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Field Trial of a *Pasteurella multocida* Extract Vaccine in Rabbits

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Pasteurella multocida* is a bacterial pathogen that can cause significant disease and subsequent effects on research activities involving rabbits. Although several vaccines have been tested under laboratory conditions, field trials of vaccines for the control of *P. multocida* in rabbits are few. We used a potassium thiocyanate extract (PTE) produced from *P. multocida* serotype D:3,12,15 to vaccinate *Pasteurella*-free rabbits at their introduction into a colony having endemic infection with *P. multocida* serotype A:3. Groups of 15 rabbits were vaccinated either SC or IN with 1.0 mg PTE once weekly for 3 wk. In addition a control group was sham-vaccinated IN with saline. After the last vaccine dose had been administered, rabbits were housed with the general colony of a facility with endemic pasteurellosis. Serum samples obtained before and 5 and 24 wk after the first dose of vaccine were evaluated by ELISA for anti-PTE IgG. Rabbits were euthanized if found in poor clinical condition, and all remaining rabbits were euthanized 24 wk after initial vaccination. Clinical disease typical of *P. multocida* infection was observed in 10 of 15 saline-vaccinated rabbits, 4 of 15 IN PTE-vaccinated rabbits, and 1 of 15 SC PTE-vaccinated rabbits. Bacterial culture of the nasopharynx at the time of necropsy was positive for *P. multocida* in 10 of 15 control rabbits, 5 of 15 IN PTE-vaccinated rabbits, and 1 of 15 SC PTE-vaccinated rabbits. Anti-PTE serum IgG activity had developed in both IN- and SC-vaccinated rabbits by 5 wk, with significantly lower activity by 24 wk after initial vaccination. IgG activity was significantly greater in rabbits vaccinated SC compared with controls or those vaccinated IN. In summary, PTE can be used to stimulate protective immunity to a heterologous strain of *P. multocida*, with stronger immunity generated by SC than IN vaccination.

Abbreviations: PTE, potassium thiocyanate extract of *P. multocida*

Pasteurella multocida is an important bacterial pathogen of domestic rabbits. Although infection may be subclinical, disease characterized by rhinitis, pneumonia, abscessation of viscera and subcutaneous sites, metritis, orchitis, septicemia, and otitis media may occur.[21,25] In most cases, the likely site of initial infection is the upper respiratory tract. Transmission occurs readily through direct contact of susceptible rabbits with carrier animals, and airborne transmission does not occur after exposure periods of 3 wk.[8] Stressors such as crowding, transportation, and high ammonia concentrations in the air often stimulate latent *P. multocida* to proliferate and cause disease.[2,9,16]

In attempts to protect rabbits from infection with *P. multocida*, a variety of vaccines have been examined, including those composed of inactivated whole bacteria,[1,15] streptomycin-dependent live *P. multocida*,[5,7] outer membrane proteins of the organism,[4,12,13] and *P. multocida* potassium thiocyanate extract (PTE)[14,20] by IM, SC, or IN routes of administration. A bivalent vaccine for pasteurellosis and rabbit hemorrhagic disease virus stimulated high antibody titers to both pathogens.[19] With respect to these various vaccine preparations, only a single field trial has been conducted in rabbits. That study evaluated the efficacy of a live streptomycin-dependent *P. multocida* serotype A:12 vaccine, which failed to protect animals from disease under field conditions.[5]

PTE vaccination of *Pasteurella*-free rabbits in the laboratory has shown promise as a means to prevent infection. Intranasal and intraconjunctival vaccination with PTE stimulated a serum IgG response against *P. multocida*, and this response was associated with protection against clinical disease and death after challenge with homologous bacteria.[20] Similar results were demonstrated for IN and IM vaccination with PTE.[14] Although these vaccines prevented colonization of the challenge organisms in the lungs, *P. multocida* still was cultured from the nasopharynx of some rabbits; however, those rabbits were challenged with a bolus of bacteria much greater than that that likely to be encountered under field conditions.[9,10,20,23] Therefore, the purpose of the present study was to evaluate the ability of PTE to prevention infection of rabbits with *P. multocida* under field conditions typical of a rabbit herd with endemic pasteurellosis.

Materials and Methods

Animals. Female New Zealand White rabbits, 2.5 to 3.0 kg, were obtained as *Pasteurella*-free animals from a commercial vendor (Covance, Denver, PA). Bacterial culture of deep nasal swabs confirmed that the rabbits were free from *Pasteurella*. The rabbits were housed at a rabbitry with a history of endemic serotype A:3 pasteurellosis (which has since been eliminated from the facility). The rabbitry comprised approximately 600 rabbits, and pathologic signs of pasteurellosis, including serous to purulent nasal discharge, pneumonia, and abscessation were noted frequently. Rabbits were fed a high-quality commercial chow (Laboratory Rabbit Diet 5326 HF, PMI, Richmond, IN), allowed ad libitum access to tap water provided via water bottles, and maintained on a 12:12-h light:dark cycle. Rabbits were euthanized by IV overdose of a commercially available euthanasia solution. All studies were approved by the institu-

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tional animal care and use committee.

Vaccine preparation. PTE of *P. multocida* (rabbit origin, serotype 3,12,15:D) prepared as previously described[20] was used as the immunogen in the current study. This strain was used to assess the possibility of successful vaccination by using a strain with a different capsular serotype, thus allowing estimation of the likelihood of success under field situations in which infection might be associated with mixed or heterologous *Pasteurella* strains. Briefly, *P. multocida* serotype 3,12,15:D was grown to confluence on tryptic soy agar containing 5% sheep blood and harvested in 6 ml of a solution containing saline and 1.0 M KSCN in equal parts. After incubation at 37 °C for 6 h, whole cells were removed by centrifugation at 8000 × *g* for 10 min, and the supernatant was dialyzed extensively against a buffer containing 0.01 M Tris-HCl, 0.32 M NaCl, and 0.01% NaN_3 (pH 8.0). The extract then was concentrated (Centriprep-10, Millipore, Temecula, CA) and sterilized by passage through a 0.45-µm filter (Amicon, Beverly, MA).

ELISA. Serum samples were assayed for anti-PTE IgG activity by ELISA as previously described.[20,23] Briefly, polystyrene microtiter wells each were coated with 10 µg PTE protein in 200 mM sodium carbonate buffer (pH 9.6). After overnight incubation, plates were washed 3 times with PBS (pH 7.4) containing 0.5% (v/v) Tween 20 and then incubated for 24 h with 3% (w/v) bovine serum albumin (Sigma Chemical, St Louis, MO). Immediately before samples were tested, wells were washed 3 times with PBS–Tween 20. Based on preliminary assays, serum samples were diluted 1:20 in PBS–Tween 20 and incubated in duplicate PTE-coated wells and uncoated wells (to control for nonspecific absorption) for 4 h. After thorough washing of the wells with PBS–Tween 20, horseradish peroxidase-conjugated goat antirabbit IgG (Sigma Chemical; 1:1000 in PBS–Tween 20) was added to the wells. After overnight incubation and washing, substrate (*o*-phenylaminediamine, Sigma Chemical) was added and the reaction stopped 30 min later by addition of an equal volume of 1 M H_2SO_4. Optical density at 490 nm was measured by using a microplate reader (Vmax Microplate Reader, Molecular Devices, Menlo Park, CA). Specific immunoglobulin activity is expressed as optical density at 490 nm per 30 min.

Evaluation of animals for *P. multocida*. At the time of euthanasia, lung, liver, and nasopharynx were cultured for the presence of *P. multocida*. In each rabbit, culture of lung was performed by vigorously swabbing a sterilely incised surface of each lung lobe with a sterile cotton-tipped bacterial swab system that included transport culture (Culturette, BD Diagnostic Systems, Franklin Lakes, NJ). The nasopharynx was sampled by passing a swab retrograde through the incised trachea for vigorous swabbing, and the liver was sampled by vigorous swabbing of 2 sterilely incised surfaces. Swabs then were streaked on tryptic soy agar containing 5% sheep blood and cultured at 37 °C for 24 h, at which time the presence or absence of *P. multocida* colonies was noted. *P. multocida* was identified as characteristic discrete, round mucoid colonies that were grayish in appearance. Gram stain of colony samples showed them to be small, gram-negative coccobacilli.

Experimental design. Prior to undergoing vaccination and introduction into the herd population, test rabbits were evaluated by deep nasal swab to confirm their *Pasteurella*-free status. This sampling was done by rotating a sterile cotton-tipped swab that was inserted approximately 1.25 in. into each nare of the rabbits after their tranquilization with acepromazine maleate (1.0 mg/kg IM). Groups of 15 rabbits were rabbits were immunized either IN or SC with 1.0 mg PTE in 0.5 ml sterile saline once weekly for 3 wk. Another group was dosed IN with sterile saline, as a control. After the last vaccine dose had been administered, rabbits were housed with the general colony of a facility that had a history of endemic pasteurellosis (which has since been eliminated). All rabbits were housed individually and did not have any direct physical contact. Test rabbits were distributed throughout the colony and located such that cages containing colony rabbits were positioned above, below, and on each side of test rabbits. Serum was sampled by auricular venipuncture before and 5 and 24 wk after initial vaccination. Serum was stored at –20 °C until evaluated by ELISA. Rabbits were euthanized if found in poor clinical condition as defined by clinical signs including lethargy, dyspnea, and inappetence; all remaining rabbits were euthanized 24 wk after initial vaccination. Means and standard errors of ELISA absorbance values were compared by using the Wilcoxon rank sum test (Instat 3.0, Graph Pad Software, San Diego, CA), with statistical significance at $P < 0.05$.

Table 1. Culture of *P. multocida* from tissues of rabbits

	Tissue sampled		
Treatment group	Nasopharynx	Lung	Liver
Saline IN	10	3	2
PTE IN	5	2	0
PTE SC	1	0	0

Results are shown as the number of rabbits that had a positive culture for *P. multocida*; each group had a total of 15 rabbits. Tissues were sampled 24 wk after initial vaccination or earlier if euthanasia was warranted in light of the clinical condition of the rabbit. Positive cultures for lung and liver came from rabbits from which *P. multocida* was cultured from the nasopharynx also.

Results

Bacterial culture of tissues and clinical observations. To determine whether vaccination with the PTE vaccine stimulated protective immunity against infection and disease associated with *P. multocida*, rabbits were observed daily for signs of clinical disease typical of pasteurellosis, and samples of nasopharynx, lung, and liver obtained from all rabbits at the time of euthanasia were cultured. Clinical disease was observed in 10 of the 15 saline-vaccinated rabbits, 4 of the 15 IN PTE-vaccinated rabbits, and 1 of the 15 SC PTE-vaccinated rabbits. All of the rabbits with clinical disease were culture-positive for *P. multocida*, and only 1 additional rabbit (in the IN PTE-vaccinated group) was free of clinical disease but culture-positive for *P. multocida*. In rabbits vaccinated either IN or SC with PTE, clinical disease was limited to mild to moderate, serous nasal discharge. In contrast, clinical disease in rabbits vaccinated with saline was characterized by serous or moderate to heavy, mucopurulent nasal discharge. In addition 4 of the 10 clinically affected control rabbits were dyspneic, and 2 of the 10 died acutely. Of the 10 clinically affected rabbits in the saline-vaccinated group, 5 were euthanized prior to scheduled euthanasia, whereas 1 rabbit in the IN PTE-vaccinated group and none in the SC PTE-vaccinated group was euthanized early.

Compared with saline vaccination, IN and SC vaccination with PTE conferred protection against *P. multocida* infection of the nasopharynx (Table 1). Some rabbits vaccinated IN with either saline or PTE were culture-positive for *P. multocida* in the lungs, although no organisms were cultured from the livers of PTE-vaccinated rabbits. Subcutaneous vaccination with PTE offered the greatest overall protection, in that only 1 of 15 nasopharyngeal samples was positive for *P. multocida*, and no lung or liver samples were positive.

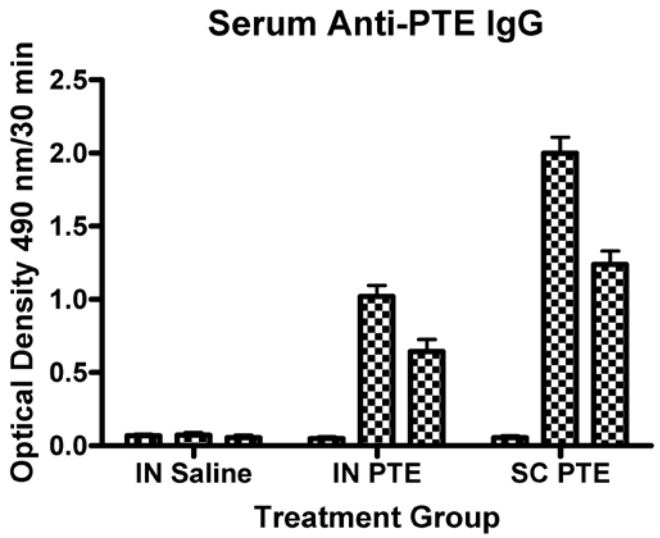

Figure 1. Effect of vaccination with PTE on serum IgG anti-PTE activity. Groups of 15 rabbits were vaccinated once weekly for 3 wk with either saline IN (control) or 1.0 mg PTE either IN or SC. Serum was sampled before vaccination (first bar for each treatment group), 5 wk after initial vaccination (second bar), and 24 wk after initial vaccination (third bar). Evaluation of serum by ELISA showed a significant ($P \leq 0.05$) increase in anti-PTE activity by 5 wk in rabbits vaccinated with PTE by either route. This activity declined by 24 wk but was still greater than that in saline controls or prevaccination samples. At both 5 and 24 wk, rabbits vaccinated SC with PTE had significantly greater ($P \leq 0.05$) anti-PTE activity than did rabbits vaccinated IN with PTE.

Serum IgG response to vaccination. As a measure of the ability of vaccination to stimulate humoral immunity, ELISA for serum IgG to PTE was performed on samples obtained before and 5 and 24 wk after initial vaccination. Both IN and SC vaccination with PTE stimulated humoral immunity by 5 wk after initial vaccination (Figure 1); this response decreased by 24 wk afterward. Samples obtained from saline-vaccinated rabbits or from rabbits prior to initial vaccination with PTE did not demonstrate appreciable anti-PTE serum IgG. At both 5 and 24 wk, antibody responses of rabbits vaccinated SC with PTE were significantly ($P \leq 0.01$) greater than those of rabbits vaccinated IN with PTE. For both treatment groups, antibody responses at both time points were significantly ($P \leq 0.001$) greater than those of prevaccination samples or samples from saline-vaccinated control rabbits. The decline in the antibody response between 5 and 24 wk after initial vaccination was significant ($P \leq 0.05$) for both groups vaccinated with PTE.

Discussion

In the current study, protective immunity developed in rabbits after either IN or SC vaccination with PTE. PTE is a lysate that contains a mixture of soluble cellular components, some of which are presumed to be of significance for bacterial virulence. Samples obtained after vaccination showed significant increases in anti-PTE serum antibody activity. These findings are in agreement with earlier studies demonstrating development of anti-PTE serum antibody activity after conjunctival and IN vaccination with PTE of rabbits which then were protected against challenge with a fixed number of *P. multocida* organisms[20] and with a study demonstrating development of protective immunity to homologous challenge after IN or IM vaccination with PTE.[14]

P. multocida is a bacterial pathogen of rabbits that generally accesses the body at mucosal surfaces, particularly the nasopharynx. The humoral immune system protects such surfaces by production of secretory IgA, and vaccination with PTE has been demonstrated to stimulate anti-PTE IgA production in the nasopharynx. This production of nasopharyngeal anti-PTE IgA is enhanced by coadministration of the adjuvant cholera toxin[23] and by incorporation of PTE into alginate microspheres.[10,24,26]

Although the amount of anti-PTE antibody present on mucosal surfaces was not measured in this study, increased resistance to *P. multocida* infection likely would result from antigen-specific secretory IgA.[30] Although vaccination typically leads to the production of secretory IgA at mucosal surfaces,[11,17] we noted an increase in anti-PTE serum IgG associated with resistance to infection, and parenteral vaccination led to greater protection than did IN vaccination. This result is in contrast to earlier findings,[14] in which IN vaccination with PTE resulted in greater protection to homologous challenge than did IM vaccination.

Serum IgG has been demonstrated to confer mucosal immunity to a variety of pathogens, including rotavirus and influenza virus.[28,29] This effect may occur due to transudation of IgG from the serum to the respiratory tract mucosa.[28] Therefore, in our study, SC vaccination with PTE resulted in protective immunity, likely at least in part by means of serum-derived IgG. The decline in serum IgG anti-PTE activity by 24 wk suggests that continued protective immunity to *P. multocida* would require additional booster vaccinations.

Sham-vaccinated control rabbits did not develop appreciable serum antibody responses to PTE, even though they were exposed to *P. multocida* and most became infected with the organism. The most likely explanation for this finding is that the *P. multocida* strain endemic to the colony did not express the dominant antigen measured by our ELISA. Although the PTE used was derived from a bacterial strain that had the same somatic serotype 12 antigen as the endemic strain, the vaccine strain had additional somatic serotype and capsular type D antigens. Further, the strain used to produce PTE produces heat-labile toxin, a virulence factor associated with some isolates of *P. multocida*. In an earlier study,[26] approximately 25% of type D isolates were toxigenic, whereas none of 109 type A isolates was toxigenic; the strain we used to produce PTE was determined to be toxigenic in the earlier study. *P. multocida* heat-labile toxin is a potent immunogen, and heat-inactivated toxin conferred protective immunity to challenge with native toxin in rabbits.[22,23] Heat-labile toxin may have been the immunodominant antigen in our vaccine, and the sensitivity of our ELISA was calibrated for antibody to heat-labile toxin. The correlation of ELISA results with bacterial culture and clinical disease findings suggests either that antibody to heat-labile toxin had an unexpected cross-protective effect or that other anti-*P. multocida* antibodies associated with vaccination, and possibly infection, were generated in concert with anti-heat–labile toxin antibodies but were not detected because our ELISA was insensitive to antibodies to antigens other than heat-labile toxin.

Although none of the vaccine preparations we tested resulted in complete protection against *P. multocida* infection, disease was uncommon in PTE-vaccinated rabbits and markedly less severe than that in saline-vaccinated control animals. The vaccine was produced from a *P. multocida* isolate having a different capsular serotype (type D) than the bacteria that historically had been associated with endemic disease at the facility (type A). However, both isolates shared a somatic serotype antigen (type 3), and it is possible that the immune response directed to this antigen was sufficient to yield protective immunity to rabbits in the PTE vaccination groups. Infection of rabbits with *P. multocida*

of different somatic serotypes resulted in serologic antibody responses to polypeptides present in both serotypes;[15,31] in addition, vaccination of rabbits with a streptomycin-dependent mutant of *P. multocida* serotype A:12 induced antibody to somatic antigens of both A:12 and A:3 strains.[18] Further, *P. multocida* of capsular types A and D stimulated cross-immunity by passive protection in sheep.[3] Our results extend to rabbits the idea that immunization with vaccines derived from a particular *P. multocida* serotype can stimulate protective cross-immunity to heterologous serotypes.

P. multocida remains an important pathogen of rabbits. Although control measures such as optimizing the environment of the rabbitry and culling of symptomatic animals reduce morbidity,[6] endemic infection represents a source of considerable potential loss. Our results demonstrate the possibility of using vaccination to greatly limit infection and disease under field conditions.

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