JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1995, p. 2297–2303 Vol. 33, No. 9
0095-1137/95/$04.00+0

Simplified Procedure for Preparation of Sensitized Latex Particles To Detect Capsular Polysaccharides: Application to Typing and Diagnosis of *Actinobacillus pleuropneumoniae*

THOMAS J. INZANA*

Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0342

Received 19 January 1995/Returned for modification 27 March 1995/Accepted 7 June 1995

A novel, inexpensive method for obtaining immunoglobulin G (IgG) specific for capsular antigen is described for use in latex agglutination tests. Hyperimmune rabbit serum against encapsulated *Actinobacillus pleuropneumoniae* was thoroughly adsorbed with a nonencapsulated mutant. The capsule titer of the adsorbed serum was unaffected, whereas reactivity to nonencapsulated cells was reduced to background levels, as determined by enzyme immunoassay. The IgG component of the adsorbed serum was recovered by protein A chromatography and was covalently coupled through a water-soluble carbodiimide to carboxylate latex beads. The sensitized latex particles (SLP) were agglutinated by 10 ng of homologous capsule or more per ml, were not agglutinated by heterologous capsules at concentrations of <10 μg/ml, and were stable for over 1 year at 4°C without loss of sensitivity. There was no difference in the sensitivity or specificity of latex particles coupled with IgG purified by capsule affinity chromatography. The SLP were agglutinated by all strains of bacteria of the homologous serotype but not by heterologous serotypes or strains of *Pasteurella multocida*, *Actinobacillus suis*, or *Haemophilus parasuis* tested at a density equivalent to a 0.5 McFarland standard. The SLP detected homologous capsule in lung tissue, nasal swabs, and concentrated urine samples from all pigs culture positive for *A. pleuropneumoniae* but one. Precoating of carboxylate latex particles with avidin followed by conjugation of biotin-hydrazide-labelled IgG to capsule increased the sensitivity of the assay approximately 10-fold. Adsorption of serum with nonencapsulated mutants may be used to prepare SLP with optimum sensitivity and specificity without the need to purify capsule or couple capsule to affinity columns.

Actinobacillus (*Haemophilus*) *pleuropneumoniae* is the etiologic agent of porcine pleuropneumonia, which is a highly contagious and economically devastating disease throughout the world. There are 12 serotypes of *A. pleuropneumoniae*, but predominant serotypes usually vary between countries. Pleuropneumonia is usually spread by a carrier animal which has recovered from clinical disease or has subclinical infection (12). Once an outbreak occurs, rapid diagnosis is essential to minimize losses. Confirmed identification of the agent and serotype may require several days. As for most encapsulated bacteria, the capsular polysaccharide of *A. pleuropneumoniae* is the predominant serotype-specific antigen (7). A rapid antigen detection assay to identify serotype-specific capsule from lung tissue of dead animals or nasal swabs from live carrier animals would be beneficial for making a rapid diagnosis and containing the infective agent.

Diagnosis of systemic infections caused by encapsulated bacteria or serotyping of such bacteria can be accomplished by various antigen detection assays. Latex agglutination, among all antigen detection assays, is the most rapid and simplest to perform. Sensitized latex particles (SLP) have been prepared for the detection of capsular polysaccharides shed from *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Cryptococcus neoformans* into the cerebrospinal fluid or urine of patients (16). Because of the simplicity and minimal amount of materials required for latex agglutination tests, their sensitivity and specificity are particularly dependent on the affinity, specificity, and class of antibodies used (2, 10, 16). As sensitivity increases, specificity may decrease if nonspecific antibodies on the latex particles that cross-react with other bacterial or host components are present (7, 8). Poor specificity may be a particular problem when testing for poorly immunogenic capsular antigens because the entire bacterium must be used for immunization. Immunization with whole bacteria is often required to induce high titers of immunoglobulin G (IgG) to capsule but also induces antibodies to potentially cross-reactive somatic antigens (7). Therefore, to produce antibodies with optimum specificity, the capsule should be purified and conjugated to a solid support and the specific antibody should be isolated by affinity chromatography. Additional procedures may also be required to purify the IgG component. However, purification of capsules and continuous preparation and regeneration of columns are time-consuming and expensive.

There is therefore a need to rapidly diagnose *A. pleuropneumoniae* infections in the field and to simplify the preparation of SLP while maintaining optimum specificity. To accomplish this, hyperimmune serum against *A. pleuropneumoniae* was adsorbed with a nonencapsulated mutant identical to the wild type in somatic antigen composition to produce a monospecific antiserum to capsule. Although antibodies can be passively adsorbed through their Fc region to unmodified polystyrene particles, the antibodies elute from the surface rapidly, resulting in a reagent with a short shelf life that is unsuitable for commercialization (2). Carboxylate-modified and amide-modified latex particles are superior to unmodified latex particles for agglutination tests because they are more hydrophilic, are more stable in aqueous solutions, and contain reactive sites for covalent conjugation of proteins (2). The IgG component of the adsorbed serum was therefore covalently conjugated to carboxylate latex particles, and the sensitivity and specificity of

* Phone: (703) 231-5095. Fax: (703) 231-3426. Electronic mail address: CVMTJI@VTVM1.CC.VT.EDU.

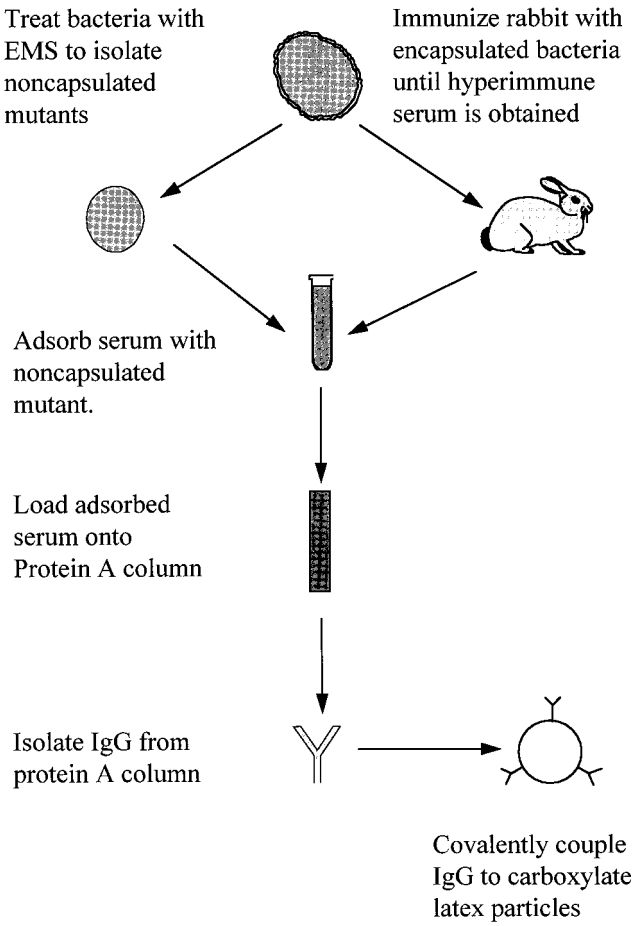

FIG. 1. Procedure for preparation of latex particles sensitized with monospecific IgG to capsular polysaccharide.

these SLP were compared with those of IgG to capsule isolated by affinity chromatography.

MATERIALS AND METHODS

The overall scheme for preparation of SLP containing monospecific IgG antibody to capsular polysaccharide is shown in Fig. 1. The details of each step are described below.

Bacterial strains and growth conditions. Primary cultures and maintenance of *A. pleuropneumoniae* serotype 1 and 5 strains have been previously described (7). Serotype 9 strains were provided by Jacques Nicolet, University of Bern, Bern, Switzerland. *A. pleuropneumoniae* serotypes 8 and 10 to 12 were obtained from Brad Fenwick, Kansas State University; serotype 7 strains were obtained from Lorraine Hoffman, Iowa State University; and serotypes 2 to 4 and 6, *Actinobacillus suis*, and *Haemophilus parasuis* were obtained from The American Type Culture Collection, Rockville, Md. Field isolates of *H. parasuis* and *Pasteurella multocida* were provided by Karen Post, Rollins Animal Disease Diagnostic Laboratory, Raleigh, N.C. Nonencapsulated mutants of *A. pleuropneumoniae* were isolated following chemical mutagenesis with ethylmethane sulfonic acid, as previously described (9). The hemolytic activity, electrophoretic outer membrane protein profiles, and electrophoretic lipopolysaccharide profiles of the parent and mutant strains were identical (reference 9 and data not shown). Capsule content was undetectable (<4 ng/10^9 CFU) in the mutant strains, as determined by inhibition radioimmunoassay (5).

Bacterial cultures were shaken rapidly in brain heart infusion broth supplemented with 10 μg of NAD per ml at 37°C until growth reached late log-early stationary phase. The bacteria were washed twice in excess 0.1 M phosphate-buffered saline (pH 7.4) (PBS) and used immediately or frozen at −20°C until needed (within 2 weeks).

Capsule purification. Capsular polysaccharides from serotype 1 (CP1), serotype 5 (CP5), and serotype 9 (CP9) were purified from stationary-phase culture supernatants of strains 4045, J45, and X1, respectively, as previously described (4), except that ultracentrifugation (4 h at 115,000 × *g*) was used in place of gel filtration to remove endotoxin.

Serum and IgG. Hyperimmune rabbit serum against *A. pleuropneumoniae* was obtained by injecting rabbits twice with formalin-fixed whole cells in adjuvant (first in Freund's complete adjuvant and then in Freund's incomplete adjuvant) and then administering weekly intravenous injections of whole cells until the titer of antibody to purified capsule was >1:12,500, as determined by enzyme-linked immunosorbent assay (ELISA) (see below). A portion of the hyperimmune serum against each serotype was adsorbed twice with an excess of the homologous isogenic nonencapsulated mutant at 0°C. A pellet from 500 ml of stationary-phase bacteria was resuspended with 5 to 10 ml of serum and mixed overnight at 4°C, the mixture was centrifuged at 10,000 × *g* for 10 min, and the clarified serum was mixed with a fresh bacterial pellet for 4 h at 4°C. The mixture was centrifuged again, and the serum was filter sterilized.

The IgG component of the adsorbed serum was isolated by protein A affinity chromatography (Pharmacia Biotech, Piscataway, N.J.) with PBS running buffer. The IgG was eluted by reverse flow with 0.1 M glycine-HCl (pH 2.8) and collected in tubes containing 0.1 M Trizma base (pH 9.0) to neutralize acidity. The IgG was immediately dialyzed against PBS and concentrated by ultrafiltration to approximately 10 mg/ml with a Minicon ultrafiltration device (Amicon Corp., Danvers, Mass.). Typically, 15 to 20 mg of IgG was isolated per 5 ml of adsorbed immune serum, as determined by bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.).

IgG to *A. pleuropneumoniae* serotype 9 capsule was isolated by affinity chromatography only. Purified serotype 9 capsule was coupled to EAH-Sepharose (Pharmacia) as described by Lucas (11). The IgG component from nonadsorbed immune serum was isolated by protein A chromatography, and the IgG was loaded onto the capsule-EAH-Sepharose column. The capsule-specific IgG was eluted from the affinity column by reverse flow with 3.5 M $MgCl_2$, dialyzed against PBS, and concentrated as described above.

Immunological assays. For the ELISA, formalin-killed nonencapsulated bacteria or purified capsule was incubated in microtiter plate wells (Costar, Cambridge, Mass.) at 10^9 CFU/ml or 10 μg/ml, respectively, overnight at 4°C in carbonate coating buffer, pH 9.6. The wells were washed three times with PBS containing 0.05% Tween 20 by using a Nunc-Immunowash 12 Autoclavable manual plate washer (Nunc, Naperville, Ill.) and incubated with PBS containing 2% bovine serum albumin (BSA) (blocking buffer) for 1 h at 37°C. The wells were washed, unadsorbed or adsorbed rabbit antiserum to whole cells diluted in blocking buffer was added, and the mixture was incubated for 1 h at 37°C. The wells were thoroughly washed, a 1:2,000 dilution of goat anti-rabbit IgG (heavy and light chains; Jackson Immunoresearch Laboratories, West Grove, Pa.) conjugated to horseradish peroxidase in blocking buffer was added, and the mixture was incubated for 1 h at 37°C. The wells were thoroughly washed again, and 5′-aminosalicylic acid substrate was added. The plates were incubated for 30 min at room temperature, the reaction was stopped with 1 M NaOH, and the A_{450} was determined with a microplate reader (Molecular Devices Corp., Menlo Park, Calif.). Controls included buffer in place of cells or capsule and preimmune serum in place of antiserum. The absorbance of control wells with the highest background (preimmune serum) was subtracted from the absorbance of test wells. The endpoint titer was considered the dilution of antiserum whose absorbance was greater than the same dilution of preimmune serum plus 3 standard deviations.

The inhibition radioimmunoassay for quantitation of capsular polysaccharide was done as described elsewhere (5).

Preparation of SLP. Affinity-purified antibody to capsule was coupled to carboxylated latex particles 0.55, 0.75, and 1.0 μm in diameter (Polysciences, Inc., Warrington, Pa.) by modification of procedures recommended by the manufacturer. The latex particles (0.5 ml) were washed three times in 0.1 M carbonate buffer (pH 9.6) and then three times in 0.02 M phosphate buffer (pH 4.7) in 1.5-ml microcentrifuge tubes. All washes were done at 14,000 × *g* for 6 min at room temperature unless otherwise stated. Following the final wash, the particles were suspended in 0.625 ml of the phosphate buffer and transferred to 1.8-ml conical, screw-cap cryovials (Nalgene, Rochester, N.Y.), and 0.625 ml of a freshly prepared solution of 2% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide · HCl (EDC; Bio-Rad Laboratories, Richmond, Calif.) was added dropwise while the solution was slowly vortexed. For some experiments, 5 to 10 mM *N*-hydroxysulfosuccinimide (NHSS) or 5 to 10 mM *N*-hydroxysuccinimide (NHS) was added to the tubes at the time EDC was added or when the IgG was added because 5 to 10 mM NHSS has been shown to enhance the EDC-mediated coupling of carbohydrates to proteins (15). The tubes were rotated slowly end-over-end for 4 h at room temperature, and the SLP were transferred to microcentrifuge tubes and washed three times with 0.01 M borate buffer, pH 8.0. The SLP were resuspended in 1.2 ml of borate buffer and transferred to clean 1.8-ml cryovials. Concentrations of IgG ranging from 200 to 2,400 μg/0.5 ml of particles were added to the SLP, which were then rotated end-over-end overnight at room temperature. To block nonspecific binding, 50 μl of 0.25 M ethanolamine was added to the SLP and rotation was continued for 30 min. The SLP were transferred to 1.5-ml microcentrifuge tubes and centrifuged for 10 min at 14,000 × *g*. The supernatant was saved for protein determination, and the SLP were resuspended in 1% BSA in borate buffer and rotated for 30 min at room temperature. The SLP were washed one additional time in BSA and resuspended in 0.5 ml of

TABLE 1. Reactivity of *A. pleuropneumoniae* antiserum with nonencapsulated bacteria or purified capsule before and after adsorption with nonencapsulated mutants

Antiserum[a]	Adsorption[b]	Antigen	Absorbance[c]
Type 5 cells	No	Nonencapsulated type 5 cells	1.50 ± 0.16
	Yes	Nonencapsulated type 5 cells	0.10 ± 0.01
	No	Type 5 capsule	2.63 ± 0.32
	Yes	Type 5 capsule	2.71 ± 0.38
Type 1 cells	No	Nonencapsulated type 1 cells	1.80 ± 0.14
	Yes	Nonencapsulated type 1 cells	0.003 ± 0.002
	No	Type 1 capsule	1.42 ± 0.04
	Yes	Type 1 capsule	1.54 ± 0.18

[a] Rabbit antiserum to live, encapsulated *A. pleuropneumoniae.*

[b] Antiserum (10 ml) was adsorbed with a resuspended pellet of washed, nonencapsulated bacteria obtained from 500 ml of culture for 18 h at 0°C. The adsorption was repeated with a fresh resuspended pellet from 500 ml of culture but mixed for 4 h at 4°C.

[c] Means ± standard deviations for sera diluted 1:100.

latex storage buffer (1% [wt/vol] BSA, 5% [vol/vol] glycerol, and 0.1% (wt/vol] NaN_3 in PBS).

For some experiments, latex particles were conjugated to avidin. Concentrations of avidin ranging from 300 to 900 μg/0.5 ml of particles were conjugated to carboxylate latex beads as described above for IgG. Any remaining reactive sites were blocked with ethanolamine, and the latex particles were washed twice with 1 ml of BSA (10 mg/ml) in borate buffer. The particles were then washed three times in PBS and sonicated briefly to disrupt any aggregates. Biotin was conjugated to IgG by modification of the procedure described by O'Shannessy et al. (13). One milliliter of cold, fresh 20 mM sodium metaperiodate was added to 2 mg of IgG in 1 ml of cold 0.1 M sodium acetate buffer (pH 5.5), and the tube was immediately covered with aluminum foil. The tube was mixed gently and kept on ice for 20 min. Glycerol was then added to a final concentration of 15 mM, and the tube was mixed and incubated on ice for 5 min. The mixture was dialyzed against 2 liters of 0.1 M sodium acetate, pH 5.5, overnight at 4°C. Dry biotin-LC-hydrazide (Pierce) was added to the oxidized IgG to a final concentration of 5 to 10 mM in a 2-ml screw-cap tube and rotated end-over-end for 2 h at room temperature. The mixture was dialyzed against 2 liters of PBS overnight at 4°C. The SLP were prepared by mixing 300 to 900 μg of biotin-hydrazide-labelled IgG (determined by bicinchoninic acid assay) with the avidin-coated latex particles for 1 h at room temperature by end-over-end rotation. The SLP were washed three times in PBS and suspended to 2.5% (wt/vol) in latex storage buffer (0.02 M phosphate buffer [pH 7.4], 0.9% NaCl, 1% BSA, 5% glycerol, and 0.1% NaN_3).

Test samples. Practical test samples for diagnosis of swine pleuropneumonia include lung specimens from necropsy and nasal swabs from live pigs. Although less easy to obtain, urine specimens were also collected to determine if capsule was disseminated systemically and shed in the urine. Lung specimens were obtained from mice inoculated with a 50% lethal dose of *A. pleuropneumoniae* serotype 5 by intranasal challenge as previously described (6). Lung and urine specimens from pigs were obtained after intratracheal inoculation with 5×10^7 CFU of *A. pleuropneumoniae* per 5 ml (9). Lung specimens from mice and pigs were obtained aseptically at necropsy. To prepare lung extracts, a piece of tissue in a minimal amount of PBS was macerated by hand in a Whirl-Pak bag (NASCO, Ft. Atkinson, Wis.). Urine was collected by cystocentesis and was concentrated to 1 to 2 ml with a Centriprep concentrator (Amicon). Nasal swabs from pigs challenged intranasally with *A. pleuropneumoniae* were obtained 1 day or 1 week after intranasal challenge and were provided by Paula Fedorka-Cray (National Animal Disease Center, Ames, Iowa). Nasal swabs were treated with 0.5% *N*-acetyl-L-cysteine (NALC) to prevent nonspecific agglutination. Each specimen was cultured on a selective medium (3) for confirmation of *A. pleuropneumoniae.*

Latex agglutination assay. The latex agglutination test was performed by mixing 10 μl of SLP with 25 μl of sample on a black-coated, rimmed agglutination slide (Polysciences, Inc.) with a wooden applicator stick and rotating the slide manually or on a rotary shaker for up to 1 min. Dilutions of sample, if necessary, were made in PBS. Test results were scored as follows: 4+, rapid agglutination (within 10 s) of 100% of SLP, with formation of a ring; 3+, agglutination of >75% of SLP, with some ring formation; 2+, agglutination of ≥50% of SLP, with no ring formation; 1+, fine particle agglutination usually involving ≤25% of SLP and interpretation questionable; and −, no visible agglutination greater than that of the negative control sample. Negative controls used included the same sample or a similar sample without specific capsule or samples which were culture negative for *A. pleuropneumoniae*. For maximum specificity, only 2+, 3+, or 4+ agglutination was considered a positive result.

Statistics. Standard deviations were calculated from the means of assays done at least three times. Significance values were calculated by the Student *t* test using InStat computer software (GraphPad Software, San Diego, Calif.).

RESULTS

Optimization of the SLP assay. Following adsorption of whole-cell antiserum with a nonencapsulated mutant of the immunizing strain, the serum was no longer reactive with nonencapsulated whole cells as determined by ELISA. However, no loss of reactivity to capsule occurred (Table 1). Covalent coupling of IgG from the adsorbed serum to latex particles 0.55 and 0.75 μm in diameter resulted in reagents essentially equivalent in sensitivity (10 ng/ml), but agglutination of the 0.75-μm-diameter particles was easier to read. In contrast, the SLP of 1.0 μm were about 10-fold less sensitive than the smaller particles (data not shown). All test assays were therefore done with SLP of 0.75 μm.

Titrations of various concentrations of capsular IgG and latex particles indicated that about 800 to 1,000 μg of IgG/0.5 ml of 2% (wt/vol) 0.75-μm-diameter latex particles was optimal for obtaining a maximum sensitivity of 10 ng/ml (Table 2). Therefore, 800 μg of IgG was used for additional test assays. The sensitivity of the reagent also increased as the percentage of IgG bound to the latex particles increased but not as the total amount of IgG bound increased. When 800 μg of IgG was added, 324 ± 19 μg of IgG (mean ± standard deviation) was bound, or 40% ± 2%, which was significantly greater than the percentage of IgG bound when other concentrations of IgG were tested ($P < 0.01$ for 400 μg and $P < 0.001$ for 1,600 and 2,400 μg). The percentage of IgG bound diminished to 25% ± 0.5% and 23% ± 1% when 1,600 and 2,400 μg of IgG were added, respectively, even though the total amount of IgG bound increased. When 400 μg of IgG was added to the latex particles, 136 ± 9 μg of IgG was bound (34% ± 2%) (Table 3).

TABLE 2. Effects of IgG concentration, NHS, and avidin-biotin on sensitivity of SLP for *A. pleuropneumoniae* serotype 5 capsule

Capsule concn (ng/ml)	Agglutination reaction with:					
	IgG at the indicated concn[a] (with 10 mM NHS)[b]					Avidin-biotin[c]
	200	400	800	1,200	2,400	
1,000	2+ (3+)	4+ (4+)	4+ (4+)	4+ (4+)	4+ (4+)	4+
100	1+ (1+)	2+ (2+)	4+ (4+)	3+ (3+)	2+ (2+)	4+
10	− (−)	1+ (1+)	2+ (2+)	1+ (1+)	1+ (1+)	3+
1	− (−)	− (−)	1+ (−)	− (−)	− (−)	2+
0	− (−)	− (−)	− (−)	− (−)	− (−)	−

[a] In micrograms per 0.5 ml of 2% 0.75-μm-diameter latex particles.

[b] Five to 10 mM NHS was optimal. Higher concentrations appeared to cause inhibition. NHS improved sensitivity slightly more than NHSS.

[c] Carboxylate latex particles (0.5 ml; 2%) were conjugated with 600 μg of avidin and 900 μg of biotinylated IgG. Avidin-biotin coupled particles needed to be sonicated after coupling to avoid aggregation and false-positive interpretations.

TABLE 3. Binding of IgG to carboxylate latex particles

IgG concn (μg)[a]	Total protein bound (μg)[b]	% of protein bound (mean ± SD)
400	136 ± 9	34 ± 2
800	324 ± 19	40 ± 2
1,600	394 ± 11	25 ± 0.5
2,400	552 ± 21	23 ± 1

[a] Total protein added per 0.5 ml of 2% 0.75-μm-diameter latex particles.
[b] Determined indirectly by assaying the amount of protein not bound to latex particles.

The reagents had a shelf life of at least 1.5 years with no loss in sensitivity. Therefore, adsorption of hyperimmune serum with nonencapsulated mutants was an inexpensive and effective way to prepare SLP with optimal sensitivity and specificity.

The addition of various concentrations of NHS to the EDC conjugation step or at the time antibody was added did not substantially enhance sensitivity of the SLP when used at a concentration of 5 to 10 mM (wt/vol) (Table 2). Higher concentrations of NHS reduced the sensitivity of the assay. In addition, when 5 to 10 mM NHS was added at the same time as EDC, loss of sensitivity also occurred (data not shown). Similar results were obtained when NHSS was used in place of NHS (data not shown). Therefore, neither NHS nor NHSS was used for preparation of SLP.

Sensitivity and specificity of SLP. One hundred nanograms of purified capsule per ml (2.5 ng total in our assay) resulted in 4+ agglutination of the homologous SLP, and 10 ng of capsule per ml (250 pg total in our assay) resulted in a positive 2+ agglutination of the homologous SLP. Identical sensitivity results were obtained with SLP coupled to antibody to CP1 (CP1-SLP). In experiments using avidin-coated particles bound with biotin-hydrazide-modified IgG (ABH SLP), agglutination of purified capsule was rapidly detected at a concentration of 10 ng/ml (3+), and a 2+ agglutination occurred at 1 ng of antigen per ml. Thus, the ABH SLP were up to 10-fold more sensitive than particles conjugated directly with IgG (Table 2). The optimum ratio of avidin to biotin ranged from 1:2 to 2:3 at concentrations between 300 and 600 μg of avidin and 600 to 900 μg of biotinylated IgG, respectively, per 0.5 ml of 2% latex particles. However, because the use of ABH reagents required three additional steps (conjugation of avidin to latex beads, oxidation of IgG, and conjugation of biotin-hydrazide to IgG), the serotyping and antigen detection assay results were obtained with IgG conjugated directly to latex particles.

The SLP reagent coupled to serotype 1-specific IgG (CP1-SLP) agglutinated (2+) when mixed with 10 μg of purified CP5 per ml but did not agglutinate at lower CP5 concentrations. In contrast, 1,000 μg of CP1 per ml was required to cause a 2+ agglutination of the CP5-SLP reagent (Fig. 2). There was no agglutination of CP9-SLP by up to 1 mg of CP1 or CP5 per ml, nor was there any agglutination of CP1-SLP or CP5-SLP by up to 1 mg of CP9 per ml.

The quality of the capsular antibody prepared by adsorption with nonencapsulated bacteria was compared with that of IgG isolated by affinity chromatography through a purified capsule-Sephadex affinity column. *A. pleuropneumoniae* CP5-SLP, CP1-SLP, and CP9-SLP reagents had identical sensitivities and specificities in latex agglutination tests with purified capsules (data not shown) and whole bacteria (Table 4). There was no cross-reactivity between serotype 1 and 9 SLP reagents and

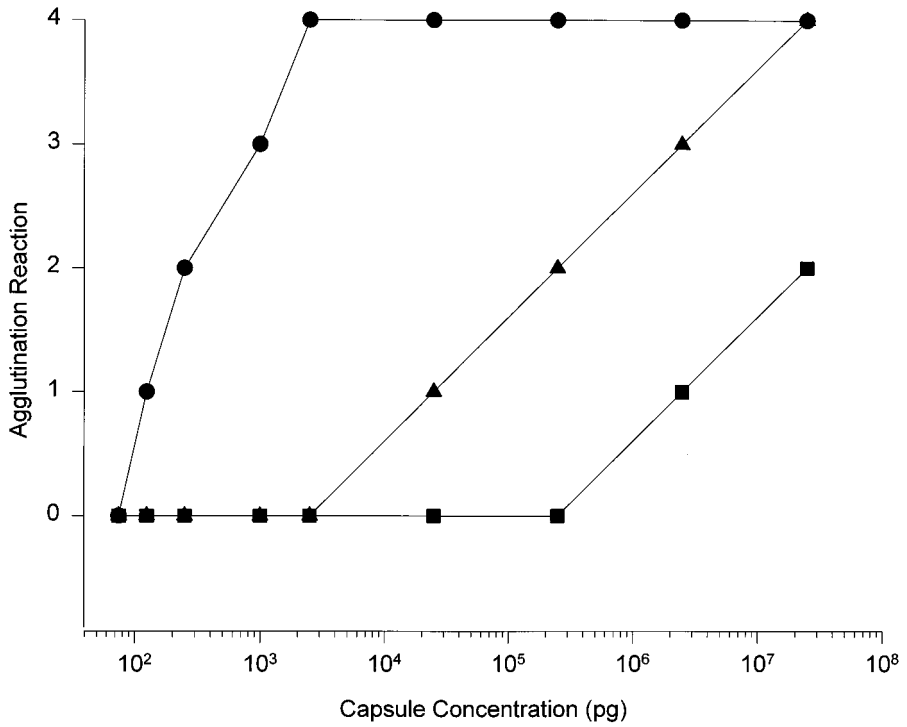

FIG. 2. Agglutination of SLP reagents with various concentrations of homologous or heterologous capsules. ●, serotype 5 capsule mixed with CP5-SLP; ■, serotype 1 capsule mixed with CP5-SLP; ▲, serotype 5 capsule mixed with CP1-SLP. The agglutination reactions of serotype 1 and serotype 9 capsules with CP1-SLP and CP9-SLP, respectively, were identical to that of the serotype 5 capsule with CP5-SLP and therefore are not shown. CP9-SLP was prepared with IgG isolated by affinity chromatography rather than adsorption. The agglutination reaction is defined in Materials and Methods.

TABLE 4. Serotyping of *A. pleuropneumoniae* isolates by latex agglutination

Serotype or species tested[a]	No. of strains[b]	Agglutination reaction with the indicated latex reagent		
		CP1-SLP	CP5-SLP	CP9-SLP
Serotypes				
1	10	4+	−	−
2	2	−	−	−
3	1	−	−	−
4	1	−	−	−
5	5	−	4+	−
6	1	−	−	−
7	6	−	−	−
8	1	−	−	−
9	7	−	−	4+
10	1	−	−	−
11	3	−	−	−
12	1	−	−	−
Nontypeable	3	−	−	−
Species				
P. multocida	5	−	−	−
A. suis	1	−	−	−
H. parasuis	4	−	−	−

[a] Strains were swabbed from overnight plates of brain heart infusion agar containing NAD and suspended in PBS (pH 7.4) to match the optical density of a McFarland 0.5 standard. Serotypes and strains were as follows: serotype 1, strains 4074 and 4045 and eight field isolates; serotype 2, strain S 1536 and one field isolate; serotype 3, strain S 1421; serotype 4, strain M62; serotype 5, strains K17, J45, 178, and B8 and one field isolate; serotype 6, strain FEMO; serotype 7, strain 29628 and five field isolates; serotype 8, strain 405; serotype 9, strain X1 and six field isolates; serotype 10, strain 13039; serotype 11, strain 56153 and two field isolates; serotype 12, strain 8329; *P. multocida*, five field isolates; *A. suis*, strain 1208; *H. parasuis*, strain 1374 and three field isolates. Nontypeable strains used were 4074-C, K17-C, and J45-C (-C, nonencapsulated mutant of the parent strain).

[b] Multiple strains of the same serotype or species reacted identically.

TABLE 5. Detection of *A. pleuropneumoniae* serotype 5 capsule in clinical specimens by latex agglutination

Animal	Specimen type (no. of specimens)	Challenge strain[b]	Reaction (no. of specimens) with:	
			CP1-SLP	CP5-SLP
Mouse	Lung (3)	None	− (3)	− (3)
	Lung (3)	Serotype 5	− (3)	4+ (3)
Pig	Lung (5)	None	− (5)	− (5)
	Lung (15)	Serotype 5	− (15)	4+ (15)
	Lung (5)	Serotype 1	3+ or 4+ (4)	− (5)
	Urine[c] (2)	Serotype 5	− (2)	4+ (2)
	Urine (2)	None	− (2)	− (2)
	Nasal swabs[d] (7)	Serotype 1	3+ or 4+ (5)	− (7)
	Nasal swabs[e] (4)	Serotype 1	− (4)	− (4)
	Nasal swabs[f] (3)	Serotype 5	− (3)	3+ or 4+ (3)

[a] Specimens were undiluted or diluted with PBS up to 1:10.

[b] Serotype 5, strain J45; serotype 1, strain 4074. In all cases, challenge was by the respiratory route (intranasal or intratracheal).

[c] Urine was concentrated in an Amicon concentrator and centrifuged at 900 rpm for 2 min to remove particulate matter before testing.

[d] Obtained 1 day after intranasal challenge with *A. pleuropneumoniae* serotype 1. The extracts were treated with NALC as a mucolytic agent to prevent nonspecific agglutination.

[e] Obtained 1 week after challenge with *A. pleuropneumoniae* serotype 1. Extracts were treated with NALC.

[f] Obtained from symptomatic pigs 3 days after challenge with *A. pleuropneumoniae* serotype 5. Extracts were treated with NALC.

capsules tested at 1 mg/ml, even though these two serotypes cross-react in some assays (8).

Application of SLP to serotyping and antigen detection. When CP1-SLP and CP5-SLP were used for serotyping, using a bacterial density equivalent to a 0.5 McFarland standard, the reagents were found to be 100% sensitive and specific when tested with both established laboratory strains and field strains. Furthermore, cross-reactions with encapsulated species commonly isolated from swine (*P. multocida*, *H. parasuis*, and *A. suis*) did not occur. Cross-reactions (e.g., between CP1-SLP and serotype 5) did occur if the density of the bacterial suspension was too high (about a no. 5 McFarland standard or greater). All serotyping agglutination reactions were either strong (4+) or negative (Table 4). The sensitivity and specificity of all the SLP reagents remained unchanged more than 1.5 years after preparation and storage at 4°C.

The SLP reagents gave a 3+ or 4+ reaction when mixed with lung extracts from mice and pigs infected with the homologous serotype of *A. pleuropneumoniae* (Table 5). None of the specimens cross-reacted with SLP reagents specific for the heterologous serotype. However, lung extracts from one of five culture-positive pigs challenged with serotype 1 did not agglutinate the CP1-SLP reagent, whereas lung extracts from all 15 culture-positive pigs challenged with serotype 5 agglutinated the CP5-SLP reagent. Lung extracts from all normal and immunized pigs were culture negative for *A. pleuropneumoniae* and were also latex agglutination negative. Thus, the overall sensitivity of the assay for detecting capsule in mouse and pig lung extracts was 96%, and the specificity was 100%. Nonspecific agglutination did not occur when lung extracts were tested undiluted. Extracts could also be diluted at least 1:10 without any reduction in the degree of agglutination. These extracts contained at least 150 ng of capsule per ml, as determined by inhibition radioimmunoassay (5). Lung extracts stored at −20°C for 3 years still resulted in a 4+ agglutination reaction with the specific SLP reagent.

Clarified, concentrated urine specimens from two of these infected pigs were also positive with the homologous SLP reagent. Urine samples from the other pigs were not tested. Concentrated urine from noninfected pigs was negative. Five of seven NALC-digested nasal swab extracts from pigs intranasally challenged the previous day with *A. pleuropneumoniae* serotype 1 were positive (3+ or 4+ agglutination) with the CP1-SLP reagent (sensitivity, 71%). However, bacterial culture for *A. pleuropneumoniae* was positive only for the five swabs that were positive by latex agglutination, resulting in a sensitivity of 100% compared with culture. If NALC was not used to digest nasal swab extracts, nonspecific agglutination occurred with extracts of all the swabs obtained, including those from noninfected pigs. None of four nasal swabs from pigs intranasally challenged with serotype 1 1 week earlier were positive for the presence of homologous capsule (Table 5), and all four swabs were culture negative for *A. pleuropneumoniae*. Nasal swabs were obtained from three pigs with severe clinical symptoms 3 days after intratracheal challenge with *A. pleuropneumoniae* serotype 5. All three swabs were culture positive and positive by latex agglutination with only the CP5-SLP reagent. Nasal swab extracts from noninfected pigs were uniformly negative with the CP1-SLP and CP5-SLP reagents.

DISCUSSION

Agglutination tests using SLP are commonly used for the diagnosis of infectious diseases. Antigen detection tests specific for carbohydrate antigens, such as the capsules of *H. influenzae*, *N. meningitidis*, *Streptococcus* spp., and *C. neofor-*

mans and the group-specific carbohydrate antigens of streptococci, have been particularly successful (10, 16).

To maximize the sensitivity of SLP assays, hyperimmune serum is desirable because IgG of high affinity (determined by ELISA) conjugated to the latex particles results in the greatest sensitivity (unpublished data). However, false-positive results may occur if cross-reactive antibodies are present on the SLP. Since carbohydrate antigens are often nonimmunogenic or poorly immunogenic in animals, whole bacteria need to be used for immunization (7), which can decrease specificity. To maximize specificity, potentially nonspecific antibodies to bacterial somatic antigens should be removed from the serum. Thorough adsorption of the immune serum with a nonencapsulated mutant of the immunizing strain was an efficient and effective method for removal of cross-reactive antibodies. An ELISA using the adsorbing mutant or purified capsule as an antigen indicated that antibodies to surface antigens of whole cells were adequately removed from the serum, whereas the titer of antibody to capsule was not affected. In addition, the sensitivity and specificity of SLP with IgG obtained by affinity chromatography and those with IgG obtained by adsorption were identical (data not shown).

The sensitivity of the SLP reagents was affected by several variables, including IgG concentration, particle size, and the addition of avidin-biotin reagents. Maximum sensitivity was obtained when the greatest percentage of IgG was bound to the latex beads, which occurred at about 800 μg of IgG per 0.5 ml of 2% latex particles. Although the total amount of IgG bound to latex particles increased when more IgG was added, agglutination significantly diminished when 1,600 μg of IgG or more per 0.5 ml of latex particles was added. Efficient agglutination is dependent upon an optimum amount of IgG on the surface of the SLP and the correct orientation of Fab antigen binding regions to obtain cross-linking of particles (2). If too much IgG is present, the antigen may interact with adjacent antibodies on the same particle, diminishing cross-linking and agglutination. If the IgG is in the incorrect orientation, the Fab sites may be inaccessible for antigen binding or for cross-linking. Thus, the amount of IgG must be optimized for agglutination, not for the total amount of IgG bound. The size of the particles was also important. Sensitivity diminished with particles of $\geq$1.0 μm, and agglutination started to become too difficult to interpret with particles of $\leq$0.5 μm.

The addition of 5 to 10 mM NHSS has been shown to enhance the EDC-mediated coupling of carbohydrates to proteins (15). However, neither NHSS nor NHS substantially enhanced the sensitivity of these SLP reagents. In fact, concentrations above 10 mM NHSS or NHS decreased the SLP sensitivity, although the total amount of protein bound increased (data not shown). Again, it is likely that coupling of IgG in the presence of NHS or NHSS adversely affected the cross-linking capability of the particles. In contrast, the use of avidin particles conjugated to biotin-hydrazide-linked antibodies improved sensitivity up to 10-fold. The enhanced sensitivity of SLP prepared with ABH reagents is probably due to several factors: (i) the IgG is bound farther away from the latex particles by biotin-hydrazide, diminishing steric interference; (ii) the glycose-rich Fc fragment is oxidized and coupled to biotin, favoring binding of Fc to the particles and leaving the Fab regions more accessible to binding with antigen and cross-linking with other particles; and (iii) the spacing provided by the biotin-hydrazide reagents allows more three-dimensional and rotational movement of the accessible Fab regions, enabling more contact with antigen and other particles.

The standard SLP reagents detected 10 ng of homologous capsule per ml. In the case of *A. pleuropneumoniae*, specificity depended on the concentration of capsule tested. At concentrations of 10 μg/ml or greater, the serotype 5 capsule agglutinated the CP1-SLP. However, 1 mg of serotype 1 capsule per ml was required to agglutinate the CP5-SLP. In contrast, even 1 mg of CP5 or CP1 per ml did not agglutinate the CP9-SLP. These results were not surprising, as similar results have been obtained for serotype 5 capsule and antibodies to CP1 in radioimmunoassays and ELISAs but not for CP1 and antibodies to CP5 (5). This cross-reactivity may be due to the *N*-acetylglucosamine residue shared by the two capsules (14). It is possible that the *N*-acetylglucosamine residue or linkage is immunogenic in CP1 but remains an accessible (though poorly immunogenic) epitope in CP5. The CP9-SLP reagent prepared with IgG isolated by affinity chromatography was identical in sensitivity and specificity to the reagents prepared with IgG from adsorbed serum. Although serotypes 1 and 9 have been reported to cross-react in some assays, I have shown here and previously (8) that the capsules do not cross-react as determined by latex agglutination, although they share α-D-galactose residues (14). This absence of cross-reactivity is unlikely to be due to the affinity-purified IgG in the CP9-SLP reagent, because the CP1-SLP reagent was prepared with IgG from adsorbed serum and also did not cross-react with CP9.

Cross-reactivity between serotypes 1 and 5 did not occur when bacterial isolates were serotyped with each reagent. The sensitivity and specificity of the SLP reagents were 100% with all isolates tested at a turbidity equivalent to a 0.5 McFarland standard. False-positive results were obtained for serotype 5 and CP1-SLP when the bacteria were suspended at a high density (about a no. 5 McFarland standard or greater), which corresponds to the results obtained with purified capsule.

When each SLP reagent was tested with culture-positive lung extracts from infected mice or pigs, all extracts but one were positive only for the homologous serotype. Radioimmunoassays of some of the extracts confirmed that the amount of capsule present was in the range that would provide a 4+ latex agglutination reaction but would not cross-react with heterologous capsules. It is not clear why one sample was negative in the assay, because another sample that yielded only 1+ growth of *A. pleuropneumoniae* (<50 colonies) had a 3+ reaction with the specific CP1-SLP. The number of CFU of bacteria that the SLP reagents could detect was not determined because the amount of capsule present varies greatly depending on the strain, stage of growth, and growth conditions (1, 4). All culture-negative lung extracts were also negative by latex agglutination. Although the extracts were very bloody, neither dilution nor centrifugation was required to interpret the results correctly. Extracts could be diluted at least 1:10, however, with no loss in reactivity.

The SLP reagents were sensitive enough to detect specific capsule in concentrated urine specimens and nasal swabs. All culture-positive nasal swabs were also positive by latex agglutination. Since none of the swabs from pigs challenged intranasally 1 week earlier were positive, it is unlikely that latex agglutination or culture of nasal swabs would be a productive means of screening pigs for chronic *A. pleuropneumoniae* infection. In pigs that are clinically ill, latex agglutination of nasal swabs may be a rapid method of diagnosis that approaches the sensitivity of culture. Unlike lung extracts, however, all nasal swabs had to be treated with NALC to prevent nonspecific agglutination.

In summary, the use of nonencapsulated mutants to obtain IgG to capsule enables the preparation of latex reagents that are sensitive, specific, rapid, and well suited to field or office testing. The technology avoids the time and expense of antigen purification and coupling of antigens to affinity columns, which

may be difficult with carbohydrate antigens. At the same time, the sensitivity and specificity of the latex reagent prepared with IgG from adsorbed serum remain uncompromised.

ACKNOWLEDGMENTS

I thank Carol Koch and Gretchen Glindemann for excellent technical assistance; Chris Wakely and Barb Dryman for assistance with handling animals; Jacques Nicolet for latex agglutination testing of some isolates of serotypes 1, 9, and 11; Paula Fedorka-Cray for providing nasal swabs from infected swine; and David Lyerly for review of the manuscript.

This work was supported in part by funds from the Virginia Center for Innovative Technology and from Diagnostic Products Corp. and by grant 88-34116-3641 from the U.S. Department of Agriculture.

REFERENCES

1. **Anderson, P., J. Pitt, and D. H. Smith.** 1976. Synthesis and release of polyribophosphate by *Haemophilus influenzae* type b in vitro. Infect. Immun. **13:**581–589.
2. **Bangs, L. B.** 1987. Uniform latex particles. Seradyn, Inc., Indianapolis, Ind.
3. **Gilbride, K. A., and S. Rosendal.** 1983. Evaluation of a selective medium for isolation of *Haemophilus pleuropneumoniae*. Can. J. Comp. Med. **47:**445–450.
4. **Inzana, T. J.** 1987. Purification and partial characterization of the capsular polymer of *Haemophilus pleuropneumoniae* serotype 5. Infect. Immun. **55:** 1573–1579.
5. **Inzana, T. J., G. F. Clark, and J. Todd.** 1990. Detection of serotype-specific antibodies or capsular antigen of *Actinobacillus pleuropneumoniae* by a double-label radioimmunoassay. J. Clin. Microbiol. **28:**312–318.
6. **Inzana, T. J., J. Ma, T. Workman, R. P. Gogolewski, and P. Anderson.** 1988. Virulence properties and protective efficacy of the capsular polymer of *Haemophilus* (*Actinobacillus*) *pleuropneumoniae* serotype 5. Infect. Immun. **56:**1880–1889.
7. **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.
8. **Inzana, T. J., J. Todd, C. Koch, and J. Nicolet.** 1992. Serotype specificity of immunological assays for the capsular polymer of *Actinobacillus pleuropneumoniae* serotypes 1 and 9. Vet. Microbiol. **31:**351–362.
9. **Inzana, T. J., J. Todd, and H. P. Veit.** 1993. Safety, stability, and efficacy of noncapsulated mutants of *Actinobacillus pleuropneumoniae* for use in live vaccines. Infect. Immun. **61:**1682–1686.
10. **James, K.** 1990. Immunoserology of infectious diseases. J. Clin. Microbiol. **3:**132–152.
11. **Lucas, A. H.** 1988. Expression of cross reactive idiotypes by human antibodies specific for the capsular polysaccharide of *Haemophilus influenzae* type b. J. Clin. Invest. **81:**480–486.
12. **Nicolet, J.** 1992. *Actinobacillus pleuropneumoniae*, p. 401–408. *In* A. D. Leman, B. E. Straw, W. L. Mengeling, S. D'Allaire, and D. J. Taylor (ed.), Diseases of swine. Iowa State University Press, Ames.
13. **O'Shannessy, D. O., M. J. Dobersen, and R. H. Quarles.** 1984. A novel procedure for labelling immunoglobulins by conjugation to oligosaccharide moieties. Immunol. Lett. **8:**273–277.
14. **Perry, M. B., E. Altman, J.-R. Brisson, L. M. Beynon, and J. C. Richards.** 1990. Structural characteristics of the antigenic capsular polysaccharides and lipopolysaccharides involved in the serological classification of *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* strains. Serodiag. Immunother. Infect. Dis. **4:**299–308.
15. **Staros, J. V., R. W. Wright, and D. M. Swingle.** 1986. Enhancement by *N*-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. Anal. Biochem. **156:**220–222.
16. **Tinghitella, T. J., and S. C. Edberg.** 1991. Agglutination tests and *Limulus* assay for the diagnosis of infectious diseases, p. 61–72. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.