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Protection of Rabbits Against Experimental Pasteurellosis by a Streptomycin-Dependent *Pasteurella multocida* Serotype 3:A Live Mutant Vaccine

YUE-SHOUNG LU* AND STEVEN P. PAKES

Division of Comparative Medicine, Department of Pathology, University of Texas Health Science Center at Dallas, Dallas, Texas 75235

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Pasteurella multocida (serotype 3:A) was isolated from a rabbit with clinical signs of suppurative rhinitis. This *P. multocida* strain was mutagenized wih *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to obtain a genetically stable streptomycin-dependent mutant, from which a live vaccine was prepared. Pasteurella-free rabbits were inoculated intranasally three times at weekly intervals and challenged intranasally with a virulent serotype 3:A rabbit *P. multocida* isolate 2 weeks after the third vaccination. The rabbits were killed 2 to 3 weeks later. The vaccine did not cause clinical disease, death, or gross or microscopic lesions. Furthermore, the vaccine protected the challenge rabbits from developing clinical disease, death, and gross lesions. However, mild focal lung lesions were noted in several of the vaccinated-challenged animals. In contrast, nonvaccinated-challenged rabbits developed pyrexia and anorexia. Furthermore, three of four of these rabbits died with severe gross lesions including pyothorax, suppurative pericarditis, and fibrinopurulent pneumonia. Microscopically, the four nonvaccinated rabbits had moderate to severe suppurative pneumonia and mild to moderate suppurative rhinitis, and two had mild tympanitis. The mutant vaccine did not appear to colonize the nasal cavities. The vaccine prevented the colonization of the virulent challenge organism in lungs, liver, spleen, genital tracts, and blood, but not the nasal cavities.

Pasteurella multocida infection is a very common and serious problem of rabbits used for biomedical research. *P. multocida* causes suppurative rhinitis (snuffles), otitis media, enzootic pneumonia, conjuctivitis, pyometra, orchitis, subcutaneous abscesses, and septicemia in rabbits (14).

Specific use of rabbits for studies involving the respiratory, genital, and sensory systems (10, 18) may be compromised because of the predilection of the bacterium for these organs. Further, *P. multocida* infection is often subclinical. Under various experimental manipulations, mild to severe clinical disease may occur, compromising the rabbits as research subjects.

Various techniques have been attempted to control and eliminate pasteurellosis. One of the more promising approaches is the development of immunization procedures which over long periods will protect animals exposed to *P. multocida*. Vaccination studies of rabbits with killed *P. multocida* have shown variable results (2, 3, 5). Alexander et al. (2) vaccinated rabbits with an autologous killed *P. multocida* isolate by repeated intravenous injections. The killed vaccine gave some protection, as mortality decreased from 14.8% (77/522) in unvaccinated groups to 8.4% (16/184) in vaccinated groups. Seavey et al. were unable to reduce the mortality of naturally infected rabbits by using a killed vaccine (E. E. Seavey, R. O. Anslow, and N. G. Heller, Abstr. Annu. Meet. Am. Assoc. Lab. Anim. Sci., 1977, abstr. no. 10).

Live vaccines including streptomycin-dependent mutants were protective against several bacterial diseases in animals and humans, particularly enteric diseases caused by *Escherichia coli*, *Salmonella typhi*, *Shigella flexneria*, etc. (11-13, 22). Recently, a live streptomycin-dependent mutant vaccine of serotype 12:A (somatic type 12 and capsular type A) *P. multocida* was reported to prevent respiratory disease in rabbits after challenge with serotype 12:A *P. multocida* (8). We have developed a live streptomycin-dependent mutant vaccine by using serotype 3:A *P. multocida* of rabbit origin. Serotypes 3:A and 12:A are the predominant isolates from rabbit respiratory pasteurellosis (4).

In this study, we evaluated the protective efficacy of the serotype 3:A streptomycin-dependent mutant vaccine against experimental rabbit pasteurellosis by using clinical param-

eters, gross and microscopic pathology, and microbiological techniques. Microbiological studies included determination of reversion frequency; time of onset, duration, and frequency of colonization of the mutant in nasal cavities; and the colonization patterns of virulent challenge *P. multocida* in nasal cavities and visceral organs.

MATERIALS AND METHODS

Animals. Pasteurella-free New Zealand white 7- to 10-month-old male and female rabbits obtained from Dutchland Laboratories, Denver, Pa., were used. Before vaccination, the nasal cavities of each rabbit were cultured three times for *P. multocida*, and the sera were tested for agar gel-precipitating antibodies to the 16 *P. multocida* somatic antigens. Only rabbits free of *P. multocida* and agar gel-precipitating antibodies were used.

Mutant isolation. The wild-type *P. multocida* strain used in these studies was isolated from a rabbit with suppurative rhinitis. The isolate is a serotype 3:A *P. multocida* (7, 16) and is streptomycin sensitive. A modification of the procedure described by Adelberg et al. (1) and Wei and Carter (23) was followed to generate streptomycin-dependent mutants. The chemical mutagenesis of the wild-type *P. multocida* was accomplished with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, followed by selection of mutants in media with and without streptomycin (20). Streptomycin-dependent mutants were selected and retested for streptomycin dependency by six successive single-colony isolations. Only genetically stable isolates were tested in mice to determine in vivo stability. The streptomycin-dependent mutants were stored immediately in stock culture agar containing 400 μg of streptomycin per ml (23). A mutant isolate (5M) genetically stable in vitro after six passages and also genetically stable in mice was chosen for rabbit vaccine experiments.

Experimental design. Rabbits were divided into three groups and treated as follows: group 1, nonvaccinated and challenged; group 2, vaccinated and challenged; group 3, vaccinated and nonchallenged. Group 1 animals (four rabbits) were challenged only, and the survivor was humanely killed 3 weeks after challenge; group 2 animals (nine rabbits) were vaccinated intranasally three times with 0.5 ml of the vaccine at weekly intervals, challenged with 0.5 ml of the challenge organism 2 weeks after the third vaccination, and humanely killed in 2 to 3 weeks after challenge; group 3 animals (two rabbits) were vaccinated like group 2 rabbits but not challenged, and they were humanely killed at the time the group 2 rabbits were killed.

Preparation of vaccine. The 5M mutant *P. multocida* organisms were grown in tryptic soy broth with 0.3% yeast extract and 500 μg of streptomycin per ml overnight in a 37°C water-bath shaker. The mutant cells were harvested in 0.01 M phosphate-buffered saline, centrifuged, washed twice, and diluted to 3.0×10^8 cells per ml as determined by the pour plate technique in tryptic soy agar with 0.3% yeast extract (TSAY) and 100 μg of streptomycin per ml. Group 2 and 3 rabbits were inoculated intranasally with the vaccine preparations (0.5 ml per rabbit) with Pasteur pipettes.

Preparation of challenge organisms. Wild-type *P. multocida* organisms were grown confluently on dextrose starch agar plates overnight at 37°C and were harvested in 0.01 M phosphate-buffered saline, centrifuged, washed twice in phosphate-buffered saline, and diluted to 3.6×10^{10} organisms per ml. Group 1 and 2 rabbits were inoculated intranasally with the challenge organisms (0.5 ml per rabbit), using Pasteur pipettes.

Bacterial isolation. The nasal cavities of rabbits were cultured periodically after the third vaccination to determine the prevalence, time of onset, and duration of nasal colonization of the mutant organisms and the virulent challenge *P. multocida*. At necropsy, bacterial cultures were collected from nasal cavities, left and right tympanic bullae, lungs, liver, spleen, genital tract, heart blood, and tissues with gross lesions. Each sample was inoculated onto two TSAY plates, one with streptomycin and the other without streptomycin, and incubated at 37°C for 24 to 72 h. Selected *P. multocida* colonies were tested for streptomycin dependency on TSAY with and without streptomycin.

Pathological evaluation. Rabbits were necropsied and examined for gross lesions. The nasal turbinates, trachea, lungs (all lobes), tympanic bullae, liver, spleen, and genital organs were fixed in 10% neutral buffered Formalin, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin-eosin, and examined for microscopic lesions. Nasal turbinates and tympanic bullae were decalcified before embedding. Microscopic lesions in the lungs, nasal turbinates, and tympanic bullae were scored according to the following schemes.

(i) Lungs. (−) Indicated no lesions recognized; (+) indicated mild lesions characterized by thickening of alveolar septa with polymorphonuclear and mononuclear inflammatory cells; (++) indicated moderate lesions characterized by inflammatory cell infiltration of alveolar and bronchiolar spaces and interstitium; and (+++) indicated severe lesions characterized by extensive necrosis, infiltration of inflammatory cells (predominantly heterophils), and accumulation of fibrin in alveolar and bronchiolar spaces, with or without pleuritis.

(ii) Nasal turbinates. Scores used were: (−) no lesions recognized; (+) mild lesions characterized by submucosal infiltration by inflammatory cells; (++) moderate lesions characterized by migration of inflammatory cells into mucosal epithelium and nasal cavities; and (+++) severe lesions characterized by mucosal necrosis and accumulation of inflammatory cells, predominantly heterophils.

(iii) Tympanic bullae. Bullae were scored as: (−) no lesions recognized; (+) lesions characterized by varying degrees of inflammatory cell infiltration.

To compare groups, the sum of lesion scores for each organ from individual animals within a group was divided by the sum of maximal possible scores to arrive at the group lesion index. A group lesion index of 1.0 is the most severe change possible for a group.

Statistic analysis. Analysis of variance was applied to determine the significant differences in lesion indices between groups.

RESULTS

Clinical observations. No clinical signs of rhinitis, pyrexia, and anorexia were observed in

vaccinated-challenged and vaccinated-nonchallenged rabbits. In contrast, nonvaccinated-challenged rabbits developed suppurative rhinitis, they became anorectic, and their body temperature increased by 0.7 to 4.1°F (ca. 0.4 to 2.3°C) above prechallenge levels. None of the nine vaccinated-challenged animals died, whereas three of four of the nonvaccinated-challenged animals died at 8 to 14 days postinoculation.

Histopathology. No gross lesions were observed in vaccinated rabbits whether challenged or not. Pyothorax, suppurative pericarditis, and suppurative pneumonia, often with pleuritis, were noted in nonvaccinated-challenged animals that died. However, no gross lesions were present in the nonvaccinated-challenged rabbit that was sacrificed. Microscopically, lesions were graded in each experimental group by the scheme outlined above (Table 1). Two of four nonvaccinated-challenged rabbits (group 1) had severe, necrotizing fibrinopurulent bronchopneumonia (Fig. 1), whereas the other two had moderate, nonnecrotizing suppurative bronchopneumonia. All animals in group 1 had mild to moderate suppurative rhinitis, and two had mild tympanitis. Lesions were not recognized in other organs. Six of nine group 2 rabbits (vaccinated-challenged) had focal to diffuse mild interstitial pneumonia (Fig. 2). The alveolar spaces were spared except in one rabbit which had moderate focal lesions in one lobe of the lungs. Three of nine rabbits had no lung lesions, and in none of the nine rabbits were lesions recognized in the nasal turbinates, tympanic bullae, and other organs. No lesions were noted in any organs of the two rabbits (group 3) that were vaccinated but not challenged.

The group lesion index as shown in Table 1 was used to measure the severity of lesions in a group. The group lesion index of pneumonia in group 1 (nonvaccinated-challenged) rabbits was 2.5 times higher than that of group 2 rabbits, and the difference is significant ($P < 0.01$). Similarly, the group lesion index of rhinitis in group 1 rabbits was 0.67 whereas the lesion index of rhinitis was 0 in group 2 rabbits, and the difference is significant ($P < 0.01$).

Isolation of streptomycin-dependent *P. multocida* mutants in nasal cavities. To determine whether the mutant vaccine can colonize and multiply in the nasal cavities, 143 sequential nasal cultures were taken from the 11 vaccinated rabbits (groups 2 and 3) at 1- to 3-day intervals, beginning day 1 after the third vaccination and continuing until the animals were killed. The streptomycin-dependent mu-

TABLE 1. *Microscopic lesions and group lesion indices of vaccinated and nonvaccinated rabbits challenged with a virulent P. multocida (serotype 3:A) strain*

Group	Treatment	Animal	Lung		Nasal turbinate		Tympanic bullae; otitis media
			Pneumonia	Group lesion index[a]	Rhinitis	Group lesion index	
1[b]	Nonvaccinated-challenged	1	+++	0.83[c]	++	0.67[d]	−
		2	+++		+		−
		3	++		+++		+
		4	++		++		+
2[e]	Vaccinated-challenged	5	+	0.26	−	0	−
		6	++		−		−
		7	+		−		−
		8	+		−		−
		9	+		−		−
		10	+		−		−
		11	−		−		−
		12	−		−		−
		13	−		−		−
3[f]	Vaccinated-nonchallenged	14	−	0	−		−
		15	−		−		−

[a] Represent the severity of lesion in the group. A lesion index of 1.0 is the most severe change possible for a group.

[b] Rabbits were challenged intranasally with 1.8×10^{10} virulent *P. multocida* organisms.

[c] Significantly higher ($P < 0.01$) than the pneumonia lesion index of group 2 rabbits.

[d] Significantly higher ($P < 0.01$) than the rhinitis lesion index of group 2 rabbits.

[e] Rabbits were inoculated intranasally with 1.5×10^8 streptomycin-dependent *P. multocida* mutant organisms each time for three times and then challenged intranasally with 1.8×10^{10} virulent *P. multocida* organisms 2 weeks after the third vaccination.

[f] Animals were vaccinated but not challenged.

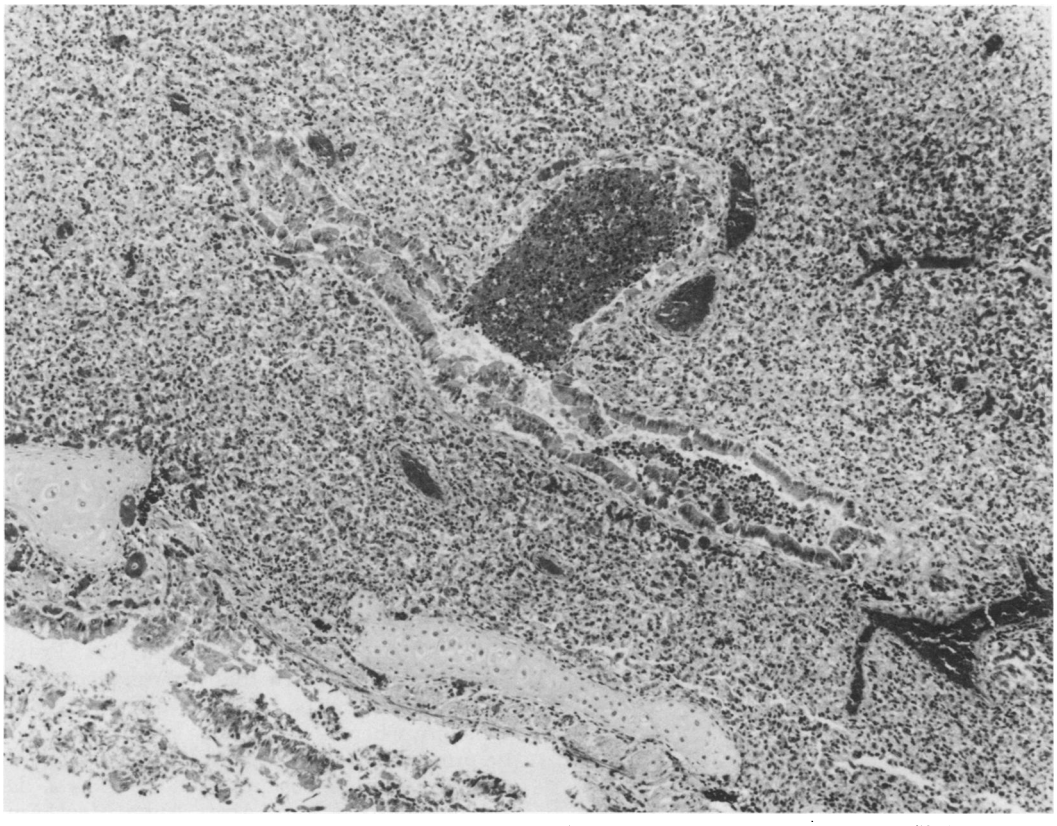

FIG. 1. *Section of lung from a nonvaccinated-challenged rabbit showing severe, necrotizing fibrinopurulent pneumonia (×300).*

tant was isolated only once, at 24 h after the third vaccination in 1 of the 11 rabbits. In vitro testing of the isolated mutant showed retention of streptomycin dependency in TSAY after six passages. It appears that the mutant vaccine does not colonize the nasal cavities of vaccinated rabbits.

Reversion of the streptomycin-dependent *P. multocida* strain in vivo. Mutant revertants (streptomycin independent) were isolated from 5 of the 143 nasal samples (3%) from 2 of the 11 rabbits. All of the positive samples were taken between 4 and 10 days after the third vaccination. One rabbit was persistently colonized by the mutant revertant for 7 days before challenge; however, no mutant revertants were isolated immediately after challenge and thereafter from this rabbit. The isolated mutant revertants were stable genetically after six in vitro passages in TSAY with and without streptomycin.

Nasal colonization of virulent challenge *P. multocida* in vaccinated and nonvaccinated rabbits. One criterion used to evaluate the efficacy of a vaccine is whether the vaccine can prevent the colonization of challenge organisms. Sequential nasal swabs were collected, 1, 2, 5, 8, 12, 17, and 21 days after challenge. The challenge organisms colonized nasal cavities of the four nonvaccinated-challenged rabbits and the nine vaccinated-challenged rabbits, beginning on day 1 after challenge and persisting until the rabbits died or were killed 14 or 21 days later. All positive isolates were the virulent wild-type *P. multocida*. These data suggest that this vaccine does not prevent nasal colonization by the wild-type virulent *P. multocida*.

Isolation of *P. multocida* from various tissues at necropsy. Virulent *P. multocida* organisms were recovered at necropsy from nasal cavities (4/4), lungs (3/4), liver (2/4), genital tract (2/4), and heart blood (2/4) of the nonvaccinated-challenged rabbits (Table 2). Conversely, challenge organisms were isolated at necropsy from nasal cavity (9/9), lung (2/9), and tympanic bullae (1/9), but were not isolated from liver, spleen, genital tract, or heart blood of the vaccinated-challenged rabbits. Neither the streptomycin-dependent mutant nor the mutant revertant was isolated at necropsy from

these rabbits. None of the wild-type challenged organisms, the streptomycin-dependent mutants, or the mutant revertants were isolated at necropsy from the vaccinated-nonchallenged rabbits. It appears that the vaccine can prevent colonization of the wild-type challenge organisms in lungs, liver, spleen, genital tract, tympanic bullae, and heart blood of a majority of rabbits, but not in nasal cavities.

DISCUSSION

We have shown that a streptomycin-dependent serotype 3:A *P. multocida* mutant vaccine is effective in preventing severe respiratory pasteurellosis in rabbits after intranasal challenge with a highly virulent wild-type serotype 3:A *P. multocida* strain. Though the vaccine did prevent the development of severe respiratory lesions in vaccinated-challenged rabbits, six of these rabbits had relatively mild pulmonary lesions characterized by alveolar thickening due to a slight, predominantly mononuclear, inflammatory infiltrate. Since rabbits were killed 2 to 3 weeks postchallenge, it is realistic to assume that these lesions were resolving. Longer-term studies are required to address this specific issue. An important characteristic of the vaccine is that it does not cause clinical disease or microscopic lesions in rabbits when repeatedly introduced intranasally. In addition, the in vivo reversion frequency of the mutant appears to be quite low, and the mutant revertant does not cause clinical disease and lesions in rabbits when repeatedly introduced intranasally (unpublished data).

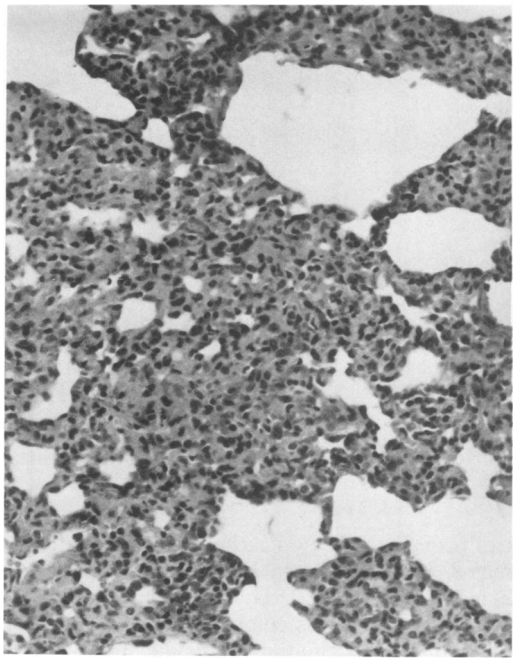

FIG. 2. *Section of lung from a vaccinated-challenged rabbit showing mild lesion, characterized by alveolar thickening due to infiltration of predominantly mononuclear inflammatory cells (×150).*

We chose serotype 3:A *P. multocida* to produce the streptomycin-dependent mutant vaccine and to challenge the animals because 3:A appears to be more virulent than 12:A. Previously, we have shown that typical respiratory pasteurellosis can be induced readily with serotype 3:A, whereas respiratory lesions could not be produced with serotype 12:A unless the animals were immunosuppressed (Y.-S. Lu, D. Ringler, and J. S. Park, Abstr. Annu. Meet. Am. Assoc. Lab. Anim. Sci. 1979, abstr. no. 51.) Consequently, it was reasoned that serotype 3:A *P. multocida* would provide the most severe test of protection provided by the live vaccine.

The intranasal route of immunization and challenge was selected in this study to simulate natural exposure to *P. multocida* (6, 9, 14). In naturally occurring rabbit pasteurellosis, there is a high nasal carrier rate in clinically healthy rabbits (19, 24), and these animals remain susceptible to severe forms of the disease, including septicemia and necrotizing pneumonia. This

TABLE 2. *Isolation of virulent challenge P. multocida (serotype 3:A) from nonvaccinated-challenged and vaccinated-challenged rabbits at necropsy*

Group		Route of challenge	No. positive/no. tested of samples taken from:						
			Nasal cavity	Lungs	Liver	Spleen	Genital tract	Tympanic bullae	Heart blood
1[a]	No	Intranasal	4/4	3/4	2/4	0/4	2/4	ND[b]	2/4
2[c]	Yes	Intranasal	9/9	2/9	0/9	0/9	0/9	1/9	0/9
3[d]	Yes	None	0/2	0/2	0/2	0/2	0/2	0/2	0/2

[a] Rabbits were challenged intranasally with 1.8×10^{10} virulent *P. multocida* organisms.

[b] ND, Not done.

[c] Rabbits were inoculated intranasally with 1.5×10^{8} streptomycin-dependent *P. multocida* organisms each time for three times and then challenged intranasally with 1.8×10^{10} homologous virulent *P. multocida* organisms 2 weeks after the third vaccination.

[d] Animals were vaccinated but not challenged.

may be due to the lack of localized and systemic humoral or cellular immunity or both in the naturally occurring disease (17)., although this has not been thoroughly investigated. This observation in the naturally occurring disease may portend similar problems as sequelae to intranasal inoculation of live vaccines. In our study, protection against challenge with a highly virulent *P. multocida* strain was attained. The vaccine did prevent colonization of the virulent *P. multocida* in the lungs and other organs but not in the nasal cavities. The protective role of systemic and local immune responses induced by the mutant vaccine is under investigation. Whether the vaccine will prevent the carrier state is yet to be determined.

Live vaccines have several advantages over killed vaccines. Live vaccines usually provide quantitatively and qualitatively better protective immunogens because chemical and physical procedures used to prepare killed vaccines may adversely alter immunogens. Also, live vaccines may stimulate longer-lasting immunity because the organisms multiply in the host and thus continue stimulation of the immune system, both locally and systematically (15, 21). We were able to recover the mutant at 24 h from 1 of 11 vaccinated rabbits, but not thereafter. The generation time of *P. multocida* is so short that the mutant may have colonized in a matter of hours and have been cleared or died due to lack of streptomycin by 24 h. Additional studies are required to test this possibility.

Chengappa et al. (8) used serotype 12:A in their live vaccine studies, and their results were similar to our observations with the serotype 3:A *P. multocida* strain. They reported complete protection in rabbits challenged with serotype 12:A organisms, citing no evidence of disease or lesions. The parameters they used to measure protection were clinical signs of disease, mortality, and gross lesions. We have included microscopic lesions, which would detect subtle changes not observable grossly. It appears that our challenge doses of the more virulent serotype 3:A *P. multocida* strain were four times greater than the dose of serotype 12:A organisms used in the experiments of Chengappa et al., thus more severely challenging the protection provided by a vaccine.

In summary, our results indicate that a live vaccine developed from a streptomycin-dependent mutant of serotype 3:A *P. multocida* provides protection in rabbits challenged with virulent serotype 3:A *P. multocida* at a level much higher than expected naturally. The vaccine protected the animals entirely from the development of gross lesions and death, but vaccinated animals did develop very mild microscopic lung lesions, which may have been resolving.

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