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Detection of Serotype-Specific Antibodies or Capsular Antigen of *Actinobacillus pleuropneumoniae* by a Double-Label Radioimmunoassay

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Diagnostic tests for *Actinobacillus pleuropneumoniae* have been problematic because current tests do not use a purified antigen and in most cases measure either antibody or antigen, but not both. We describe a Farr-type double-label radioimmunoassay that utilizes purified, serotype-specific, ^{3}H-capsule to measure antibody to capsule directly or that can measure capsule in a sample indirectly by inhibition of antibody binding. The assay could detect about 1 ng of serotype-specific antibody in serum or at least 100 pg of capsule in a sample. Due to the sensitivity of the assay, false-positive results were common with neat sera (probably due to cross-reacting antibodies to unrelated antigens), but the specificity was improved when the sera were diluted 1:100. The radioimmunoassay should prove to be a useful reference method for research and diagnostic testing and for comparison of new assays for detection of capsule or antibodies to capsule.

Actinobacillus (*Haemophilus*) *pleuropneumoniae* is the etiologic agent of swine pleuropneumonia. Infection of pigs may result in acute, subacute, or chronic disease. Severe outbreaks of the disease occur when an immune, subclinical, chronic carrier is introduced into a nonimmune herd (21). Antibodies to *A. pleuropneumoniae* are commonly found in a high proportion of pig herds (5, 20). Current diagnostic tests for pleuropneumonia involve culture and serology. A wide variety of serologic tests have been developed to detect antibodies to any of the 12 serotypes of *A. pleuropneumoniae* or for serotyping (14–17). However, only a few serotypes are commonly isolated in a given geographic region. In the United States, serotypes 1 and 5 account for 80 to 89% of reported infections (16, 19).

Detection and epidemiology of serotypes that predominate in a given region can be improved when serologic tests are serotype specific. Serotype specificity, in turn, is determined by the capsular polymer of *A. pleuropneumoniae* (10). However, the most common tests for detection of antibody to *A. pleuropneumoniae* (complement fixation) or for serotyping (slide agglutination) utilize whole cells or a crude extract or use antiserum to whole cells, respectively. Somatic antigens of different serotypes of *A. pleuropneumoniae* (10), and possibly of different species of related bacteria (17, 18), are capable of cross-reacting among themselves. As more sensitive diagnostic tests are developed for serologic testing of *A. pleuropneumoniae*, serotype specificity may decrease and false-positive tests may increase. Coagglutination has been reported to be useful for serotyping *A. pleuropneumoniae* (14), and enzyme-linked immunosorbent assay (ELISA) and dot blot ELISA have been used for sensitive detection of antibodies to capsule (10) or somatic antigens. Although both tests are currently being used successfully, the coagglutination test is not necessarily specific for capsule, and the ELISA requires that the purified antigen adhere reproducibly to a solid phase. In addition, none of the described tests have the flexibility to be easily used for detection of antibodies or antigen.

Radioimmunoassay (RIA) is an extremely sensitive method for detection of antibodies or antigen. A double-label RIA is the reference method for detection of antibodies to *Haemophilus influenzae* type b and can be used as an inhibition assay to detect picogram amounts of capsular antigen (3). We have developed a modification of the double-label RIA for detection of capsular antigen or antibodies to *A. pleuropneumoniae*. The RIA is extremely sensitive for detection of serotype-specific antibodies. In addition, it can be used to detect and quantitate capsular antigen in respiratory specimens or associated with the bacteria. The disadvantage of the assay is that its high sensitivity results in detection of cross-reacting antibodies to common antigens unless the serum sample is diluted at least 1:100.

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MATERIALS AND METHODS

Bacterial strains and growth medium. Serotype 1 strain 4045 and serotype 5 strains K17, K17-C (noncapsulated mutant of K17), J45, and 178 were grown in Casamino Acids-yeast extract medium supplemented with 5 μg of nicotinamide adenine dinucleotide per ml (CY medium). The source of the strains, medium preparation, and the growth conditions have been described previously (9, 10).

Intrinsic radiolabeling and purification of *A. pleuropneumoniae* capsule. *A. pleuropneumoniae* K17 was intrinsically labeled with D-[6-^{3}H]glucose (18 Ci/mmol) (ICN Radiochemicals, Irvine, Calif.) by modification of the procedure described by Anderson (3). Two procedures were used to radiolabel and purify capsule. In procedure A, 5 ml of bacteria was grown in CY medium to early stationary phase, the cells were washed three times with CY medium, and the pellet was suspended in 250 μl of CY medium. A 50-μl portion of this culture was transferred to a sterile tube (10 by 75 mm) containing 25 μg of dried [^{3}H]glucose (total activity, 2.5 mCi). Chloramphenicol (0.04 μg) was added, and the

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culture was shaken gently overnight at 37°C. The tube was opened in a radioisotope fume hood, and the culture was diluted with 0.8 ml of 4°C 0.1 M phosphate buffer, pH 7.0. The culture was transferred to a Microfuge tube, the bacteria were sedimented at 10,000 × *g* for 15 min, and the supernatant was transferred to a clean Microfuge tube. Hexadecyltrimethylammonium bromide (Cetavlon; Eastman Kodak Co., Rochester, N.Y.) was added to a final concentration of 0.005 M, and the mixture was incubated at 4°C for 2 h. The mixture was centrifuged at 10,000 × *g* for 10 min, the pellet was washed twice by vigorous vortexing with 1 ml of cold 0.005 M Cetavlon in phosphate buffer, and the invisible precipitate was extracted twice with 0.15 ml of cold 0.4 M NaCl. The supernatant was transferred to a clean Microfuge tube, 3 volumes of −20°C ethanol (95%) were added, and the mixture was incubated at −20°C overnight. The mixture was centrifuged for 30 min at 10,000 × *g* and extracted twice with 0.3 ml of distilled water. The pooled aqueous phases were extracted twice with an equal volume of 90% room temperature phenol by vortexing for 10 min. NaCl, 5 M, was added to the aqueous phase to a final concentration of 0.4 M, and 3 volumes of −20°C ethanol were added. After at least 3 h at −20°C, the mixture was centrifuged for 30 min at 10,000 × *g*, and the precipitate was suspended in 0.2 ml of 0.1 M NaCl. The suspended capsule was applied to a Sepharose CL-4B column (1 by 28 cm) and eluted with 0.01 M NaCl at a linear flow rate of 5 cm/h, and 1-ml fractions were collected and assayed for tritium by liquid scintillation counting. Peak fractions that were positive for tritium were pooled, and the capsule was precipitated by the addition of 3 volumes of −20°C ethanol. After the mixture had incubated for at least 3 h at −20°C, it was centrifuged at 10,000 × *g* for 30 min. The pellet was suspended in phosphate-buffered saline containing 0.01% Thimerosal, pH 7.4, to an activity of 4 to 5 μCi/ml and stored at −70°C.

In procedure B, 25 μl of a mid-log-phase culture of *A. pleuropneumoniae* in CY medium was added to 0.5 ml of CY medium in a sterile tube containing 25 μg of dried [^{3}H]glucose. The culture was shaken overnight at 37°C, the cells were sedimented at 10,000 × *g* for 15 min, the centrifugation was repeated with the supernatant, and 0.005 M (final concentration) Cetavlon was added to the supernatant. The remaining procedures were the same as those described for procedure A, except that the 0.4 M NaCl and distilled water volumes for extractions were increased 10-fold and, following phenol extraction and ethanol precipitation, the preparation was suspended in 0.4 ml of distilled water and centrifuged at 100,000 × *g* for 4 h in an Airfuge (Beckman Instruments, Inc., Palo Alto, Calif.).

RIA for antibody or antigen. The working solution containing ^{3}H-capsule and ^{36}Cl (16.1 mCi/g; ICN Radiochemicals) was prepared exactly as described by Anderson (3). The RIA for antibody, including all controls, was also done as described previously (3, 6). For determination of antigen content, samples containing standard concentrations or unknown concentrations of capsule were diluted in fetal bovine serum; 25 μl of standard hyperimmune anticapsular serum diluted to yield 50% binding in the RIA was added to 25 μl of sample and incubated overnight at 4°C. A 25-μl portion of working solution was then added, and the RIA was completed as for antibody.

The percent antibody bound was calculated as described before (3, 7), except that an IBM computer and Lotus 1-2-3 software were used for computing and storing results. The amount of capsule in a sample was calculated from the amount of inhibition of antibody binding compared with a standard curve.

Antiserum and serologic assays. Preparation of high-titer swine and rabbit antisera to capsule and the ELISA has been described previously (10). Determination of specific antibody to capsule in serum was determined by precipitation with purified capsule (8) followed by protein determination (13).

Electrophoresis and fluorography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified, ^{3}H-capsule preparations was done as described by Laemmli (12). Fluorography of the dried gel was done following treatment of the gel with Amplify per the manufacturer's instructions (Amersham Corp., Arlington Heights, Ill.) and exposure on XAR-5 film (GBX-2) (Eastman Kodak Co., Rochester, N.Y.) for 7 days at −70°C.

RESULTS

Characterization of the ^{3}H-capsule preparations. The percentage of tritium present in each purification step of the radiolabeled capsule by procedure A was calculated. Following radiolabeling, 99.6% of the total [^{3}H]glucose added to the culture was accounted for. Six percent remained in the bacterial pellet after removal of the supernatant. Some 92% remained in the supernatant after precipitation by Cetavlon.

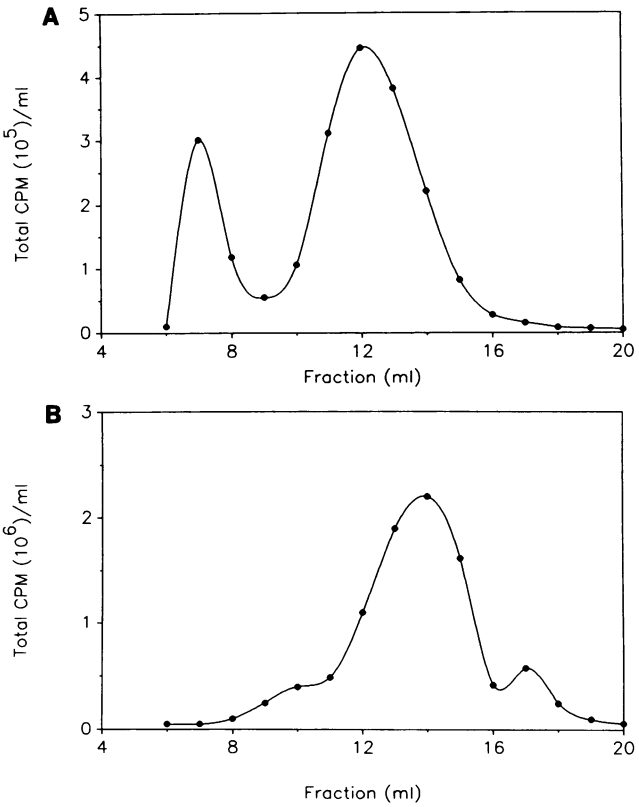

FIG. 1. Chromatographic elution profile of ^{3}H-capsule (serotype 5) from Sepharose CL-4B. The bacteria were radiolabeled with [^{3}H]glucose in the presence of chloramphenicol, and the capsule was purified by Cetavlon precipitation, NaCl, and phenol extraction (A) or grown with [^{3}H]glucose in the absence of chloramphenicol and purified as for panel A but with the addition of ultracentrifugation (B).

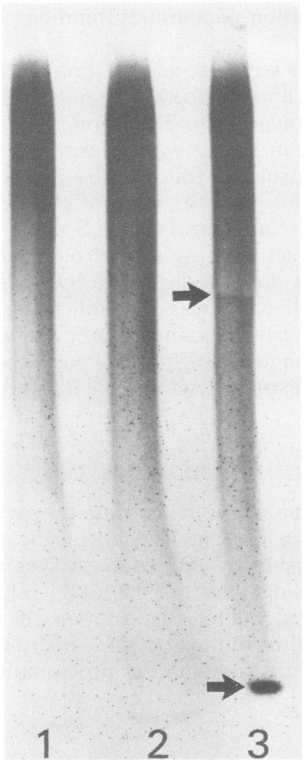

FIG. 2. Fluorography of the ^{3}H-capsule (serotype 5) prepared by procedure A. Samples of pooled fractions from peaks 1 and 2 were electrophoresed in sodium dodecyl sulfate–10% polyacrylamide gels, fixed, incubated with an amplifying fluorographic reagent, dried, and exposed to X-ray film for 7 days. Lanes: 1, 30,000 cpm of pooled fractions from peak 2; 2, 50,000 cpm of pooled fractions from peak 2; 3, 30,000 cpm of pooled fractions from peak 1. Arrows point to probable protein (upper) and lipopolysaccharide (lower) contamination.

Two percent radioactivity remained in the pooled supernatants after washing with Cetavlon. Less than 1% of the total activity was present in the ethanol phase after precipitation or in the phenol phase after extraction. The total [^{3}H]glucose recovered in the capsule preparation prior to Sepharose CL-4B chromatography was 0.18% of the total [^{3}H]glucose used for radiolabeling. Two major peaks containing tritium were eluted from the Sepharose CL-4B column (Fig. 1A). The first peak contained about 0.9×10^6 dpm, and the second peak contained about 3.2×10^6 dpm. In contrast, capsular material from procedure B (which was prepared from cells grown overnight in [^{3}H]glucose without chloramphenicol and ultracentrifuged prior to chromatography) eluted primarily as a single peak from Sepharose CL-4B; this peak was similar in profile to that of the second peak of material from procedure A (Fig. 1B). In addition, the total activity of the peak material from procedure B was 18×10^6 dpm.

Although inhibition studies indicated that peak 1 from procedure A was predominately capsule, fluorography of peaks 1 and 2 indicated that peak 1 contained some contaminating lipopolysaccharide and protein (Fig. 2, lane 3), but peak 2 did not (lanes 1 and 2). Furthermore, RIA with the peak 1 material consistently resulted in 10 to 15% protein binding with fetal bovine serum, whereas the same fetal bovine serum incubated with peak 2 material resulted in 0.1 to <2% protein binding. A standard hyperimmune swine

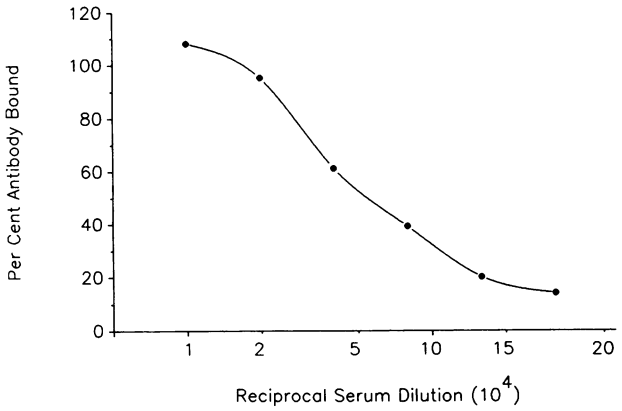

FIG. 3. Antibody binding of dilutions of swine hyperimmune antiserum to ^{3}H-capsule of serotype 5. Antiserum was diluted in fetal bovine serum and incubated with the working solution of ^{3}H-capsule, precipitated with 50% saturated ammonium sulfate; the precipitate was solubilized and counted in a liquid scintillation counter. The percent antibody bound was calculated with a Lotus 1-2-3 program and IBM computer by modification of the formula described in reference 7.

serum to serotype 5 could be diluted 1:10,000 in fetal bovine serum to obtain about 50% protein binding with the ^{3}H-capsule prepared from procedure A, but the same serum could be diluted 1:55,000 to obtain 50% binding with ^{3}H-capsule prepared by procedure B. Therefore, the specific activity of the ^{3}H-capsule prepared by procedure B was apparently higher than the preparation from procedure A. Subsequent preparations of ^{3}H-capsule from serotype 1 were made following the protocol for procedure B, and fractions 11 to 17 from the Sepharose CL-4B column were concentrated for use in the RIA. Although it is conceivable that proteins other than antibody could bind the radiolabeled capsule, if the capsule is adequately purified, binding by proteins other than antibody should be negligible. Therefore, protein bound to capsule from preparation B will subsequently be referred to as antibody bound.

Sensitivity of the RIA for antibody and antigen detection. A dilution curve of standard swine antiserum to serotype 5 was established by RIA with ^{3}H-capsule prepared by procedure B (Fig. 3). The antiserum used for this assay contained 8.4 mg of specific capsular antibody per ml, determined by complete precipitation with purified capsule and the Lowry assay (13). Therefore, using capsule from procedure B, 1.9 ng of antibody in 25 μl of our standard antiserum was measured by the RIA at 50% antibody binding. Thus, at the titer of this serum (102,400), approximately 1 ng of antibody could be detected. The RIA was eightfold more sensitive than ELISA with ^{3}H-capsule preparations of both serotypes 5 and 1 (Table 1). Antiserum to serotype 5 capsule cross-reacted weakly with ^{3}H-labeled serotype 1 capsule by RIA and ELISA. In contrast, antiserum to serotype 1 capsule did not cross-react with ^{3}H-labeled serotype 5 capsule by either of these assays.

Capsule content could be measured by inhibition of swine antibody binding to ^{3}H-capsule by dilutions of purified unlabeled serotype 5 capsule (Fig. 4). Less than 100 pg of serotype 5 capsule could be detected. Unknown quantities of capsule in samples could be calculated by comparing the percent antibody binding to the inhibition curve. Results with ^{3}H-capsule of serotype 1 for quantitation of homologous capsule were similar, although rabbit serum to serotype 1 capsule was used.

TABLE 1. Comparative reactivity and cross-reactivity of ELISA and RIA with serotype 1 and 5 capsules and antisera

Capsular antigen serotype	Antiserum serotype[a]	ELISA titer	RIA titer
5	5 (swine)	12,800	102,400
1	1 (rabbit)	12,800	102,400
5	1 (rabbit)	Negative	Negative
1	5 (swine)	8	64
1	5 (rabbit)	4	32

[a] Animals were immunized intravenously with a live culture of the indicated serotype weekly for 8 weeks.

Application of RIA for detection of antibody. Serum from pigs experimentally infected with various *A. pleuropneumoniae* serotypes was tested with ^{3}H-capsule preparations of serotypes 5 and 1 (Table 2). All serum samples tested neat had high antibody binding activity (not shown). Therefore, the sera were tested at 1:10, 1:100, and 1:1,000 dilutions. Preimmune pigs had negative levels of antibody (considered less than or equal to 10% binding) at all serum dilutions. Ten percent binding was chosen as the cutoff for a negative result based on tests with negative control samples, which ranged from <1 to 8% binding; an additional 2% was added as a margin of error. However, pigs infected with any serotype of *A. pleuropneumoniae* had positive levels of antibody at 1:10 dilutions, except serum to serotype 2 tested with ^{3}H-capsule of serotype 1. At a 1:100 dilution, one antiserum to serotype 1 remained positive for serotype 5 (23% binding), and one antiserum to serotype 3 remained positive (16% binding). The remaining sera were positive only for the homologous ^{3}H-capsule preparation. At a 1:1,000 dilution, one serum positive for serotype 5 was negative, and two sera positive for serotype 1 were borderline. Complement fixation titers, determined for some sera, confirmed that all infected pigs tested were positive for *A. pleuropneumoniae*.

Normal pigs were also examined for antibody to the capsule preparations (Fig. 5). Of 11 individual sera from the local university swine herd, 10 were positive for antibodies to serotype 5 at 1:100 by RIA (group I, closed circles). All of the sera were positive at 1:10 (open circles). The one serum that was negative for serotype 5 at 1:100 was positive with ^{3}H-capsule of serotype 1. All but two samples were positive with serotype 1 ^{3}H-capsule at a 1:100 dilution (not shown). All of these serum samples were confirmed positive by complement fixation at a titer of 1:32 or higher (results provided by the National Veterinary Services Laboratory, Ames, Iowa). For some samples, antibody binding was nearly identical for both serotypes of labeled capsule, while others were positive for only one serotype at 1:100 (e.g., 55% binding of one sample for serotype 5 and 4% binding for serotype 1; the complement fixation titer was 1:64). Sera from pigs in closed herds from Iowa and Minnesota were also tested for reactivity with serotype 5 ^{3}H-capsule (Fig. 5, groups II and III, respectively). Three of the sera from Iowa were positive for serotype 5, and six were negative at 1:100; all samples were negative for serotype 1 at 1:100 (not shown). Only three of the samples were negative for serotype 5 at 1:10. These sera were negative by complement fixation (less than 1:4 dilution) and ELISA (less than 1:200), using whole cells or lipopolysaccharide as antigen, respectively (Y.-W. Chiang, personal communication). All of the sera from the Minnesota herd were negative for antibodies to both serotype 5 and 1 capsules at 1:10 and 1:100.

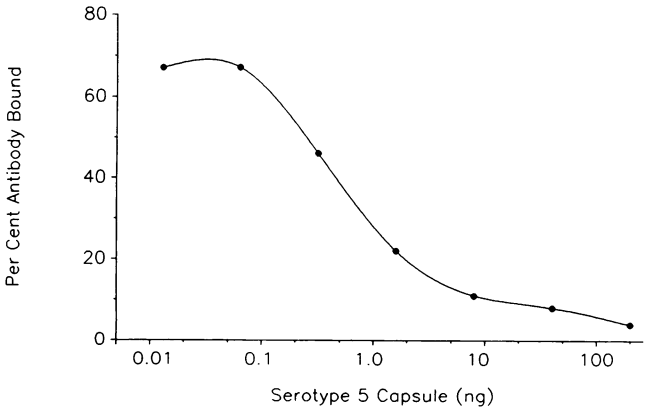

FIG. 4. Radioantigen inhibition assay curve for determination of total capsule content. Hyperimmune swine serum to serotype 5 capsule was diluted in fetal bovine serum to obtain about 60% antibody binding with ^{3}H-capsule. The serum was preincubated with various concentrations of purified serotype 5 capsule, prior to incubation with ^{3}H-capsule and completing the assay as described in the legend to Fig. 3.

TABLE 2. Reactivity of ^{3}H-capsular polysaccharides of *A. pleuropneumoniae* serotypes 1 and 5 with sera from preimmune and immune pigs

Serotype specificity of antiserum[a]	% Antibody bound by given ^{3}H-capsule						Complement fixation titer[b]
	Serotype 5			Serotype 1			
	Log 1[c]	Log 2	Log 3	Log 1	Log 2	Log 3	
Preimmune[d]	8	2	2	8	2	1	ND
1	39	8	4	91	79	36	64
1	83	23	8	62	27	9	ND
1	43	7	5	60	26	10	ND
2	11	2	1	8	2	<1	16
3	43	16	4	10	1	<1	64
4	25	4	2	20	1	<1	128
5	99	43	7	22	5	<1	64
5*	98	95	39	21	2	2	ND
7	27	4	1	31	5	1	64
7*	15	4	1	18	2	<1	ND

[a] Immune sera were collected from nonimmune pigs (complement fixation titer negative) following a single sublethal challenge.
[b] Determined at the National Veterinary Services Laboratory, Ames, Iowa. ND, Not determined.
[c] Log dilution of sera.
[d] Preimmune sera were obtained from two of the pigs later challenged with serotypes 5* and 7*.

To determine whether the antibodies detected in noninfected animals could have been present due to exposure to bacteria or antigens cross-reactive with the ^{3}H-capsule preparations, sera from other animals that are not natural hosts for *A. pleuropneumoniae* were also tested (Fig. 5). Serum samples from three cows (group IV) were positive for antibody to serotype 5 capsule at 1:100, and two of three sera from horses (group V) were positive at 1:100; one was borderline. All of the large-animal samples were positive at 1:10. Sera from three rabbits (group VI) were negative at 1:10 and 1:100, whereas serum from one mouse was positive at 1:100 and sera from two mice were positive at 1:10 (group VII).

Detection of capsular antigen by inhibition RIA. The versatility of the RIA allowed it to be used for serotype-specific antigen detection and quantitation as well as for antibody. The assay was used to quantitate the content of capsular

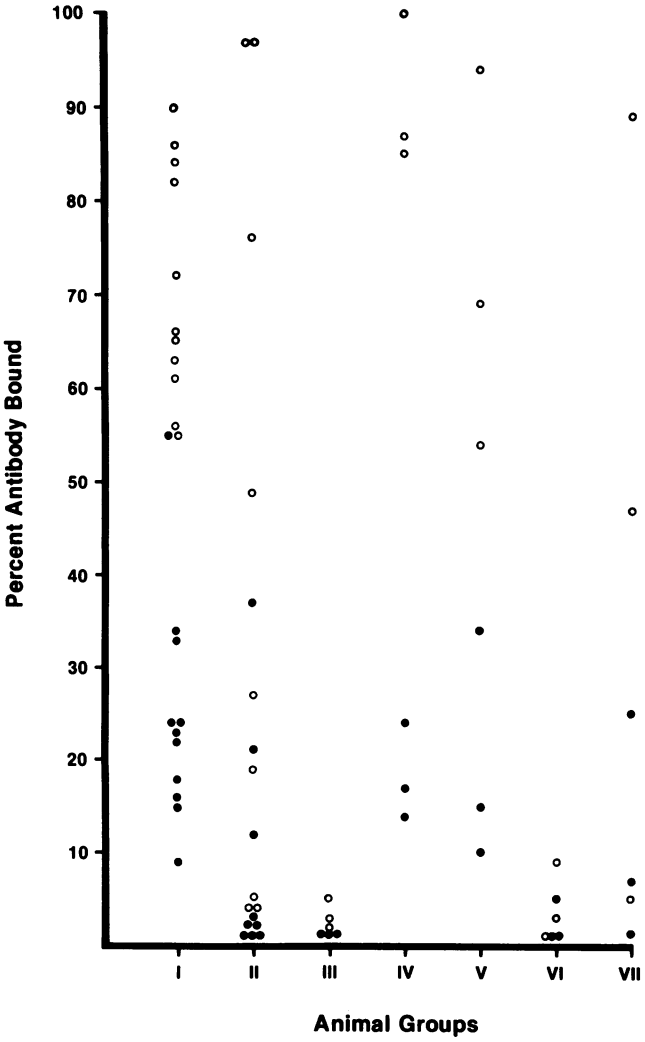

FIG. 5. Binding of antibody from various animal species to ^{3}H-capsule of serotype 5 *A. pleuropneumoniae*. Sera were diluted 1:10 (○) or 1:100 (●) in fetal bovine serum prior to testing in the RIA. Animal groups: I, swine maintained at the Virginia Tech swine center; II, swine maintained at a closed research herd in Iowa; III, swine maintained at a closed research herd in Minnesota; IV, normal cattle; V, normal horses; VI, normal rabbits; VII, normal mice.

antigen produced by three strains of serotype 5 bacteria and to confirm the lack of capsule produced by a noncapsulated mutant (10) (Table 3). In addition, at least 4 ng of capsule per ml (or 100 pg total) could be detected from nasal swabs of swine infected with strain J45 *A. pleuropneumoniae* and from lung tissue of mice and swine infected with strain J45. The capsule content ranged from 15 ng/ml in infected mouse lung to >100 ng/ml in lesions of infected swine lung.

DISCUSSION

Our ^{3}H-capsule was initially prepared by the procedure described by Anderson (3). However, the specific activity of tritium obtained in the capsule by this procedure was not as high as that reported by Anderson (3). This is possibly due to inherent differences in capsule production or release between *A. pleuropneumoniae* and *H. influenzae* type b or both. Therefore, we modified the procedure primarily by growing the cells overnight in the presence of [^{3}H]glucose and adding ultracentrifugation to the purification process. ^{3}H-capsule purified from *A. pleuropneumoniae* following overnight growth in the labeled compound greatly increased the specific activity of the capsule preparation. The addition of ultracentrifugation and recovery of selected fractions from Sepharose CL-4B chromatography resulted in at least as pure a preparation as when stationary-phase cells were incubated with [^{3}H]glucose in the presence of chloramphenicol. Ultracentrifugation removed most of the material that previously eluted in the void volume, which, in addition to capsule, contained lipopolysaccharide and protein, and material that nonspecifically reacted with fetal calf serum.

TABLE 3. Radioantigen inhibition assay for quantitation of *A. pleuropneumoniae* serotype 5 capsule

Specimen	Capsule content per ml
A. pleuropneumoniae	
J45[a]	130 ± 18 μg
178[a]	19 ± 4 μg
K17[a]	155 ± 25 μg
K17-C[b]	<4 ng
Extracts	
Infected mouse lung (J45)[c]	15.3 ± 2.5 ng
Noninfected mouse lung[c]	<4 ng
Infected swine lung (J45)[c]	107 ± 19 ng
Noninfected swine lung[c]	<4 ng
Nasal swabs from infected pig (J45)[c]	25 ± 2 ng
Nasal swabs from noninfected pigs[c]	<4 ng

[a] Serotype 5 strain.
[b] Noncapsulated mutant of strain K17.
[c] Mean capsule content of at least three different samples ± the standard deviation for sampled data.

The RIA had the flexibility of directly measuring antibody to capsule or, by inhibition, antigen. The sensitivity of the RIA exceeded that of any test for *A. pleuropneumoniae* yet described. With our standard antiserum, <100 pg of capsule, or about 1 ng of antibody to capsule, could be detected and measured. Due to the sensitivity of the RIA, it was not surprising that some weak cross-reactivity of antiserum to serotype 5 capsule occurred with serotype 1 capsule. Of interest, however, was that antiserum to serotype 1 capsule did not cross-react with serotype 5 capsule. Though somewhat less sensitive, these results were confirmed by ELISA. The serotype 1 capsule of *A. pleuropneumoniae* has the structure [(4)-β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*-(1-PO_4)]$_n$ (85% acetylated at position 6 of β-D-Glc*p*NAc), while the structure of the serotype 5a capsule is [→6)-α-D-Glc*p*NAc-(1→5)-β-D-dOclA*p*-(2→]$_n$ (1, 2); the serotype 5b capsule has the same structure as 5a except that β-D-Glc*p* is linked 1→4 to β-D-dOclA*p* (E. Altman, J.-R. Brisson, D. W. Griffith, and M. B. Perry, unpublished data). Therefore, serotype 1 and 5 capsules share *N*-acetylglucosamine as a common component. This amino sugar may compose part of a weak, but common, antigenic epitope of each capsule, which may only be detected with hyperimmune antiserum and a sensitive assay. Furthermore, the *A. pleuropneumoniae* capsules are composed of common sugars that occur in many capsular polysaccharides (11). In fact, the structure of the capsule of *A. pleuropneumoniae* serotype 1 is identical to that of *H. influenzae* type c, except that the serotype 1 capsule is acetylated at the 6-position of *N*-acetylglucosamine, rather than the 3-position in the type c capsule (11). It is difficult to compare the sensitivity and specificity of the RIA with those

of other serologic tests currently in use, except for the ELISA described previously (10), because other assays have not used purified capsule or monospecific antiserum in the assays. When whole cells are used as antigen in assays, preparation of the cells may result in decapsulation. Thus, a negative result by complement fixation, but positive by RIA, may have been due to the lower sensitivity of the assay combined with the diminished amount of capsule present. Nonetheless, the sensitivity of the RIA described is so high that cross-reactions not previously detected by other assays would not be unexpected unless the sera are properly diluted.

Most normal pigs tested for antibody to *A. pleuropneumoniae* were positive when the sera were tested neat, and many were still positive at 1:10. At 1:100, however, most sera negative for *A. pleuropneumoniae* by other assays were negative by RIA. One antiserum to serotype 1 and one antiserum to serotype 3 remained weakly positive to serotype 5 ^{3}H-capsule at 1:100. However, since we did not have preimmune sera from these pigs, it is possible they may have been exposed to serotype 5. Mittal et al. (14) have reported that pigs may be coinfected with more than one serotype, particularly serotypes 5 and 1, and that such coinfections can be detected with more sensitive assays. The positive antibody results at higher concentrations were likely due to antibodies made in response to cross-reacting antigens having components common to those in *A. pleuropneumoniae* capsules (e.g., glucose, galactose, *N*-acetylglucosamine, etc.). Our evidence that sera positive for antibodies to the capsule were not necessarily specific for *A. pleuropneumoniae* was demonstrated by the positive responses of animals that were not natural hosts for *A. pleuropneumoniae*. In addition, large animals that may naturally be exposed to more bacteria (horses and cattle) were positive at higher dilutions of antiserum than laboratory animals (rabbits and mice). Three of the antisera with known serotype specificity were negative when tested at 1:1,000 (one to serotype 5 and two to serotype 1). Therefore, dilution of sera to 1:1,000 resulted in false-negative results. It is likely that the optimum dilution of serum for testing by RIA would be 1:200 or 1:400.

A variety of variables have been identified that affect antibody binding in the RIA for *H. influenzae* type b capsule (4); these include the concentration and size distribution of the radiolabeled antigen, as well as the dilution and affinity binding of the sera. Differences in specific activity of capsules prepared by procedures A and B clearly affected the sensitivity of the RIA. Variations due to differences in size could be controlled by pooling the same fractions from Sepharose CL-4B chromatography.

In addition to antibody detection, the amount of antibody to the capsule could be quantitated by comparing the percent antibody bound in a test serum with that of a standard antiserum, in which the amount of antibody to capsule was determined by precipitation. Quantitative assays for antibody to the capsule of *H. influenzae* type b are commonly used to determine the antibody response to capsular vaccines and whether sera contain protective levels of antibody to capsule (4).

When used in combination with a standard antiserum, the competitive inhibition RIA can be used to quantitate the amount of capsule present in samples or produced by bacteria. Antigen detection is particularly well suited to the RIA because of its sensitivity. Thus, antigen could be detected in nasal swabs of pigs or lung lesions of infected mice and pigs. Detection of capsule in nasal swabs is particularly useful because the procedure is noninvasive and because recovery of *A. pleuropneumoniae* from nasal swabs may be compromised due to overgrowth by less fastidious bacteria.

The major disadvantages of the RIA are that it is not suitable for field testing and requires the use of radioisotopes, making it predominately a research tool. Furthermore, because of the widespread occurrence of *A. pleuropneumoniae* in pigs and the potential for mixed infections due to more than one serotype, we were not able to test enough known positive and negative sera to determine the exact dilution of serum required to minimize false-negative and false-positive results. However, the RIA should be highly useful as a standard assay by which to compare future diagnostic tests and for research involving the capsule and capsular antibody of *A. pleuropneumoniae*.

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LITERATURE CITED

1. **Altman, E., J.-R. Brisson, and M. B. Perry.** 1986. Structural studies of the capsular polysaccharide from *Haemophilus pleuropneumoniae* serotype 1. Biochem. Cell Biol. **64**:707–716.
2. **Altman, E., J.-R. Brisson, and M. B. Perry.** 1987. Structure of the capsular polysaccharide of *Haemophilus pleuropneumoniae* serotype 5. Eur. J. Biochem. **170**:185–192.
3. **Anderson, P.** 1978. Intrinsic tritium labeling of the capsular polysaccharide antigen of *Haemophilus influenzae* type b. J. Immunol. **120**:866–870.
4. **Anderson, P., R. A. Insel, S. Porcelli, and J. I. Ward.** 1987. Immunochemical variables affecting radioantigen-binding assays of antibody to *Haemophilus influenzae* type b capsular polysaccharide in childrens' sera. J. Infect. Dis. **156**:582–590.
5. **Brandreth, S. R., and I. M. Smith.** 1985. Prevalence of pig herds affected by pleuropneumonia associated with *Haemophilus pleuropneumoniae* in eastern England. Vet. Rec. **117**:143–147.
6. **Farr, R. S.** 1958. A quantitative immunochemical measure of the primary interaction between I*BSA and antibody. J. Infect. Dis. **103**:239–262.
7. **Gotschlich, E. C.** 1971. A simplification of the radioactive antigen binding test by a double label technique. J. Immunol. **107**:910–911.
8. **Heildelberger, M., C. M. MacLeod, S. J. Kaiser, and B. Robinson.** 1946. Antibody formation in volunteers following injection of pneumococci or their type-specific polysaccharides. J. Exp. Med. **82**:303–320.
9. **Inzana, T. J.** 1987. Purification and partial characterization of the capsular polymer of *Haemophilus pleuropneumoniae* serotype 5. Infect. Immun. **55**:1573–1579.
10. **Inzana, T. J., and B. Mathison.** 1987. Serotype specificity and immunogenicity of the capsular polymer of *Haemophilus pleuropneumoniae* serotype 5. Infect. Immun. **55**:1580–1587.
11. **Kenne, L., and B. Lindberg.** 1983. Bacterial polysaccharides, p. 287–363. *In* G. R. Aspinall (ed.), The polysaccharides, vol. 2. Academic Press, Inc., New York.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. **Mittal, K. R., R. Higgins, and S. Larivière.** 1983. Identification and serotyping of *Haemophilus pleuropneumoniae* by coagglu-

tination test. J. Clin. Microbiol. **18**:1351–1354.
15. **Nielsen, R.** 1986. Serological characterization of *Actinobacillus pleuropneumoniae* strains and proposal of a new serotype: serotype 12. Acta Vet. Scand. **27**:452–455.
16. **Rapp, V. J., R. F. Ross, and B. Z. Erickson.** 1985. Serotyping of *Haemophilus pleuropneumoniae* by a rapid slide agglutination and indirect fluorescent antibody tests in swine. Am. J. Vet. Res. **46**:185–192.
17. **Rapp, V. J., R. F. Ross, and T. F. Young.** 1985. Characterization of *Haemophilus* spp. isolated from healthy swine and evaluation of cross-reactivity of complement-fixing antibodies to *Haemophilus pleuropneumoniae* and *Haemophilus* taxon "minor group." J. Clin. Microbiol. **22**:945–950.
18. **Rosendal, S., and K. R. Mittal.** 1985. Serological cross-reactivity between a porcine *Actinobacillus* strain and *Haemophilus pleuropneumoniae*. Can. J. Comp. Med. **49**:164–170.
19. **Schultz, R. A., R. F. Ross, A. Gunnarsson, and R. Nielsen.** 1983. Serotyping of 50 different isolates of *Haemophilus pleuropneumoniae* from swine pneumonia in Iowa and surrounding states. Vet. Med. Small Anim. Clin. **78**:1451–1453.
20. **Schultz, R. A., T. F. Young, R. F. Ross, and D. R. Jeske.** 1982. Prevalence of antibodies to *Haemophilus pleuropneumoniae* in Iowa swine. Am. J. Vet. Res. **43**:1848–1851.
21. **Sebunya, T. N. K., and J. R. Saunders.** 1982. *Haemophilus pleuropneumoniae* infections in swine: a review. Am. J. Vet. Res. **182**:1331–1337.