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EFFICACY OF VACCINATION OF RABBITS WITH LIVE PASTEURELLA MULTOCIDA, SEROTYPE 7,3,12, INTRANASALLY ALONE AND WHEN COMBINED WITH INTRAMUSCULAR VACCINATION WITH A KILLED P. MULTOCIDA HOMOGENATE

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Abstract

Three trials were conducted to investigate the efficacy of use of a live, serotype 7,3,12 field strain of Pasteurella multocida to vaccinate four to five-week-old rabbits intranasally (IN) in a dose that could be cleared by the rabbits. Trial 1 determined the dose of P. multocida in colony forming units per milliliter that could be cleared by rabbits while still eliciting a local immune response. Local immune response was decided by detection of changes in sIgA from nasal lavage samples. In Trial 2, live vaccine strain P. multocida was introduced into the nares of rabbits. Rabbits clearing the vaccine strain were challenged with heterologous P. multocida, serotype 3. In the third trial, rabbits were vaccinated IN as in Trial 2 or IN with live vaccine strain P. multocida and intramuscularly with a sonicated P. multocida homogenate using DEAE-dextran as an adjuvant. Again, rabbits clearing the vaccine strain were challenged with heterologous P. multocida. None of the vaccination procedures in Trials 2 or 3 proved efficacious in protecting rabbits from challenge.

Introduction

Pasteurella multocida is a common agent of disease in domestic rabbits in commercial rabbitries. Pasteurella has been implicated in respiratory disease and can cause abscesses, otitis media, reproductive organ infections and septicemia (Flatt, 1974). Control of pasteurellosis would eliminate many of the problems encountered by rabbit producers, but experimental use of P. multocida vaccines in rabbits reported to date have provided inadequate protection against colonization and disease, especially from infection with serotypes heterologous to the vaccine strain (Ringler, et al., 1985; Al-Lebban, et al., 1988; Deeb, et al., 1989). In cattle and turkeys, field strains of live Pasteurella have been used as vaccines with good success (Newman, et al., 1980; Olson and Schlink, 1986). Therefore, vaccination with a field strain of live, multi-serotype P. multocida in a dose lower than that needed to cause long-term infection might stimulate an immune response that will prove to be protective against further infection in rabbits.

With this aim, three trials were conducted. The object of Trial 1 was to determine the dose in colony forming units per milliliter (CFU/ml) of P. multocida, serotype 7,3,12, that would not result in long-term infection, but would elicit an immune response. In Trial 2 the object was to find if the live culture of P. multocida introduced into the nares and cleared by rabbits would provide protection against heterologous challenge. In Trial 3 rabbits were vaccinated intranasally (IN) with live P. multocida as well as intramuscularly (IM) with a whole-cell homogenate to boost serum antibody titer and thus, perhaps, provide additional protection from challenge.

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Materials and Methods

Rabbits All rabbits used in these trials were New Zealand White and New Zealand White-Californian crossbred rabbits (Oryctolagus cuniculus) from the University of Arkansas, Department of Animal Sciences' rabbitry. Experimental rabbits were housed individually in stainless steel cages in vertical racks in an isolation room and were fed and watered ad libitum.

Bacteriology The vaccine strain of P. multocida was cross-reactive with antisera in an agar gel precipitin test (AGPT) detecting somatic antigens (Heddleson, et al., 1972) to three common local serotypes (7,3,12) of P. multocida found in rabbits in acute disease states. The culture originated from the lung of a rabbit. The challenge strain, P. multocida serotype 3, originated from the lung of a rabbit with pneumonia and septicemia.

Pasteurella for use as live vaccines and challenge cultures were grown in tryptose broth overnight at 37°C, centrifuged to pellet the cells at 2000 x g for 20 minutes and the pellet resuspended in sterile phosphate buffered saline, pH 7.1-7.2 (PBS). Appropriate dilutions were then made to achieve the desired quantity of bacteria in CFU/ml. Approximate CFU/ml for the particular cultures had been previously determined by making 10-fold dilutions of the suspensions of bacteria as prepared above in PBS, plating 0.5 ml of each dilution onto brain heart infusion agar and incubating for 24 hours at 37°C. Counts of colonies were then made. Before inoculation of rabbits with vaccine or challenge strain of P. multocida suspended in PBS, duplicate 10-fold dilutions and plate counts were carried out as above.

Inocula from lavage or tissues for bacterial culture were plated onto 5% sheep blood agar, incubated from 24 to 48 hours at 37°C, and bacterial growth identified by standard methods.

Preparation of Killed Vaccine A heavy inoculum of P. multocida was swabbed onto two tryptose agar plates which were incubated overnight at 37° C. Bacterial growth was harvested from the plates with a bent glass rod after pipeting 7.5 ml PBS onto the surface. This bacterial suspension was sonicated for 20 minutes at 35% power using the medium tip of a sonic dismembrato. The sonicate was centrifuged three times for 20 minutes each time at 2000 x g retaining the supernatant and discarding the pellet each time. The supernatant was then passed through a sterile 0.45 micron filter. Total protein was found to be 44 µg/ml using Bio-Rad protein reagent and bovine serum albumin standards (Bio-Rad Laboratories, Richmond, CA). To 5 ml of supernatant was added 0.125g diethylaminoethyl-dextran, chloride form (DEAE-dextran, Sigma Chemical Company, St. Louis, MO). To five ml of sterile PBS was added 0.125g DEAE-dextran to use to sham inoculate control rabbits.

ELISA for serum IgG and nasal sIgA ELISA was performed as previously reported on sera harvested from the blood to detect IgG against P. multocida (Glass and Beasley, 1989).

ELISA was performed on nasal lavage fluid to detect secretory IgA against P. multocida to determine if a local immunological response had occurred. As no standards were available, means of absorbances of pre-inoculation and post-inoculation nasal lavage fluids were calculated for each experimental group, and statistics were performed (Wilcoxon Signed Rank Test) to determine if there was a significant difference between these values within an experimental group. To perform the test, antigen-coated plates prepared as those used to detect serum IgG were used. No lavage fluid was added to three wells on each plate for use as conjugate controls. Each sample of undiluted nasal lavage fluid was added to wells at 50 µl/well in triplicate and incubated at room temperature for one hour. Plates were washed three

times with PBS containing 5% tween-20. Goat anti-rabbit sIgA (Organon Teknika Corp., West Chester, PA) diluted 1:2000 in PBS was added to all wells at 50 µl/well and incubated at room temperature for 1.5 hours. Plates were washed three times as above, and peroxidase-conjugated rabbit anti-goat IgG (Organon Teknika Corp.) diluted 1:10,000 in PBS was added to each well at 50 µl/well and incubated for two hours. Plates were washed as above, and O-phenylenediamine substrate dissolved in phosphate-citrate buffer, pH 5.0, was added at 50 µl/well. Plates were incubated at room temperature for 20 minutes, and 25 µl 2.5M sulfuric acid was added to all wells to stop the reaction. Absorbance of wells was read on an Agrichek Minireader II (Dynatech Laboratories, Inc., Alexandria VA) at OD_{490}. Average absorbance of conjugate control wells was subtracted from average absorbances of each lavage sample.

Trial 1: Four-week-old rabbits were weaned and transported to the isolation room. Two days later they were anesthetized with ketamine hydrochloride (30mg/kg), blood was collected via heart puncture and nasal lavages were performed by injecting 2 ml sterile PBS into the nares with a sterile syringe and collecting the lavage fluid in sterile petri plates. Sera were harvested from the blood and frozen at -20°C. A portion of the lavage fluid was used for bacterial culture, and the remainder was frozen at -20°C.

Two days later, after determining there were no *P. multocida* in the lavage fluid, rabbits were divided into groups and inoculated with 0.05 ml per nostril with dilutions of *P. multocida* suspended in sterile PBS. Since each rabbit received 0.1 ml of each dilution of bacteria, the following amounts reflect the actual CFU of bacteria received rather than the dilution made which would be ten-fold more. Group A, n=5, received a dose of $9x10^6$ CFU, group B, n=5, received a dose of $9x10^5$ CFU, group C, n=4, received $9x10^4$ CFU, group D, n=5, received $9x10^3$ CFU and group E, n=5, received $9x10^2$ CFU. Four rabbits were uninoculated controls.

Dead or moribund rabbits were necropsied when identified. Eleven days post-inoculation, remaining rabbits were anesthetized with ketamine hydrochloride, blood was collected and nasal lavages performed as above. Rabbits were then euthanized in a CO_2 tank, and inoculum was collected from tissues to culture for bacteria from the lungs, tracheas, middle ears, livers and spleens with calcium alginate swabs.

ELISAs were performed to detect anti-*Pasteurella* IgG from the sera and sIgA from nasal lavage fluids.

Trial 2: Handling of rabbits previous to vaccination was conducted as in Trial 1. After determining that there were no *P. multocida* in the nasal lavage fluid, rabbits were divided into three groups. Groups 1 and 2 were uninoculated. Rabbits in Group 3, n=12, were inoculated with 0.05 ml/nostril with $3x10^7$ CFU/ml vaccine strain of *P. multocida*. Seventeen days post vaccination, rabbits were anesthetized with ketamine hydrochloride, blood was collected via heart puncture and nasal lavages were performed as previous with a portion used for bacterial culture and the rest frozen as above. After removing rabbits retaining *P. multocida*, Groups 3, now n=7, and 2, n=7, were challenged with *P. multocida*. Each rabbit was inoculated with 0.05 ml in each nostril using a suspension of *P. multocida* in sterile PBS of $7.6x10^8$ CFU/ml. Group 1, n=5, was an uninoculated control group. Twelve days post-challenge, all rabbits were bled, nasal lavage fluid collected and necropsy performed as in Trial 1.

ELISAs were performed to detect serum IgG and sIgA from lavage fluid against *P. multocida*.

Trial 3: Five-week-old rabbits from seven litters were weaned and transferred to the isolation room. Rabbits were bled, nasal lavages done as in Trials 1

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and 2, and the litter from which each rabbit came was noted. After screening for P. multocida in the lavage fluid, rabbits were randomly divided into groups and vaccinated.

Vaccination Rabbits were vaccinated as follows: Group 1, n=5, unvaccinated control; Group 2, n=5, DEAE-dextran control, inoculated IM in biceps femoris with DEAE-dextran solution only; Group 3, n=5, challenged control with DEAE-dextran control, vaccinated IM with 0.5ml DEAE-dextran only; Group 4, n=5, unvaccinated, challenged control; Group 5, n=10, vaccinated in each nostril with 0.05 ml P. multocida, $4.2x10^6$ CFU/ml (actual dose=$4.2x10^5$CFU); and Group 6, n=10, vaccinated with live P. multocida as above and IM with 0.25mL sonicated Pasteurella and DEAE-dextran solution.

Challenge Three weeks post-vaccination rabbits were bled and nasal lavages performed as previously. Rabbits that had cleared the live vaccine were challenged with serotype 3 P. multocida. Five microliters of $5x10^{10}$ CFU/ml P. multocida in sterile PBS was inoculated into each nostril of rabbits in Groups 3 (n=5), 4 (n=5), 5 (n=5) and 6 (n=8).

Two weeks post-challenge blood was collected and nasal lavages and culture for bacteria performed as previously. Three weeks post-challenge rabbits were anesthetized and bled as before, euthanized in a CO_2 chamber and necropsied. A lung lavage was carried out by injecting 10 ml sterile PBS from a 10cc sterile syringe through a food intubation canula inserted into a slit made near the distal end of the trachea. Fluid was then pulled back into the syringe, decanted into a sterile tube and a portion used for inoculum to culture for bacteria. Middle ears and tracheas were swabbed with sterile calcium alginate swabs for bacterial culture. Tissues, including nasal turbinates and lungs were collected in 10% buffered formalin, embedded in paraffin, sectioned at 6 microns and stained with hematoxylin and eosin for histological examination.

Serotyping for somatic antigens using an AGPT was carried out on some P. multocida isolates from necropsy, each from an individual rabbit, including five from Groups 3 and 4 (one from middle ear and 4 from lungs), two from Group 6 (both from lungs) and three from Group 5 (one middle ear and two lungs).

ELISAs were performed to test for serum IgG and nasal lavage sIgA against P. multocida. A Kruskal-Wallis test was used to determine if a significant difference ($p < 0.05$) existed between mean ELISA absorbances for IgG in post-challenge sera from Groups 5 and 6.

Results and Discussion

Trial 1 Seven days post-inoculation, one rabbit in Group E was found dead and another from Group D was moribund. Neither had consumed water or food for 24 hours. Pasteurella multocida was isolated from lungs, tracheas, livers and middle ears of both rabbits indicating a septicemic condition.

In Group A, no P. multocida was isolated from any tissues cultured In Group B, P. multocida was isolated from 2/5 rabbits, one from sinuses only and one from sinuses and middle ears. In Group C no P. multocida was isolated. In Group D, P. multocida was only isolated from the rabbit that died. In Group E, no P. multocida was isolated except from the sick rabbit.

ELISA Results None of the sera collected pre- or post- inoculation were positive for IgG against P. multocida.

Differences in average absorbances in ELISA for sIgA between pre- and post- inoculation lavage samples in Group A were significantly different at $p < 0.1$. There was no significant difference in any other group.

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Discussion Eighty-three percent of rabbits were able to clear Pasteurella administered in varying doses. Possible reasons for rabbits continuing to harbor P. multocida are decreased genetic resistance and inability of a rabbit's immune system to recognize the bacteria when given in low doses allowing the bacteria to rapidly multiply and then overwhelm the rabbit's natural immune functions. This seems a possibility of what may sometimes happen in the field.

From these results, it appeared that only the higher dose of P. multocida induced a significant sIgA response in rabbits, so this dose was chosen to use for vaccination.

Trial 2 Of the 12 rabbits vaccinated with live P. multocida, five harbored P. multocida in their sinuses 17 days after vaccination.

Twelve days post-challenge P. multocida was isolated from 5/7 rabbits in Group 3 and from 4/7 rabbits in Group 2. From 4/7 infected rabbits in Group 3 P. multocida was isolated from the sinuses only and in 1/7 from the sinuses, trachea and middle ear. In Group 2 one rabbit harbored Pasteurella in the sinuses and lungs, and the remaining rabbits were infected in the sinuses only. No Pasteurella was isolated from any tissues of rabbits in the unchallenged control group.

Gross Pathology Minimal lesions were observed, and differences in gross pathology among all rabbits were unremarkable.

ELISA Results No rabbits tested positive for IgG against P. multocida from any sera collected.

There were no significant differences within any group for sIgA against P. multocida in lavage samples when testing for differences in means between sera collected 17 days post-vaccination and pre-vaccination. When testing for differences in mean absorbances between post-challenge and pre-vaccination lavage samples at 12 days post challenge, there were significant differences ($p < 0.05$) in both the challenged control and the vaccinated group.

Discussion Secretory IgA response was not detected in rabbits 17 days post-vaccination from the nasal lavage samples but was demonstrated at 11 days post-inoculation in Trial 1. An assumption could be made that sIgA response did occur, but sIgA had fallen to an undetectable level when the lavage samples were taken.

DiGiacomo, et al. (1990), detected serum IgG within three weeks after initial culture of P. multocida. If live vaccination had elicited a humoral immune response, IgG against Pasteurella would most probably have been detected in the sera from post-challenge rabbits in Group 3. Therefore, it is assumed that no IgG response occurred due to vaccination.

Over 40% of rabbits were unable to clear P. multocida on initial introduction of the bacterium into the nares, and, of the rabbits that cleared the initial dose, only 29% were able to clear Pasteurella when challenged with a heterologous strain. Apparently exposure to P. multocida that elicits a local immune response does not enchance protection to later infection by Pasteurella.

Trial 3 Five of ten rabbits from Group 5 and 2/10 in Group 6 harbored P. multocida in their sinuses three weeks post-vaccination. Three of seven rabbits that did not clear the bacteria were from the same litter.

Two weeks post-challenge, P. multocida was isolated from nasal lavage fluid in 9/10 rabbits from Groups 3 and 4 (challenged controls), 5/5 rabbits in Group 5 and 6/8 rabbits in Group 6. At necropsy (three weeks post-challenge), in 5/9 Pasteurella-positive rabbits in Groups 3 and 4, the bacterium was isolated from multiple tissues. This was the case in 3/5 rabbits in Group 5 and 3/6 rabbits in Group 6. No Pasteurella was isolated

from any rabbits in Groups 1 and 2.

ELISA Results Two rabbits, both in Group 1, tested positive for serum IgG against P. multocida previous to vaccination. This was most likely due to maternal antibody, as these rabbits tested negative for the remainder of the experiment. All other rabbits tested negative for serum IgG against P. multocida previous to vaccination. All sera from rabbits three weeks post-vaccination were negative for IgG against P. multocida. Two weeks post-challenge, sera from rabbits in Groups 1, 2 and 3 were negative while 3/5 rabbits in Group 5 and 6/8 rabbits in Group 6 tested positive. At three weeks post-challenge, Groups 1, 2 and 3 were negative for IgG against P. multocida, while 1/5 rabbits in Group 4, 3/5 rabbits in Group 5 and 6/8 rabbits in Group 6 were test positive. Mean absorbances from Group 6 from sera tested at two and three weeks post-challenge were higher than those in Group 5, although the differences were not statistically significant at $p < 0.05$ (Figure 1). The relationship between P. multocida isolation and ELISA results are listed in Table 1.

Statistical significance ($p < 0.05$) was shown for differences in mean absorbances between post-vaccination and pre-vaccination, post-challenge and pre-vaccination and post-challenge and post-vaccination for sIgA in nasal lavage fluid in Group 6 rabbits. No other groups showed a significant difference.

There were no differences in isolation of P. multocida in challenged rabbits nor increased absorbances in ELISAs due to detection of serum IgG against P. multocida in Group 2 and 3 rabbits which received inoculations of DEAE-dextran solution with no antigen.

Gross Pathology Minimal lesions were observed in rabbits in Groups 1 and 2. In Group 3, one rabbit had a purulent exudate in one middle ear and a pus-filled area of necrosis in one lung. Another rabbit from this group had mild conjunctivitis. In Group 4, two rabbits had purulence unilaterally in the middle ear. In Group 5, three rabbits contained purulent exudate in their sinuses and one rabbit also had pus in one middle ear. One rabbit in Group 6 had purulence in the sinuses and one middle ear.

Histopatholgy In the lungs there were varying degrees of pneumonitis. These lesions varied from small foci of inflammatory cells and macrophages with proliferation of alveolar cells to diffuse involvement of large areas of the lung in which there was a thickening of alveolar walls by proliferation of macrophages. While there was little accumulation of fluid, heterophils and macrophages were present in alveoli and lymphoreticular cell accumulations occurred around blood vessels and bronchi and were scattered in alveolar walls through the lung. Nodular collections of lymphocytes occurred in the lamina propria of bronchi and bronchials.

In the sinuses and nasal passages lesions were mild in most rabbits and were characterized by the occurrence of nodular collections of lymphocytes in the epithelium of the turbinates. These lesions varied greatly in degree. There was little change in the epithelium.

Average lesion scores for treatment groups are given in Table 2.

AGPT Results Serotyping of isolated cultures of P. multocida following necropsy revealed that most were somatic antigen serotype 3 (the same as the challenge strain). In Group 5, a P. multocida isolate from the lung was serotype 12,7,4. In Group 6, a P. multocida from the lung of one rabbit did not react with any antisera in the AGPT, nor did sera from this rabbit test positive for IgG against P. multocida in the ELISA. Pasteurella isolates from the lung and middle ear of another rabbit in Group 6 proved to be serotypes 12,7,4 and 12, respectively. This information strongly suggests that these

rabbits harbored *P. multocida* before the experiment was begun and in tissues other than the sinuses as no *P. multocida* was isolated previous to vaccination or post-vaccination from nasal lavage fluids.

Discussion Some rabbits in Trial 1 became infected or ill when given low doses of *P. multocida*, and a high percentage of rabbits given a slightly higher dose as in Trials 2 and 3 were unable to clear the bacteria. Apparently, finding a dose of live *P. multocida* that can be cleared by most rabbits is unfeasible.

Although inoculation with a killed homogenate of *P. multocida* appears to have increased the serum IgG against that bacterium and to have possibly helped boost presence of sIgA, it did not provide sufficient additional protection from infection with *P. multocida* in the sinuses or spread of the bacterium to other tissues.

It appears from the serotyping results in Trial 3 that in using young rabbits raised in a commercial-type rabbitry as subjects for experiments dealing with vaccination for *P. multocida*, investigators need to consider the possibility of rabbits harboring the bacterium in tissues inaccessible upon initial screening of live animals.

Providing protection to rabbits against *P. multocida* has proven to be a difficult task for researchers. Some rabbits have shown an inherently better ability to resist infection with *Pasteurella*, and, if isolated from other animals, can be kept free of pasteurellosis. Unfortunately, most rabbit producers don't have facilities to effectively isolate rabbits. Perhaps the solution lies in vaccination of rabbits naturally resistant to *P. multocida*.

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Table 1. Detection of serum IgG against *Pasteurella multocida* in rabbits culture positive and negative for *P. multocida* in Trial 3

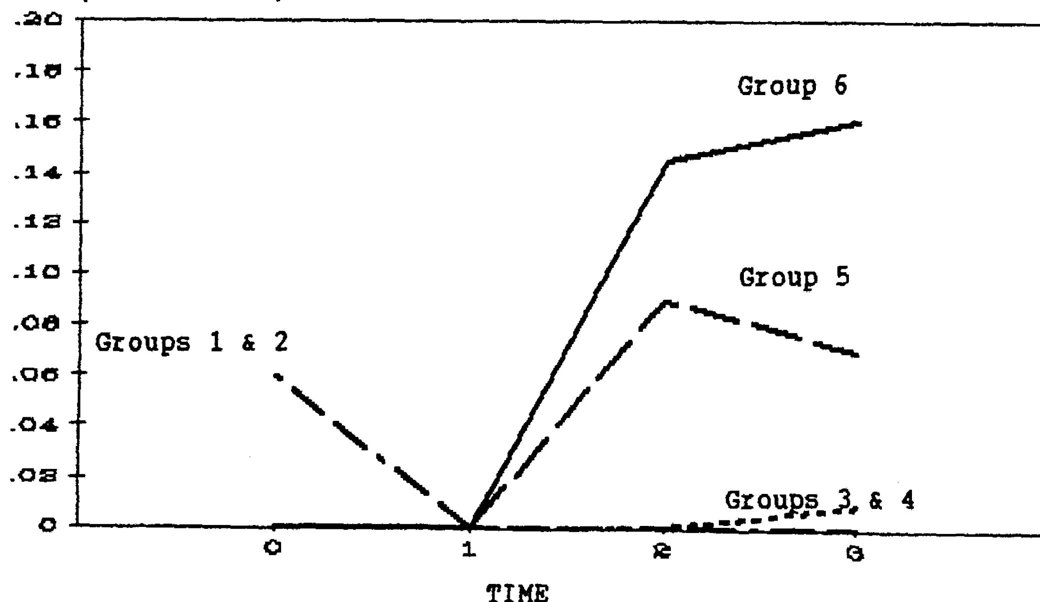
Culture	IgG	Weeks Post-Chall	Group 5 live vaccine IN	Group 6 live vaccine IN & killed IM
+	+	two	3	3
+	+	three	3	5
+	-	two	2	2
+	-	three	2	1
-	+	two	0	2
-	+	three	0	1
-	-	two	0	0
-	-	three	0	1

Table 2. Mean lesion scores* of rabbits in Trial 3.

	lungs	nasal turbinates
Groups 1 & 2	1.6	0.4
Groups 3 & 4	2.0	1.4
Group 5	3.5	2.0
Group 6	2.0	1.3

*Most severe lesions=4.0, no lesions=0.0

Figure 1. Changes in mean ELISA absorbances due to detection of serum IgG in groups of rabbits prevaccination (Time 0), post-vaccination (Time 1) and two and three weeks post-challenge (Times 2 & 3) in Trial 3



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