

Enzyme-Linked Immunosorbent Assay for Immunoglobulin G Antibody to *Pasteurella multocida* in Rabbits

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Three antigen preparations of *Pasteurella multocida*, lipopolysaccharide antigen, boiled-cell extract antigen, and boiled whole-bacterium antigen, were used in an enzyme-linked immunosorbent assay (ELISA) to detect rabbit immunoglobulin G antibody to *P. multocida*. The sensitivity of each antigen preparation was compared by using sera from *P. multocida*-infected and uninfected rabbits and sera from two rabbits immunized with different serotypes of *P. multocida*. In the ELISA, all three antigen preparations detected high titers of antibodies in infected rabbits and markedly lower levels in uninfected rabbits. When whole-bacterium or boiled-cell extract antigens were used, the ELISA detected antibodies in sera from both immunized rabbits, but with lipopolysaccharide antigen, only antibody to the homologous serotype was detected. Sera absorbed with *P. multocida* and *Bordetella bronchiseptica*, another respiratory pathogen of rabbits, revealed that antibodies detected in the ELISA did not cross-react. Since the lipopolysaccharide antigen was more difficult to prepare and may be type specific, and since the whole-bacterium antigen was the least sensitive, the boiled-cell extract was chosen as the best antigen preparation to use in the ELISA.

Pasteurellosis is a commonly encountered disease in both commercial and research rabbit colonies (7, 8). Rabbits often carry *Pasteurella multocida* in the upper respiratory tract without clinical signs (7, 12, 13), and the stress of experimental manipulation may significantly increase the occurrence of pasteurellosis (19). Culture techniques to detect infection are time consuming, and failure to isolate the organisms may be due to their low numbers or inaccessible location rather than to their absence. Although a number of procedures have been developed for the measurement of humoral immunity, recent reports (6, 14, 15) indicate that antibody detection by the enzyme-linked immunosorbent assay (ELISA) may be more suitable for assessment of pasteurella infection. We therefore explored the use of ELISA for detecting antibodies to *P. multocida* in naturally infected rabbits.

MATERIALS AND METHODS

Preparation of antigens. The strain of *P. multocida* used was isolated from an adult rabbit with mucopurulent rhinitis and was identified as serotype 12 by the gel diffusion precipitin test and as capsular type A by staphylococcal hyaluronidase decapsulation at the National Animal Disease Center, Ames, Iowa. The organism was grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) for 48 h at 37°C under a 5% CO₂ atmosphere. The broth was centrifuged to pellet the cells, which were then washed three times in phosphate-buffered saline (PBS), pH 7.2 (18). To make the whole-bacterium and boiled-cell extract antigens, bacteria were resuspended in 15 ml of PBS and placed in a boiling-water bath for 1 h. After boiling, the suspension was centrifuged and washed three times with 15 ml of carbonate-bicarbonate buffer, pH 9.6 (18). Supernatant fractions from the boiled-cell suspension and the carbonate-bicarbonate buffer washes were combined to make boiled-cell extract antigen. Bacterial cells were resuspended in carbonate-bicarbonate buffer, pH 9.6, to a no. 2 McFarland standard to

make whole-bacterium antigen. Stock antigen preparations were stored at 4°C.

Lipopolysaccharide (LPS) antigen was extracted by the procedure of Westphal and Jann (20) from cells grown and harvested as described above. Instead of undergoing dialysis, the aqueous phase was purified by gel filtration on a chromatography column with Bio-Gel P-6DG (Bio-Rad Laboratories, Richmond, Calif.). Distilled water was used as a buffer at a flow rate of approximately 90 ml/h. Fractions of the eluted sample were collected by an ISCO model 1200 fraction collector (Instrumentation Specialties Co., Lincoln, Nebr.). The LPS, first fraction, was detected with an ISCO model UA-2 UV analyzer. This fraction was lyophilized, rehydrated to a concentration of 1.0 mg/ml with carbonate-bicarbonate buffer, pH 9.6, and stored at 4°C. A Lowry protein determination (11) on the LPS preparation was negative (<10 µg/ml), and spectrophotometric analysis at 260 nm indicated the absence of RNA (9).

Preparation of sera. Uninfected sera were obtained from 28 young adult, pasteurella-free New Zealand White rabbits (Dutchland Rabbitry, Denver, Pa.) which were negative for *P. multocida* by nasal culture. Sixteen adult New Zealand White rabbits with mucopurulent rhinitis were used as a source of infected sera. Nasal cultures from these rabbits, which were clinical cases at the University of Washington, Seattle, yielded *P. multocida*. Nine of the isolates, serotyped by the National Animal Disease Center, were serotype 12, and one was serotype 3 (six failed to grow after storage). All 10 isolates were capsular type A. Sera were also obtained from two pasteurella-free New Zealand White rabbits which had been immunized with *P. multocida* by repeated intramuscular injections (12), one rabbit with serotype 3 (P-1059) and the other with serotype 12 (P-1573). Sera were initially diluted 1:10 with PBS and stored at -20°C until tested. Serial dilutions, at 0.5 log increments, were used in the ELISA.

Absorption of sera. Three aliquots of the serotype 12 and serotype 3 antisera and three aliquots of one infected and one uninfected serum sample were taken. An equal volume of a no. 10 McFarland suspension of *P. multocida* (test

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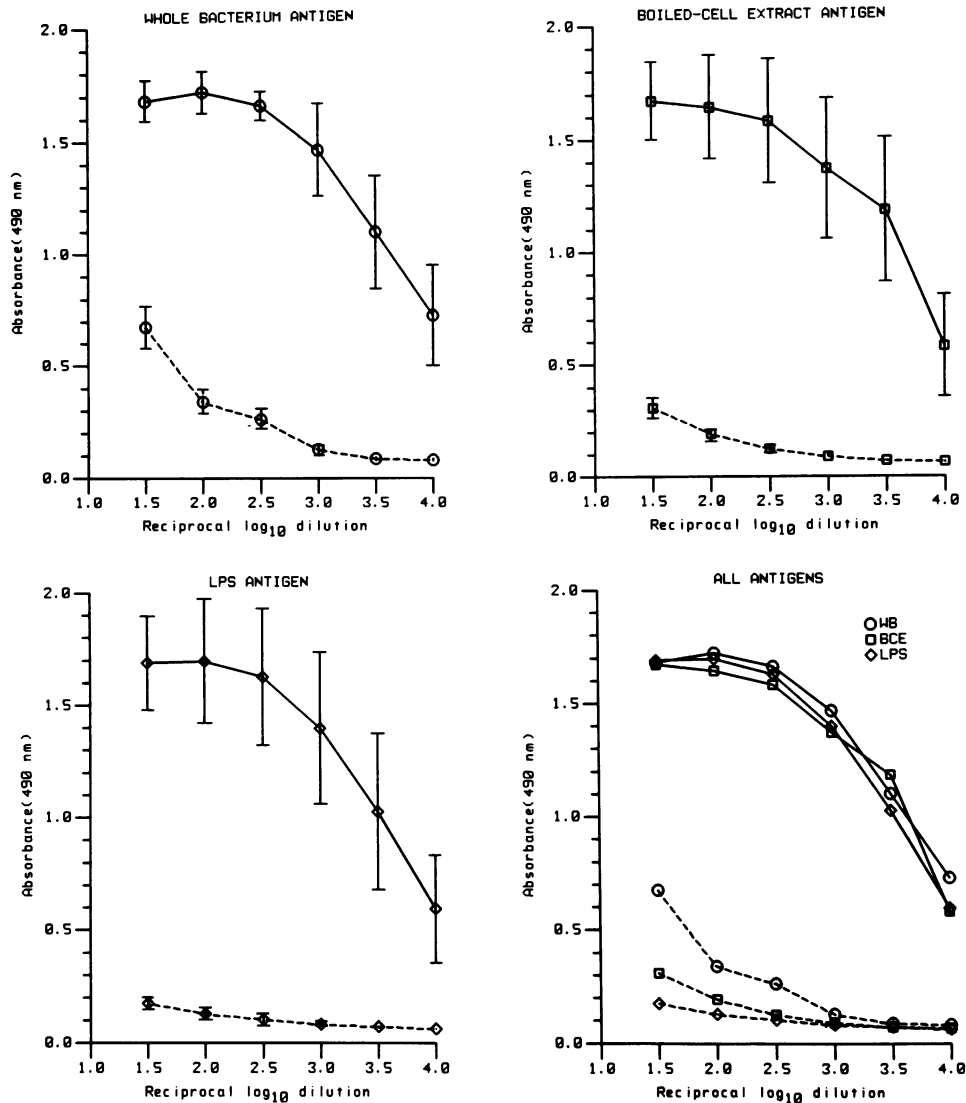


FIG. 1. Titration of sera from 16 infected (—) and 28 uninfected (---) rabbits with the use of whole bacterium (WB), boiled-cell extract (BCE), or LPS of *P. multocida* as the coating antigen in the ELISA. Values are the mean and 95% confidence intervals.

antigen) in PBS was added to one aliquot of each serum, and a no. 10 McFarland suspension of *Bordetella bronchiseptica* ATCC 785 in PBS was added to another. These mixtures were incubated overnight at room temperature. The last aliquot of each serum was used as a control with an equal volume of PBS added to give an equivalent dilution. Supernatants from the absorbed aliquots were tested in the ELISA and compared with their control aliquot.

Plate preparation. Optimum antigen concentrations for coating microtiter plates were determined by checkerboard titration. Serial dilutions (in 0.5 log increments) of each antigen preparation were tested against serial dilutions (in 0.5 log increments) of sera from two infected and two uninfected rabbits. The optimum coating concentrations for the whole-bacterium antigen, the boiled-cell extract antigen, and the LPS antigen were 1:100, 1:320, and 1:100 dilutions of the stock preparation, respectively. A 100- μ l aliquot of diluted antigen preparation was added to each well of an Immulon I microtiter plate (Dynatech Laboratories, Inc., Alexandria, Va.), placed in a moist chamber, and held at 4°C

for 48 h. Unbound antigen was discarded, and the wells were washed three times for 5 min each with PBS containing 0.1% Tween (PBST). Nonspecific binding sites were blocked by adding 200 μ l of PBST with 0.01% gelatin to each well and incubating the plates for 10 min at 37°C in a moist chamber. After the PBST-gelatin was removed, the plates were again washed with PBST and stored at 4°C for up to 1 week before use.

ELISA procedure. The general method of Voller et al. (18) was used for the ELISA. A 100- μ l aliquot of diluted serum was added to each well of an antigen-coated plate and incubated for 1 h. All incubations were done in a moist chamber at 37°C. Serum dilutions were discarded, and plates were washed three times for 5 min each with PBST. After washing, 100 μ l of peroxidase-conjugated, affinity-purified, goat anti-rabbit immunoglobulin G (IgG), whole molecule (conjugate) (Sigma Chemical Co., St. Louis, Mo.), was added to each well, and the plates were incubated for 1 h. The conjugate was diluted 1:1,000 with PBS, as recommended by the manufacturer. After the wells were incubated

TABLE 1. Absorbance of sera from 16 pasteurilla-infected and 28 uninfected rabbits with three different antigen preparations in the ELISA

Serum dilution	Absorbance (mean \pm SD) with:					
	Whole-bacterium antigen		Boiled-cell extract antigen		LPS antigen	
	I ^a	U ^a	I	U	I	U
1:32	1.68 \pm 0.17	0.68 \pm 0.24	1.67 \pm 0.32	0.31 \pm 0.11	1.69 \pm 0.39	0.17 \pm 0.08
1:100	1.72 \pm 0.17	0.34 \pm 0.13	1.64 \pm 0.42	0.19 \pm 0.07	1.70 \pm 0.52	0.13 \pm 0.06
1:320	1.66 \pm 0.25	0.26 \pm 0.13	1.58 \pm 0.51	0.12 \pm 0.04	1.63 \pm 0.57	0.10 \pm 0.05
1:1,000	1.47 \pm 0.39	0.13 \pm 0.04	1.37 \pm 0.58	0.09 \pm 0.03	1.40 \pm 0.63	0.08 \pm 0.02
1:3,200	1.10 \pm 0.47	0.09 \pm 0.02	1.18 \pm 0.60	0.07 \pm 0.01	1.02 \pm 0.65	0.07 \pm 0.01
1:10,000	0.73 \pm 0.42	0.08 \pm 0.02	0.58 \pm 0.42	0.07 \pm 0.01	0.59 \pm 0.45	0.06 \pm 0.01

^a I, Infected; U, uninfected.

and washed with PBST as before, 100 μ l of *o*-phenylenediamine substrate (Sigma) was added to each well, and the plates were incubated for 30 min. The *o*-phenylenediamine substrate was prepared by the method of Voller et al. (18). At the end of the incubation, 25 μ l of 8 N H₂SO₄ was added to stop the reaction. Absorbance was read at 490 nm on a Bio-Tek EIA Reader model EL307IP (Bio-Tek Instruments, Inc., Burlington, Vt.). Each plate contained the following control wells: no antigen, no conjugate, no serum, and no substrate. In place of the omitted component, PBST was added. All samples and controls were run in duplicate, and each experiment was performed at least twice.

RESULTS

Sensitivity. Three antigen preparations were used to detect IgG to *P. multocida* in known infected and uninfected rabbits. With each preparation there was a significant difference in reactivity between the two groups of rabbits (Fig. 1). Whole-bacterium antigen had the least separation, especially at low serum dilutions. LPS antigen had the greatest separation, with very low readings on sera from uninfected rabbits at all dilutions. Results with all three antigen preparations were similar when infected sera, including serum from a rabbit infected with a serotype 3 isolate, were tested (Fig. 1). However, with uninfected sera, results were highest with whole-bacterium antigen, lowest with LPS antigen, and intermediate with boiled-cell extract antigen. The absorbance for sera from uninfected rabbits showed little variability. The variance seen generally decreased with dilution. The variability of absorbance for sera from infected rabbits, however, increased with dilution (Table 1). The within-test variation in absorbance for infected rabbits was usually less than 10%; it was less than 4% at the 1:100 dilution.

Isotypic sensitivity was evaluated by comparing the results from antisera to specific serotypes of *P. multocida* with each antigen preparation. Antiserum to serotype 12 reacted strongly with all three antigens (Fig. 2A). Antiserum to serotype 3, however, gave different reactions with each preparation (Fig. 2B). The reactivity of serotype 3 antiserum with whole-bacterium antigen was slightly less than that seen with serotype 12 antiserum but was considerably less than that seen with serotype 12 antiserum when the boiled-cell extract preparation was used. When LPS antigen was used, serotype 3 antiserum reactions were equivalent to reactions seen with uninfected rabbit sera.

Specificity. To determine whether antibodies to other gram-negative bacteria might cross-react in the ELISA, sera from infected, uninfected, and immunized rabbits were absorbed with suspensions of *P. multocida* and *B. bronchiseptica*. When compared with the controls, the reactivity of the serum aliquots absorbed with the suspension of *P.*

multocida was reduced by an average of 66%, whereas the reactivity of *B. bronchiseptica*-absorbed sera decreased less than 3% (Fig. 3). This selective reduction in reactivity was observed with all three antigen preparations.

DISCUSSION

Previously reported serological assays for the detection of pasteurilla infection, such as agglutination and hemagglutination tests in both avian and mammalian species, have not

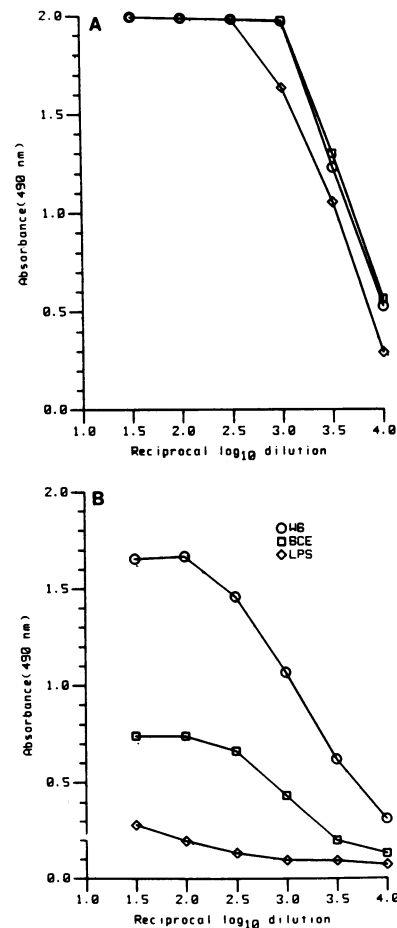


FIG. 2. Titration of sera from rabbits immunized with (A) *P. multocida* serotype 12 or (B) *P. multocida* serotype 3, by using whole bacterium (WB), boiled-cell extract (BCE), or LPS derived from *P. multocida* serotype 12 as the coating antigen in the ELISA.

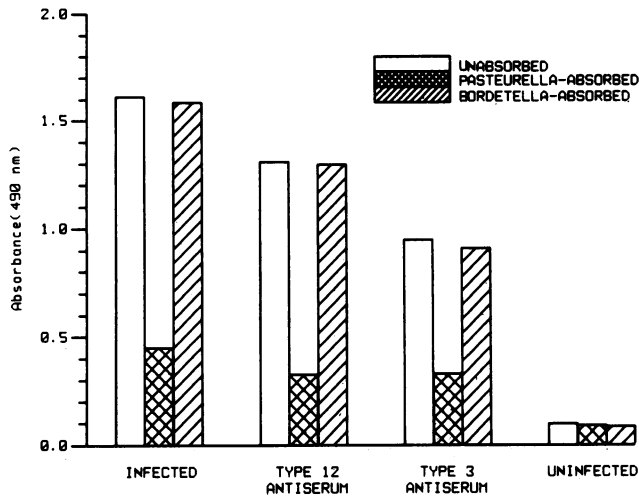


FIG. 3. Reactivity at the 1:1,000 dilution of sera, after absorption with *P. multocida* (test antigen) or *B. bronchiseptica* ATCC 785 with boiled-cell extract as the coating antigen in the ELISA.

been widely used for a variety of reasons (3). The indirect hemagglutination assay, one of the more frequently used tests, lacks sensitivity (14). Comparison of the agglutination test and the ELISA for measuring antibody responses in turkeys receiving oral vaccination against *P. multocida* revealed that the ELISA was more sensitive and correlated with protection against oral challenge with virulent *P. multocida* (15).

Use of the ELISA to measure antibody levels to pasteurilla in rabbits has recently been reported (4, 6, 14). In studies of immunity to pasteurellosis in rabbits immunosuppressed with a malignant variant of Shope fibroma virus, Corbeil et al. (6) measured antibody titers by the ELISA with boiled whole cells as antigen. The ELISA has also been used to measure antibody levels in immunized rabbits by using either boiled cells (14) or LPS (4, 14) of *P. multocida* as antigen.

In this study, three antigen preparations of *P. multocida* were used in the ELISA to detect IgG to *P. multocida*. Preliminary analysis of the antigen preparations by polyacrylamide gel electrophoresis revealed that both the whole-bacterium and the boiled-cell extract antigens contained many protein bands when stained with Coomassie Blue (Bio-Rad Laboratories), whereas no protein was detected in the LPS antigen ($<5 \mu\text{g}/\text{cm}^2$). Silver staining with periodate oxidation (17) revealed several more bands in the whole-bacterium and boiled-cell extract antigens but only one band in the LPS preparation, at about 14,500 daltons, indicating that the LPS preparation was relatively pure.

With this ELISA, high titers ($>1:10,000$) of IgG were found in samples from rabbits clinically infected with *P. multocida*. Rabbits without infection showed very little reactivity with any of the antigen preparations. The variability of titers in sera from pasteurilla-infected rabbits was most probably due to the severity and duration of infection or differences in antigenicity between the infecting organism and the test organism. One limitation of this study was that most pasteurilla isolates from infected rabbits were serotype 12, the predominant type in this area (7). Therefore, infection due to other serotypes, such as type 3, the second most prevalent serotype (1, 5, 12, 16), might be missed. In the absence of large numbers of sera from rabbits naturally

infected with various serotypes, antisera from two immunized rabbits were evaluated. Titration of serum from rabbits immunized with serotype 3 or 12 of *P. multocida* in the ELISA, with serotype 12 as the coating antigen, revealed that the whole-bacterium and boiled-cell extract antigens detected antibodies against both serotypes, whereas the LPS antigen detected antibodies specific to serotype 12. This pattern was different from that of the rabbit naturally infected with *P. multocida* serotype 3, which had shown high titers with all three antigen preparations. The difference may be due to the type of exposure (natural infection versus immunization) or the length of time after exposure that the sera were obtained. It was also not known whether the naturally infected rabbit had been previously exposed to *P. multocida* serotype 12.

We did not expect the boiled-cell extract antigen, derived from a *P. multocida* serotype 12, to detect antibodies in rabbits naturally infected or immunized with serotype 3, since the antigen was prepared in a manner similar to that for the heat-stable antigen used in the gel diffusion precipitin test described by Heddleston et al. (10). In the diffusion test this antigen preparation is type specific, and it has been shown that LPS, derived by the Westphal procedure, appears to be a major component of the heat-stable antigen responsible for the specificity (2). The only difference between antigens was the absence of Formalin in the preparation of our antigen. However, preparation of a boiled-cell extract antigen with Formalin (heat-stable antigen) resulted in the same reaction with antiserum from the rabbit immunized with serotype 3. The discrepancy may be due to the higher sensitivity of ELISA methods compared with gel diffusion (14).

To assess the specificity of the ELISA, representative sera were absorbed with *P. multocida* and another common gram-negative respiratory pathogen of rabbits, *B. bronchiseptica* (8). Like *P. multocida*, *B. bronchiseptica* can be found in the upper respiratory passages of clinically normal rabbits and can be isolated from rabbits with mucopurulent rhinitis. It was therefore essential that this ELISA be able to differentiate rhinitis caused by *P. multocida* from that caused by *B. bronchiseptica*. After absorption, the reactivity of *P. multocida*-absorbed sera decreased significantly and that of *B. bronchiseptica*-absorbed sera did not, demonstrating that the IgG antibodies detected by this method did not cross-react with *B. bronchiseptica*.

Since the boiled-cell extract antigen was easier to prepare than the LPS antigen, was more sensitive than the whole-bacterium antigen in the ELISA, and showed cross-reactivity between serotypes, it was chosen as the preparation for routine colony screening with the ELISA. A serum dilution of 1:100 was chosen for screening, since it had clear separation between sera from infected and uninfected rabbits and showed the least within-test variability of absorbance values. On the basis of results obtained in uninfected rabbits, an absorbance reading of 0.250 was used as the cutoff point to determine antibody titer when testing serial dilutions of serum. We expect that this test will be useful in detecting *P. multocida* infection in rabbits with or without signs of disease.

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