stock cultures were stored at -70° C in skim milk and glycerol. For routine growth, a loopful of thawed stock culture was streaked on trypticase soy agar plates supplemented with 5% heated bovine blood and 0.01% nicotinamide adenine dinucleotide (NAD) and incubated overnight at 37° C in 5% CO_2.

ANTIGEN PREPARATION

Capsular polysaccharides (CPS) were extracted from broth cultures of each strain by the method of Inzana (19), with some modifications (18). Polyanionic material was precipitated from cell-free culture supernatants using 10 mM hexadecyltrimethylammonium bromide and extracted with 0.4 M NaCl, reprecipitated in two volumes of 95% ethanol at -20° C, and then phenol extracted repeatedly until no protein could be detected by the method of Bradford (20), using bovine serum albumin (Sigma Chemical Co., St Louis, Missouri) as the standard. These extracts were further purified by ultracentrifugation for 4 h at 100,000 *g* to remove lipopolysaccharide (LPS).

Antigen concentrations for ELISA were determined by checkerboard titration, such that the positive reference serum diluted 1:200 gave an OD_{405} = 1.000 15 min after addition of the substrate/indicator solution. These concentrations corresponded to 0.5 μg/mL glucosamine for the serotype 1 and 5 antigens as previously reported (18). The concentration of the serotype 7 antigen corresponded to 0.5 μg/mL galactose using the phenol-H_2SO_4 carbohydrate assay described by Dubois *et al* (21), with D(+) galactose (Sigma Chemical Co., St. Louis, Missouri) as the standard. The serotype 2 antigen was not standardized to an individual sugar component of the capsule, as were the other serotype antigens, due to insufficient quantities. However, the concentration of the serotype 2 antigen necessary to produce an OD_{405} = 1.000 with homologous serum was equivalent to 10 μg/mL (w/v) of purified serotype 2 CPS (kindly provided by M. Perry, National Research Council, Ottawa, Ontario). The chemical structures of these capsular polysaccharides have been characterized by Altman *et al* (22-25).

SERA

Negative reference sera were collected from 16 pigs from the research herd of the Ontario Ministry of Agriculture and Food at Arkell, Ontario. This herd was free of *A. pleuropneumoniae* and *A. suis* infections. Positive reference sera specific for each of the four serotypes of *A. pleuropneumoniae* were collected from individual pigs three to four weeks following aerosol exposure, as previously described (26). The animal experiments followed the guidelines of the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.

Field sera were collected from 21 farrow-to-finish herds in Ontario having more than 100 pigs. Fifteen pigs, ages three to five months, were randomly selected from each herd for sampling. Some samples were used for other purposes and were not available for ELISA testing. Ten of the herds (designated 1-10) were part of the Ontario Swine Herd Health Program and free of pleuropneumonia. These were closed herds, inspected regularly by veterinarians for any clinical signs of pleuropneumonia. Also, farmers in this program were required to submit heads and lungs from 20 pigs three times annually for postmortem exami-

TABLE I. Average OD_{405} nm for replicate[a] samples of swine sera raised against serotypes 1, 2, 5 and 7 of *Actinobacillus pleuropneumoniae*, *Actinobacillus suis* and negative sera, tested at a 1:200 dilution against capsular antigens from the four serotypes of *Actinobacillus pleuropneumoniae*

Antiserum to	Antigen			
	Serotype 1	Serotype 2	Serotype 5	Serotype 7
Serotype 1	**1.137 ± 0.131**[b]	0.260 ± 0.078	0.149 ± 0.079	0.074 ± 0.025
Serotype 2	0.053 ± 0.009	**1.085 ± 0.131**	0.202 ± 0.098	0.123 ± 0.038
Serotype 5	0.023 ± 0.022	0.613 ± 0.180	**1.187 ± 0.153**	0.100 ± 0.035
Serotype 7	0.079 ± 0.039	0.298 ± 0.143	0.115 ± 0.063	**1.185 ± 0.218**
A. suis	0.338 ± 0.077	0.443 ± 0.143	0.226 ± 0.123	0.129 ± 0.048
Negative	0.023 ± 0.026	0.054 ± 0.049	0.022 ± 0.025	0.016 ± 0.018

[a]Homologous antisera to each serotype of *A. pleuropneumoniae* were tested 48 times (3 replicates/plate, 16 plates, 5 plates/day); heterologous antisera were tested 16 times. *A. suis* antisera were tested in quadruplicate. Negative sera were tested in duplicate
[b]Mean ± SD. Homologous reactions are shown in bold type

nation, including bacteriological culture of lung tissue when indicated. The other 11 herds, designated A-K, had histories of pleuropneumonia. There had been previous sporadic cases of pleuropneumonia in herds A and B. Herds C and E had experienced previous acute outbreaks, and in herds G-J acute cases of pleuropneumonia were occurring at the time of sampling. Herds D, F and K were deemed chronically infected based on clinical signs of unthriftiness and sporadic mortality diagnosed as pleuropneumonia by necrospy and bacteriological examination.

In addition, four *A. suis* reference sera were collected from a herd with sporadic cases of *A. suis* septicemia, but free of pleuropneumonia. *Actinobacillus suis* was isolated from the tonsils of the sampled pigs. All serum samples were stored at -20° C until used.

ELISA

Enzyme-linked immunosorbent assays were performed as described previously (18), with the following exceptions. Other than initial overnight coating at 4° C, all incubations were at 22° C. Plates were not blocked, as the use of various blocking agents did not appear to reduce the low levels of background seen when only phosphate buffered saline with 0.05% Tween 20 (PBS-T) was used. Each serum sample was diluted 1:200 in PBS-T, and 100 μL were added to one well in each of four quadrants (27). Controls included in each plate were: positive (homologous) serum, in triplicate; one replicate of each heterologous serum; negative serum, in duplicate; and no serum (PBS-T only, used as a blank in eight wells).

All field sera were assigned numbers, and testing was done in a single blind manner. The means and standard deviations (SD) were calculated for all replicate samples. Negative cut-off values for each serotype were calculated as the mean OD_{405} + 3 SD for the 16 negative sera tested. The diagnostic quality of the ELISA was determined on a herd basis. A herd was considered positive if at least one serum sample gave a positive reaction. The ELISA results were also compared on a group basis, according to the pleuropneumonia history of the herds.

RESULTS

The serotype 1, 5 and 7 antigens gave specific reactions when tested against a 1:200 dilution of sera from experimentally infected pigs; the serotype 2 antigen, however, was cross-reactive (Table I). When the highly purified serotype 2 CPS provided by M. Perry was tested in an ELISA against each of the reference sera, the pattern of cross-reactivity was the same (data not shown). All antigens reacted minimally with the negative reference sera, but each reacted to varying extents with sera from pigs infected with *A. suis* (Table I), giving OD values intermediate between those for negative and positive *A. pleuropneumoniae* reference sera. Therefore, two different cut-off values were calculated for each serotype: the mean OD_{405} + 3 SD for the 16 negative sera, below which test sera were considered negative for pleuropneumonia; the mean OD_{405} + 2 SD for the *A. suis* sera tested, above which test sera were considered positive for antibodies to *A. pleuropneumoniae*.

The number of pigs per herd detected as positive by ELISA are shown in Tables II and III. Some herds had reactors to more than one serotype, and some sera were positive for more than one serotype.

DISCUSSION

The low OD values obtained with the 16 sera from pigs free from *A. pleuropneumoniae* and *A. suis* infections indicated that antibodies to bacteria of the normal flora were not detected in the ELISAs using CPS antigens from serotypes 1, 2, 5 and 7 of *A. pleuropneumoniae*. The results obtained with these sera were used to

TABLE II. ELISA results for sera from clinically negative swine herds tested against capsular antigens from serotypes 1, 2, 5 and 7 of *Actinobacillus pleuropneumoniae*

Ag[a]	OD_{405}[b]	Herd										Total[c]
		1	2	3	4	5	6	7	8	9	10	
1	< 0.101	1[d]	4	1	14	10	12	14	10	5	0	**71**
	0.010-0.492	14	11	14	1	1	3	0	4	10	14	**72**
	> 0.492	0	0	0	0	0	0	0	0	0	1	**1**
2	< 0.202	0	2	5	13	1	0	6	7	0	0	**34**
	0.202-0.729	11	13	10	2	10	15	8	7	14	15	**105**
	> 0.729	4	0	0	0	0	0	0	0	1	0	**5**
5	< 0.098	1	0	2	13	11	11	14	6	1	1	**59**
	0.098-0.512	14	15	13	2	0	4	0	8	14	14	**85**
	> 0.512	0	0	0	0	0	0	0	0	0	0	**0**
7	< 0.070	13	10	4	14	6	9	11	14	15	2	**98**
	0.070-0.225	2	5	11	1	5	6	3	0	0	13	**46**
	> 0.225	0	0	0	0	0	0	0	0	0	0	**0**

[a]Capsular antigens of *A. pleuropneumoniae* serotypes 1, 2, 5 and 7
[b]Optical density at 405 nm
[c]Total number of sera with OD values in each range
[d]Number of sera/OD range/herd

TABLE III. ELISA results for sera from clinically positive swine herds tested against capsular antigens from serotypes 1, 2, 5 and 7 of *Actinobacillus pleuropneumoniae*

		Herd											
Ag[a]	OD405[b]	A	B	C	D	E	F	G	H	I	J	K	Total[c]
	< 0.101	6[d]	1	13	13	13	14	15	5	14	3	0	**97**
1	0.101-0.492	1	9	1	1	2	2	0	9	1	9	0	**35**
	> 0.492	0	5	1	0	1	0	0	1	0	1	14	**23**
	< 0.202	7	13	13	9	12	0	9	9	7	1	2	**82**
2	0.202-0.729	0	2	2	5	4	16	6	6	8	5	12	**66**
	> 0.729	0	0	0	0	0	0	0	0	0	7	0	**7**
	< 0.098	2	6	12	0	9	3	15	4	11	0	1	**63**
5	0.098-0.512	5	9	3	0	6	13	0	11	4	3	12	**66**
	> 0.512	0	0	0	14	1	0	0	0	0	10	0	**26**
	< 0.070	6	8	10	8	9	0	9	5	12	4	0	**71**
7	0.070-0.225	0	6	3	6	6	0	6	6	3	8	5	**49**
	> 0.225	1	1	2	0	1	16	0	4	0	1	9	**35**

[a]Capsular antigens of *A. pleuropneumoniae* serotypes 1, 2, 5 and 7
[b]Optical density at 405 nm
[c]Total number of sera with OD values in each range
[d]Number of sera/optical density range/herd

calculate individual negative cut-off values for each serotype assay. The serotype 1, 5 and 7 CPS antigens were specific, their reactivity with heterologous antisera being similar to the negative reference sera. In contrast, the serotype 2 CPS showed considerable cross-reactivity with the antiserotype 5 serum. This cross-reactivity was probably not due to contaminating antigens, as highly purified serotype 2 CPS provided by M. Perry (NRC, Ottawa, Ontario) gave similar results. However, since the antigens were not tested for residual LPS, it is not possible to rule out this antigen as a source of cross-reactivity.

Sera from pigs infected with *A. suis* cross-reacted slightly with the serotype 1 and 5 CPS antigens, and to a higher degree with the serotype 2 CPS. The serotype 7 CPS showed little cross-reactivity with the antisera against *A. suis*. These results corroborate those of Rosendal and Mittal (6), who found that pigs hyperimmunized against *A. pleuropneumoniae* serotypes 1-5 had antibody titers to an *A. suis* strain as well as homologous antibody titers, whereas a serotype 7 pig serum had only the homologous antibody titer. They also found that, in immunodiffusion, sera against serotypes 1, 2 and 7 of *A. pleuropneumoniae* reacted with antigens in the *A. suis* strain. However, lines of identity were seen only with serum against serotype 2. Based on the levels of cross-reactivity of antisera to *A. suis* with the *A. pleuropneumoniae* CPS antigens, second cut-off values were calculated for each serotype assay in order to reduce the number of false reactors due to possible *A. suis* infection. The results of the field survey indicate that by using this second cut-off level, the ELISA using CPS antigens was able to discriminate between the clinically positive and negative herds. Due to the cross-reactivity of serotype 2 antigen, the second cut-off value for this antigen was relatively high. This may have resulted in a false low number of serotype 2 reactors detected in the clinically positive herds. Since the serotype of infection was not known for any of the clinically positive herds, sensitivity and specificity values based on the two different cut-off levels could not be determined. Further evaluation of these cut-off values with a large number of antisera from pigs known to be infected with specific serotypes of *A. pleuropneumoniae*, as well as *A. suis*, is necessary.

Among the herds classified as positive, those chronically infected (D, F and K) were readily identified by the ELISA, with all sampled pigs showing high levels of reactivity. Variable numbers of positive reactors were detected in the herds which had previously experienced acute cases of pleuropneumonia (C and E), and in those experiencing acute cases at the time of sampling (G-J). It is possible that the infection had not spread through the entire herd and that the pigs sampled had not been exposed, or had not yet mounted an immune response.

In conclusion, diagnosis of pleuropneumonia caused by *A. pleuropneumoniae* serotypes 1, 5 and 7 can be made by screening for antibodies using CPS antigens in ELISA. This test may be more effective for diagnosing chronically infected herds. Due to cross-reactivity with other serotypes and *A. suis*, serodiagnosis of *A. pleuropneumoniae* serotype 2 infections cannot be made with certainty with this test.

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