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Detection of Antibodies to *Pasteurella multocida* by Capture Enzyme Immunoassay Using a Monoclonal Antibody against P37 Antigen

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As infection with *Pasteurella multocida* is common in rabbits, an enzyme immunoassay (EIA) was developed for its detection. A murine immunoglobulin G monoclonal antibody was used to capture a 37-kDa polypeptide of *P. multocida* serotype A:12 in an EIA to detect antibodies to *P. multocida*. The 37-kDa antigen was selected since it was previously shown to be a major immunogen during *P. multocida* infection in rabbits. The sensitivity of the P37 EIA, determined with sera from 56 rabbits infected with *P. multocida*, was 98%. Specificity, evaluated with sera from 62 rabbits from colonies free of *P. multocida*, was 92%. Titration curves of sera from rabbits immunized with *P. multocida* serotype A:3 or A:12 coincided, indicating that the P37 EIA was equally efficient in detecting antibodies to the two major serotypes of the organism. Comparison of the P37 EIA with the current serodiagnostic test, a bacterial lysate EIA, revealed relatively good correlation ($r = 0.68$). However, specificity was greatly improved, as 34% of uninfected rabbits were falsely positive by the lysate EIA whereas only 3% of uninfected rabbits were falsely positive by the P37 EIA. The coefficient of variation for same-day tests was 10%, and that for interday tests was 15%, indicating good reproducibility. The greater sensitivity and specificity of the P37 EIA should significantly enhance diagnostic capability to identify rabbits infected with *P. multocida*.

Infection with *Pasteurella multocida* is a significant cause of clinical disease in rabbits (5, 6, 9, 11–13, 16, 22, 23). The most frequently occurring clinical syndrome involves infection of the upper respiratory tract. Although nasal cultures are frequently performed to identify respiratory infections, they are of limited value since they can be negative, even in rabbits known to be infected (6, 8, 10, 16, 27). Furthermore, *P. multocida* infection in many organ systems, including middle ears, lungs, paranasal sinuses, reproductive organs, kidneys, liver, lymph nodes, and bones (2, 3, 8, 11, 14, 15, 21, 30, 33), may not be amenable to culture. Occult infections often lead to disease when rabbits are stressed (5, 8, 14). Therefore, the need for a rapid and simple method for detecting current infection is indicated.

Even though rabbits produce antibodies to *P. multocida*, chronic infections usually result (8). Most serologic tests for detection of *P. multocida* infection have depended on the use of whole-cell lysates or components of the bacterial outer membrane as antigens to detect the presence of antibodies in the sera of infected rabbits (6, 7, 17, 18, 20, 26–28, 35). These tests have limitations in that they may detect only the specific serotype of the bacterium used in development of the assay or they lack specificity since they measure antibodies to cross-reacting antigens. Assays that use antigenic mixtures have a greater possibility of cross-reactions and, hence, false-positive results. Thus, we developed a test using a monoclonal antibody directed against a 37-kDa protein found in serotypes of *P. multocida* commonly found in rabbits (36). The monoclonal antibody was used to capture the 37-kDa antigen, providing an antigen-specific assay. The test is specific and sensitive in the identification of rabbits infected with *P. multocida.*

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MATERIALS AND METHODS

Preparation of antigen. Isolates 602 (serotype A:3) and 892 (serotype A:12) of *P. multocida* (12) were grown in 1-liter flasks of brain heart infusion broth (BBL) for 48 h at 37°C. Cultures were centrifuged, washed three times in phosphate-buffered saline (PBS; pH 7.4), suspended in PBS, and stored frozen in 1-ml aliquots. Aliquots were sonicated for 30 s with a Brandon sonicator cell disrupter, model 200, equipped with a microtip probe six times on ice. Antigens to be used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were boiled for 5 min in sample buffer (36) and stored at −20°C.

SDS-PAGE and Western blotting (immunoblotting). SDS-PAGE was performed with a 1.5-mm-thick, discontinuous gel of 5% polyacrylamide for stacking and 10% polyacrylamide for separation. A total of 500 μg of protein of bacterial lysate was added to each gel. After separation, proteins were transferred to nitrocellulose paper (Bio-Rad, Richmond, Calif.) and stored in blocking buffer (32) at 4°C. Strips, cut in 5-mm widths, were developed by incubation in rabbit serum diluted 1:500 in PBS or in undiluted murine cell culture supernatant for 1 h and washed four times with PBS containing 0.1% Tween 20 (PBST) at 5-min intervals. Strips were incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated goat antibody specific for either mouse (Antibodies Inc., Davis, Calif.) or rabbit (KPL, Gaithersburg, Md.) immunoglobulin G (IgG) for 1 h and washed three times with PBST and once with Tris-buffered saline. Color was developed with a 0.05% solution of 4-chloro-1-naphthol (Sigma, St. Louis, Mo.) and H_2O_2 for 10 min.

Immunization of mice. Four-week-old female BALB/c mice (B&K, Everett, Wash.) were immunized by a modification of the method of Coghlan and Hanausek (4). Sonicated antigens from *P. multocida* were separated by SDS-PAGE and transferred to nitrocellulose paper. Standard molecular mass references (Sigma) were used as guides to cut out a strip of nitrocellulose paper containing polypeptides in the range of 37 to 38 kDa. The paper strip was put in a microcentrifuge tube, submerged in liquid nitrogen, and ground into a coarse powder with a measuring spatula. The powder was emulsified with PBS-Freund's incomplete adjuvant and put in a 1-ml syringe with the tip of the barrel removed. The syringe was used to aseptically deliver the paper into a subcutaneous tunnel above the shoulders of the mice, which had been anesthetized with 0.2 mg of ketamine and 0.3 mg of xylazine intraperitoneally (i.p.). The procedure was repeated 3 weeks later. Two weeks after the second immunization, mice were euthanized and their spleens were aseptically removed.

Fusion. Spleens from immunized mice were placed in RPMI 1640 cell culture medium (BioWhittaker, Walkersville, Md.). Splenic cells were teased from the fibrous capsule and filtered through nylon mesh. Cells were washed twice in RPMI 1640 medium. A non-Ig-producing myeloma cell line, NS-1, was added at a ratio of 1 myeloma cell to 7.5 spleen cells and fused with 1 ml of 40% polyethylene glycol in RPMI 1640 medium. Fused cells were resuspended in RPMI 1640 medium supplemented with hypoxanthine, aminopterin, and thymidine, and this suspension was plated in the wells of 96-well culture plates, with approximately 200 cells per well. Medium in the wells was tested for antibody

production by EIA, with sonicated bacteria as the antigen. Positive cells were cloned twice by serial dilution in RPMI 1640 medium supplemented with hypoxanthine and thymidine and tested for antibodies by EIA and Western blot analysis. Only one clone produced the desired antibody and was used to produce ascites in BALB/c mice. The monoclonal antibody was typed with an Immuno-Type mouse monoclonal antibody isotyping kit (Sigma).

Ascites production. Six-week-old female BALB/c mice were primed by injecting them with 0.4 ml of Pristane (Sigma) i.p. Ten days later, 10^6 hybridoma cells in 0.5 ml of RPMI 1640 medium were injected i.p. into mice. Ascites fluid was collected twice by inserting a 20-gauge needle into the peritoneal cavity and withdrawing fluid. Cells in the fluid were removed by centrifugation in a serum separator tube. The antibody was purified with an ImmunoPure IgG purification kit (Pierce Chemical Company, Rockford, Ill.).

EIA. An enzyme immunoassay (EIA) was developed with the monoclonal antibody to capture the 37-kDa polypeptide antigen from lysed preparations of *P. multocida*. The optimal concentrations of antibody and antigen were determined by checkerboard titrations on microtiter plates (Maxi Sorb C bottom; VWR, Seattle, Wash.). The monoclonal antibody was serially diluted from 1:100 to 1:204,800 against the antigen, which had been serially diluted from 1:50 to 1:6,400. Protein concentrations of stock solutions were determined with a microtiter protein assay kit (Pierce Chemical Company), and the final concentrations were calculated based on dilutions used. The monoclonal antibody was diluted with 100 μl of carbonate buffer (pH 9.6) per well and adsorbed for 2 days at 4°C. Wells were washed with PBST and blocked for 1 h with PBST containing 5% (wt/vol) nonfat dry milk (PBST-M). Wells were washed with PBST and incubated at 37°C for 1 h with antigen diluted in PBST-M. After being washed, wells were used immediately or stored at −70°C. To measure antibodies, 90 μl of PBST-M and then 10 μl of a 1:10 dilution of test serum in PBST were added. After incubation for 1 h at 37°C, plates were washed and 100 μl of a 1:2,000 dilution of mouse-adsorbed goat anti-rabbit antibody conjugated with horseradish peroxidase (Antibodies Inc.) was added to each well. Wells were incubated for 30 min at 37°C and washed, 100 μl of *o*-phenylenediamine substrate (Sigma)-H_2O_2 was added to the wells, and color was allowed to develop for 10 min. The reaction was stopped by adding 25 μl of 8 N H_2SO_4. Absorbance values were determined on an EIA reader (model EL307IP; Bio-Tek Instruments, Burlington, Vt.) at a wavelength of 492 nm.

Rabbit sera. Sera used for *Pasteurella*-free controls were obtained from three rabbit colonies (Hare-Marland, Hewitt, N.J.; Baylor College of Medicine, Houston, Tex.; and the University of Washington, Seattle) maintained under specific-pathogen-free conditions. These rabbits were monitored by repeated culture of the nares and were free of clinical signs of pasteurellosis. Sera used for *Pasteurella*-positive controls were obtained from adult rabbits which were culture positive by nasal swab at the time of blood collection or were positive from any site of necropsy (6, 10, 12). Rabbits were either naturally infected, from rabbitries where either serotype A:3 or A:12 was endemic, or experimentally infected by intranasal inoculation with serotype A:3 or A:12. Sera used to compare the P37 EIA and lysate EIA were selected based on absorbance values previously determined by the lysate EIA. These sera were known to have high or low absorbance values, and 11 sera with equivocal values were from *Pasteurella*-free rabbits.

Immunization of rabbits. Four female New Zealand White crossed with Florida White rabbits were obtained from a commercial rabbitry (R&R, Stanwood, Wash.). These rabbits were determined to be free of *P. multocida* by lack of growth of *P. multocida* on swabs taken from the nares obtained three times at 14-day intervals and streaked onto agar plates with 5% sheep blood agar and by the absence of antibodies reactive in the EIA (6, 20) and Western blot analysis. Two rabbits each were inoculated with isolate 602 or 892 of *P. multocida* killed by exposure to cobalt radiation for 4 h. Bacteria were shown to be killed by absence of growth on sheep blood agar. A total of 2 mg of protein (dry weight) of killed cells was emulsified in 1 ml of PBS containing Freund's complete adjuvant (Sigma) and injected subcutaneously in 0.1-ml amounts above both flanks. This was followed by a booster injection 3 weeks later of the same isolate of bacteria in Freund's incomplete adjuvant (Sigma), injected in the same manner. Seven days after the booster injections, rabbits were anesthetized with acepromazine maleate (5 mg), xylazine (5 mg/kg of body weight), and ketamine (30 mg/kg) and exsanguinated. After separation from blood cells, sera were aliquoted into 1.5-ml amounts and frozen at −20°C.

RESULTS

Characterization of monoclonal antibody. Only 1 of 768 wells of fused spleen cells produced the desired antibody. From that well, 1 murine monoclonal antibody was produced. The monoclonal antibody produced a single, strong band, by Western blot analysis against lysed bacteria, at 37 kDa when assayed against antigens of strains 602 and 892 (Fig. 1). Strain 602 also showed two light bands at 30 and 40 kDa, while strain 892 showed light bands at 31 and 71 kDa. Isotyping revealed that the monoclonal antibody was of the IgG1 class of antibody.

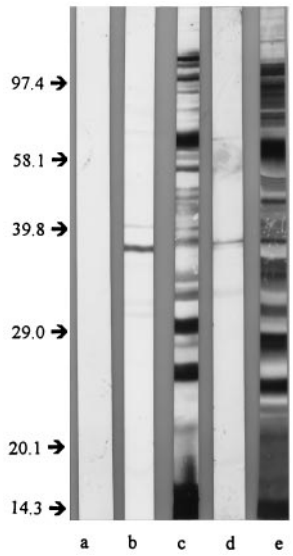

FIG. 1. Immunoblot reactions with monoclonal antibody P37. Lane a, control strip incubated with culture medium without monoclonal antibody; lanes b and d, strips incubated with P37 monoclonal antibody from culture supernatant; lanes c and e, strips incubated with serum from a rabbit experimentally immunized with serotype A:12. Antigens in lanes b and c were separated proteins from serotype A:3, and antigens in lanes d and e were separated proteins from serotype A:12. Molecular mass markers (in kilodaltons) are indicated at the left.

Development of EIA. The P37 monoclonal antibody was used to capture the 37-kDa antigen. Titrations of monoclonal antibody and antigen by checkerboard titrations gave the widest separation of absorbance values for positive and negative controls at a 1:200 dilution of both antibody and antigen. The protein concentrations of purified antibody and antigen at this dilution were 18.5 and 52.0 μg/ml, respectively. Serial dilutions of sera from *P. multocida*-positive and *P. multocida*-negative rabbits were assayed. At a serum dilution of 1:100, absorbance values from *Pasteurella*-positive and *Pasteurella*-negative rabbits were widely separated (Fig. 2). This was the dilution of sera used for antibody testing. Sera from four rabbits immunized with serotype A:3 or A:12 of *P. multocida* were assayed to determine if antibody to the P37 antigen was present in both

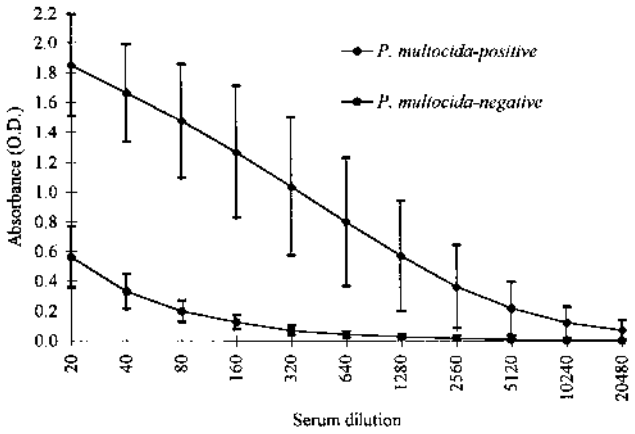

FIG. 2. Serial dilutions of sera from rabbits naturally infected with *P. multocida* (n = 27) were compared to those from *Pasteurella*-negative rabbits (n = 32) by the P37 EIA. Points indicate mean values, and bars represent 1 standard deviation. O.D., optical density.

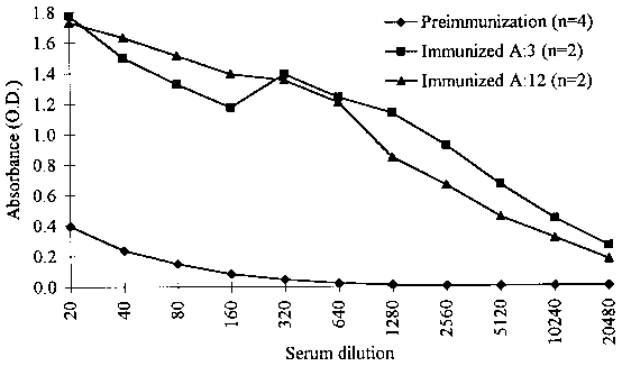

FIG. 3. Serial dilutions of sera from four rabbits before and after immunization with either serotype A:3 or A:12 of *P. multocida*. Points indicate mean values. O.D., optical density.

serotypes. The absorbance values at different dilutions were similar for rabbits immunized with either serotype (Fig. 3).

Establishing limits. Sera of 62 rabbits from *P. multocida*-free rabbit colonies were tested at a 1:100 dilution by the P37 EIA. A mean absorbance value of 0.24 with a standard deviation of 0.12 was found for 59 rabbits. Three rabbits with absorbance values greater than 1.20 were considered outliers and were excluded from the calculation. Western blots revealed that these rabbits reacted with the 37-kDa polypeptide but not with other antigens usually detected in *P. multocida* infection (36). While these rabbits were excluded from establishing the mean value for *Pasteurella*-negative rabbits, they were included in the calculation of the specificity of the EIA. As 2 standard deviations above the mean absorbance of *Pasteurella*-negative rabbits was 0.48, an absorbance value of 0.50 was selected as the cutoff value between negative and positive sera. The mean absorbance value of 56 rabbits with clinical and cultural evidence of *P. multocida* infection was 1.23, with a standard deviation of 0.40.

Sensitivity and specificity. Sensitivity was established with sera from 56 rabbits which had clinical signs of pasteurellosis and at least one positive culture for *P. multocida*. Sera from 1 rabbit had a value below the 0.50 absorbance cutoff value, giving a sensitivity of 98%. Specificity was determined with sera from 62 rabbits maintained in colonies that were free of *P. multocida*. Sera from five *Pasteurella*-negative rabbits had absorbance values above the 0.50 cutoff, giving a specificity of 92% (Table 1).

Comparison of tests. The P37 EIA was compared with a bacterial lysate EIA currently used for serodiagnosis (6, 20) with sera from 12 *P. multocida*-infected and 31 uninfected rabbits (Fig. 4). Pearson's correlation coefficient for the tests

TABLE 1. Sensitivity and specificity of the monoclonal antibody P37 capture EIA in detecting antibodies to *P. multocida* in rabbits

P37 EIA result	No. of rabbits with indicated *P. multocida* antibody test result[a]		Total
	Positive	Negative	
Positive	55	5	60
Negative	1	57	58
Total	56	62	

[a] Based on cultural results. With 55 of 56 rabbits testing positive, the sensitivity of the assay is 0.98. With 57 of 62 rabbits testing negative, the specificity of the assay is 0.92.

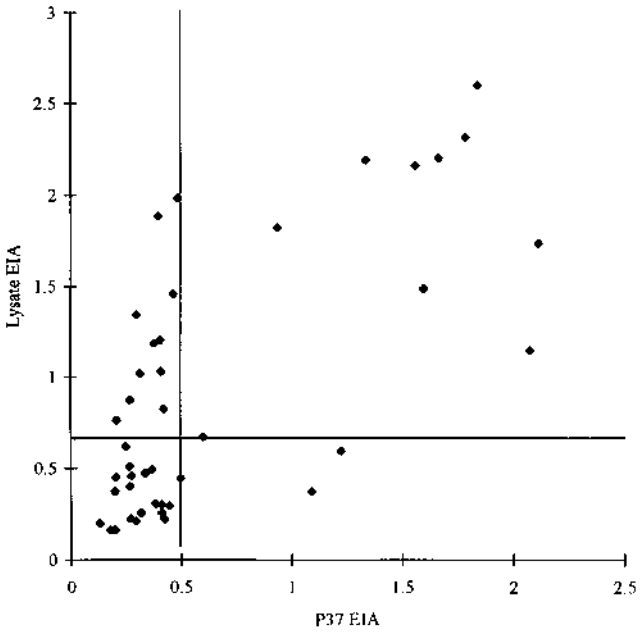

FIG. 4. Comparison of results obtained with rabbit sera by the P37 EIA and lysate EIA. Each axis shows absorbance values for each test. Lines indicate the cutoff value for each test.

was 0.68. Eleven rabbits known to be *Pasteurella* free tested negative by the P37 EIA but positive by the lysate EIA. Only one known *Pasteurella*-free rabbit was positive by the P37 EIA but negative by the lysate EIA. Another rabbit, culture positive for *P. multocida*, was positive by the P37 EIA but negative by the lysate EIA. A third rabbit, also culture positive for *P. multocida*, was negative by both tests.

Reproducibility. The consistency of the EIA was evaluated by testing replicates of seven sera with various absorbance values (Table 2). Six replicates were tested on one day (same-day tests) and on each of three different days (interday tests). The average coefficient of variation for same-day tests was 9.9% (range, 3.7 to 18.2%), whereas that for interday tests was 14.5% (range, 7.4 to 30.8%). There was an inverse relationship between absorbance values and coefficients of variation in that the highest coefficients of variation occurred with sera from *Pasteurella*-negative rabbits. The reproducibility of the P37 EIA was also assessed with a microtiter plate the same day it was prepared and with a plate prepared and frozen at −70°C

TABLE 2. Reproducibility of P37 EIA results in detecting antibodies to *P. multocida* in rabbits

Rabbit	Mean absorbance value ± SD[a]	
	Same-day test[b]	Interday test[c]
1462	0.22 ± 0.04 (18.2)	0.26 ± 0.08 (30.8)
6932	0.29 ± 0.04 (13.8)	0.28 ± 0.04 (14.3)
780-F	1.14 ± 0.12 (10.5)	1.06 ± 0.14 (13.2)
1150-F	1.31 ± 0.10 (7.6)	1.18 ± 0.14 (11.9)
1357	1.35 ± 0.05 (3.7)	1.23 ± 0.16 (13.0)
66053-18	1.37 ± 0.12 (8.8)	1.35 ± 0.15 (11.1)
1421	1.48 ± 0.10 (6.8)	1.49 ± 0.11 (7.4)

[a] Numbers in parentheses are coefficients of variation.
[b] Six replicates of each serum sample on a single plate were tested on the same day.
[c] Sera were tested on three different days.

TABLE 3. Reproducibility of results of P37 EIA using fresh or frozen antigen-coated plates in detecting antibodies to *P. multocida* in rabbits

P. multocida status of rabbits	Total no. of rabbits	Mean absorbance value ± SD from plate with indicated type of antigen		Correlation coefficient
		Fresh	Frozen	
Uninfected	12	0.39 ± 0.09	0.37 ± 0.13	0.91
Infected	9	1.55 ± 0.53	1.69 ± 0.60	0.98

1 month previously. Absorbance values were similar, and Pearson's correlation coefficient was 0.98 for *P. multocida*-infected rabbits and 0.91 for uninfected rabbits (Table 3).

DISCUSSION

Serologic tests for *P. multocida* infection using whole-cell lysates as test antigens (6, 17, 20, 26, 27, 28) are confounded by cross-reactions with antibodies induced by infection with related bacteria. Assays using lipopolysaccharides, which are primary somatic markers (7), are strain specific; therefore, antibodies to one serotype do not react, or react poorly, to the lipopolysaccharides of other serotypes (20). Serotypes of *P. multocida* commonly infecting rabbits are A:3 and A:12 (22, 23, 36). Both serotypes are responsible for the majority of morbidity caused by *P. multocida* in rabbits (9, 12, 13, 22, 23). Zimmerman et al. (36) showed that antibodies were consistently produced by rabbits infected with either serotype of *P. multocida* to proteins of approximately 28, 30, and 37 kDa. In our test, the 37-kDa protein is captured in plate wells by a mouse monoclonal antibody specifically directed against the antigen. The antigen binds any antibodies directed against it in the rabbit sera being tested. By using a defined antigen rather than a bacterial lysate, there is less chance of cross-reactive antibodies influencing the results. The mouse monoclonal antibody was produced in a unique manner. Nitrocellulose strips have been used to immunize rabbits to microgram quantities of antigen (4); however, this is the first report of mice immunized in this manner with the intent of developing a monoclonal antibody.

A cutoff value for determining whether absorbance values are positive or negative was established with sera from rabbits maintained in *Pasteurella*-free colonies. These rabbits had no clinical signs of pasteurellosis, and *P. multocida* was not detected in the colony during routine quality assurance testing. The mean and standard deviation of the absorbance values for these rabbits were calculated at the dilution of serum to be used in testing. The cutoff value of 0.50 was more than twice the standard deviation above the mean. This ensures that greater than 95% of rabbits uninfected with *P. multocida* will give a negative test result. Sera from three rabbits reacted strongly in the P37 EIA. Since these rabbits had absorbance values in excess of those of other rabbits, they were considered falsely positive and were not used to establish the cutoff value. Inclusion of these rabbits in the calculation of the cutoff value would have resulted in an unreasonably high value, decreasing the sensitivity below acceptable limits. A sensitivity of 98% and a specificity of 92% for the P37 EIA are within the generally accepted range for clinical tests. The three *P. multocida*-negative rabbits that were not included in calculating the cutoff value were included with the *P. multocida*-negative rabbits used to calculate specificity.

The ability of the P37 EIA to discriminate between rabbits which produce falsely positive absorbance values by the lysate EIA (6, 20) from true *P. multocida*-negative rabbits was shown in a direct comparison of the tests. Of 31 *Pasteurella*-negative rabbits, 11 rabbits gave a positive reaction by the lysate EIA but a negative reaction by the P37 EIA. Serum of one rabbit from which *P. multocida* was recovered from the paranasal sinuses at necropsy gave a negative reaction by the P37 EIA. Since this rabbit also had a negative reaction by the lysate EIA, it may not yet have developed antibodies to *P. multocida* following infection. Further studies are needed to determine when antibodies to the P37 antigen appear in rabbit serum after infection and whether they disappear after elimination of infection.

Several monoclonal antibodies have been developed for the purpose of classifying different *Pasteurella* species, serotypes, and toxins (1, 19, 24, 25, 29, 31, 34). Lu et al. (24) developed a monoclonal antibody directed against an outer membrane protein of *P. multocida* estimated to be 37.5 kDa. The 37.5-kDa protein was detected on both serotypes A:3 and A:12 as well as other serotypes of *P. multocida*. The antibody protected mice and rabbits from respiratory disease when they were experimentally challenged with *P. multocida* (25).

This is the first report of a murine monoclonal antibody used to capture a specific antigen for the purpose of testing for serologic evidence of *P. multocida* infection in rabbits. The monoclonal antibody was produced against a 37-kDa antigen shown by Zimmerman et al. (36) to be conserved in capsular type A and somatic types 3 and 12 of *P. multocida*. Rabbits infected with either of these serotypes produce antibodies to this polypeptide antigen. Use of the P37 capture EIA should provide a more accurate diagnosis of *P. multocida* infection in rabbits than serologic methods currently available.

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