Evaluation of a vaccine against *Mannheimia haemolytica* and *Pasteurella multocida* in sheep

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Of the 20 currently identified species in the genus Pasteurella, *Mannheimia haemolytica* (formerly called *Pasteurella haemolytica*) and *Pasteurella multocida* are the most important respiratory pathogens affecting domestic ruminants, especially in sheep and cattle, together causing fibrinous, necrotic pneumonia. This disease, commonly called "shipping fever", is a leading cause of economic loss in the sheep industry. Factors such as transportation, viral infection, and overcrowded housing, may predispose the opportunistic infection by the pathogen. At present, several commercial vaccines have been developed to control pasteurellosis in sheep, including types such as bacterins, live attenuated, leukotoxin, capsule, lipopolysaccaride, subunit vaccines, sodium salicilate extract and potassium thiocyanate. Previous studies have shown that effective specific antibody response against *M. haemolytica* whole-cell antigen could be achieved via vaccination. However, for *P. multocida*, vaccines did not induce strong specific antibody response and some reports even suggested an increase in severity of infection by *P. multocida* after vaccination.

In this study, the efficacy of an autogenous vaccine made from antigens from both *M. haemolytica* and *P. multocida*, was evaluated by measuring specific serum antibody titers produced against both bacteria in immunized sheep. Lambs were vaccinated on Nov. 23 and sera were obtained from all control (unvaccinated) and vaccinated animals on Nov. 16, Nov. 30, Dec. 7 and Dec. 14. The results showed that the vaccine induced significant antibody response against *M. haemolytica* in both ewes and rams after 7 days post vaccination. For the response to *P. multocida*, specific antibodies were induced in ewes; however, the vaccine failed to stimulate specific antibody production against *P. multocida* in rams.

Acknowledgements

This work was completed under the supervision of Dr. Jerrie Gavalchin at Cornell University. Without her patient guidance and consistent support, the completion of this work could not be possible.

We would like to thank Dr. Michael L. Thonney for providing the lamb serum samples and Dr. Yung-Fu Chang for providing the vaccine.

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1. Introduction

Mannheimia haemolytica (formerly called *Pasteurella haemolytica*) and *Pasteurella multocida* are known to be the most prominent pathogens in the family Pasteurellaceae causing great economic losses in the domestic animal industry **[1] [2]**. Of the various *M. haemolytica* strains, *M. haemolytica* serotype A2 is the major pathogen responsible for diseases in sheep, causing a fibrinous, necrotic pneumonia, also called "shipping fever". But this strain was not as well-characterized as strain A1, which mainly causes pneumonia in cattle **[2]**. Factors such as transportation, viral infection, and overcrowded housing, may also facilitate the opportunistic infection by the pathogen **[3]**. *P. multocida* was found present in lung lesions of sheep affected by pneumonia as well **[4] [5]**, but due to its high antigenic variability, its molecular pathogenic mechanisms still remain to be investigated **[6]**.

Our experiment was part of a long-term project to develop and tested the effectiveness of an autogenous vaccine to prevent pneumonia in young lambs. The vaccine was developed from antigens of both *M. haemolytica* and *P. multocida*. The goal of this experiment was to test this vaccine's efficacy in inducing specific antibody responses to the two bacteria in both ewe lambs and ram lambs.

Currently there are two types of serological tests for evaluating vaccine efficacy against *M. haemolytica* and *P. multocida*. One is the leukotoxin neutralization test (LNT) and the other is an ELISA test. **Mosier et al. 1986 [14]** showed that ELISA was a more convenient and faster tool compared with LNT. In this experiment, we measured the level of serum antibodies against both *M. haemolytica* and *P. multocida* using a whole-cell ELISA assay **[15]**.

2. Review of Literature

At present, several commercial vaccines have been developed to control pasteurellosis in sheep. For example, Once PMH, One Shot, Presponse, use antigens including bacterins, leukotoxin, capsule, different surface antigens and lipopolysaccaride **[7] [8] [15]**. However, researchers found that each vaccine induced variable levels of protection in response to different bacteria strains. **Diker et al. 2000 [15]** reported monovalent combined immunogens induced high antibody titers against homologous serotypes, but low titers against heterologous serotypes; and antigens from *M. haemolytica* Serotype A1 and A7 were found to be more antigenic than A2 and T4. There were also species differences in vaccine efficacy with variability in the induction of protective response in sheep and cattle seen **[12]**.

Previous studies have shown that effective specific antibody response against *M. haemolytica* whole-cell antigen could also be achieved via vaccination. **Mehmet Akan et al. 2006 [9]** reported One Shot Ultra 8 vaccine induced specific antibody production against *M. haemolytica* using ELISA tests, with higher specific antibody titers in vaccinated groups than in control groups. In addition, LNT (leukotoxin neutralization test) also revealed a higher specific antibody titer in vaccinated sheep compared to unvaccinated sheep. The study also evaluated lung lesion scores of both vaccinated and control animals and statistically significant differences ($P<0.001$) were observed between the vaccinated and control groups, with fewer lesion in the vaccinated groups. **S. Srinand et al 1996 [10]** examined the efficacy of four vaccines: ‘One Shot’ (SmithKline Beecham, West Chester, PA, a bacterin-toxoid), ‘Presponse’ (Langford Laboratories, Guelph, Ontario, an Lkt-rich culture supernatant), ‘Once PMH’ (BioCor Inc., Omaha, NE, a modified live vaccine), and ‘Septimune’ (Fort Dodge laboratories, Fort Dodge, IA, an outer membrane extract). The study showed that ‘One Shot’, and ‘Once PMH’ vaccinated animals showed a significant ($P < 0.05$) increase in antibody levels against leukotoxin at 28 days post vaccination. ‘Once PMH’ vaccinated animals also showed significant ($P < 0.05$) increase in antibody levels against IROMPs (iron regulated outer membrane proteins) at 28 days after vaccination compared to the other groups. ‘Presponse’, ‘Once PMH’, and ‘One Shot’ vaccinated animals showed a significant ($P < 0.05$) increase in antibody levels against CP (capsular polysaccharide) over time. These groups also had significantly higher antibody levels against CP, compared to controls and ‘Septimune’ vaccinated at 14 and 28 days ($P < 0.05$).

However, other studies suggested that vaccination did not effectively protect sheep from infection. **Chandrasekaran S et al. 1991 [11]** tested an oil adjuvant vaccine incorporating locally isolated strains of *M. haemolytica* type 7 and *P. multocida* types A and D, and found that the vaccine significantly reduced the lung lesions at $P<0.05$ level in vaccinated sheep compared with the control groups when all animals were challenged with *M. haemolytica* alone. However, when animals were challenged with *P. multocida* or the combination of *M. haemolytica* and *P. multocida*, the vaccine failed to induce significant levels of protection in vaccinated animals. Interestingly, the study also compared the efficacy of a commercial vaccine named Carovax (Wellcome Laboratories, UK, antigen information unavailable, protecting sheep and pigs from *M. haemolytica*) with this oil adjuvant vaccine containing locally isolated bacteria strains and found the Carovax vaccine did not produce any significant reduction in lung lesions caused by *M. haemolytica* and/or *P. multocida.* This observation indicated that geological differences in terms of isolation of bacteria strains could also influence the efficacy of vaccine, which would further complicate the development of a vaccine which could protect sheep from diverse strains of the bacteria. **E. F. Cassirer et al. 2001 [12]** tested whether a combination of an experimental *P. trehalosi* and *M. haemolytica* vaccine and a commercially-available bovine *P. multocida* and *M. haemolytica* vaccine would increase lamb survival following a pneumonia epidemic. They found that lamb survival differed among flocks (range 22% to 100%), and, unexpectedly, survival of lambs born to vaccinated ewe lambs was lower (P = 0.08) than survival of lambs born to unvaccinated ones. Antibody titers were high in ewes prior to vaccination, and vaccines failed to enhance antibody titers in treated ewes. None of the lambs born to vaccinated ewe lambs survived. These data suggested that, using existing technology, vaccinating ewe lambs following pneumonia epidemics would have little chance of increasing neonatal survival and population recovery, for example, in bighorn sheep.

Currently there is no effective vaccine that protects sheep from strains of both *M. haemolytica* and *P. multocida* or strains derived from different geological origins. However, given the significant economic losses due to sheep pneumonia that is caused by these two bacteria, it is necessary to develop such a vaccine for the benefits of sheep industry. It is likely that this can be realized. **Jerry K. McVicker et al. 2002 [13]** indicated the development of an effective vaccine could be possible based on the fact that animals that were naturally infected did develop resistance to subsequent infection.

3. Materials and Methods

3.1 Animals

Sheep were from flocks at the Cornell Sheep Farm, composed of Dorsets and Finnsheep. Twenty ewe lambs and twenty ram lambs born in August 2005 were randomly selected and weaned on October, 2005. Blood samples were obtained from each animal on November 16 before vaccination. On November 23, 10 randomly selected ewes and 10 randomly selected rams were vaccinated, while an additional 10 animals of each sex comprised control groups. Blood samples were obtained from both control and vaccinated animals on November 30, December 7 and December 14.

3.2 *M. haemolytica* and *P. multocida* vaccine preparation

The vaccine used in this study was prepared by Dr. Yung-Fu Chang at the Animal Health Diagnostic Center of Cornell University. Isolates of *Mannheimia haemolytica* and *Pasteurella multocida* were obtained from Cornell lambs dying of pneumonia. They were grown in culture and then killed and enriched for leukotoxin (the toxin produced by the bacteria that causes lung damage) to create the bacterin. The vaccine was expected to produce antibodies and prime cell-mediated immune responses against both bacterial antigens and leukotoxin.

3.3 *M. haemolytica* and *P. multocida* antigen preparation for ELISA tests

We prepared whole-cell antigens of both bacteria for ELISA testing. Both bacteria were streaked onto BHI (Brain heart infusion broth) agar and then collected separately into formaldehyde (4%) containing peptone water. After being washed three times in phosphate buffered saline (PBS), the cell collection was diluted to10^8 bacteria/ml for use in the assay **[9] [15]**.

3.4 ELISA tests for specific antibody

Bacteria prepared in 3.3 were diluted to 10^6 cells per ml into carbonate coating buffer (0.05M Na_2CO_3, 0.05M $NaHCO_3$, pH 9.6). Each was then dispensed into the wells of an Immunolon 1B plate (Thermo Fisher Scientific Inc, Waltham, MA) in a volume of 50µl per well. After overnight incubation at 4°C, the plates were washed twice with PBS containing 1% Tween20 and twice with PBS. Then serum samples that had been serially diluted from 1/40 to 1/1280 into PBS containing 0.05% Tween 20, were added in duplicate to the wells, at 50µl per well. After overnight incubation at 4°C, the plates were washed as described above. Then 50µl anti-sheep IgG conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) diluted 1:1000, was added to each well **[9] [15]**. The plates were incubated overnight again and then washed as before. Finally, p-nitrophenyl phosphate substrate (Sigma Chemical Co., St Louis, MO), with 1mM of $MgCl_2$ was added to each well in a volume of 50µl per well. Absorbance values were read at OD 405 nm after 20 min incubation by an ELISA reader.

3.5 Statistical analysis

Specific antibody titers in control and vaccinated sera were evaluated. We used JMP data-analysis software to analyze our data; Fit Model tests examined the regression relationship between two variables. The null hypothesis of the Fit Model is that two variables are independent of each other. When $P<0.05$, the null hypothesis is rejected.

4 Results and Discussions

4.1 Determination of specific antibody titers

We did not have access to serum samples from known affected lambs; therefore we had to determine ELISA titers using another approach. We reasoned that if the vaccine was effective in inducing specific antibody, we would expect that vaccinated animals would have higher specific antibody titers than control animals would. Similar to the methods described by **Mehmet et al. [9]**, we determined the specific antibody titer as the reciprocal of the last dilution of each serum sample that gave an OD that was greater than three standard deviations above the mean value of the OD of sera from control sheep at the 1: 1280 dilution (Table 1-8).

Table 1 Development of specific antibody titers against *M. haemolytica* over the four sampling dates in control ewes

Dates/Control ewes' ID	1	2	5	9	10	12	15	19	24	35
Nov. 16	160	640	640	320	320	640	160	640	640	160
Nov. 30	160	80	320	160	160	320	160	80	640	80
Dec. 7	40	40	640	160	80	640	40	80	80	40
Dec. 14	40	160	80	80	80	160	80	160	640	80

Table 2 Development of specific antibody titers against *M. haemolytica* over the four sampling dates in vaccinated ewes

Dates/Vaccinated ewes' ID	17	20	21	23	25	26	27	30	34	37
Nov. 16	320	1280	1280	640	320	320	320	320	160	320
Nov. 30	640	640	1280	1280	1280	1280	1280	1280	1280	1280
Dec. 7	160	320	1280	1280	1280	1280	640	160	40	320
Dec. 14	1280	640	640	640	640	640	640	320	640	320

Table 3 Development of specific antibody titers against *M. haemolytica* over the four sampling dates in control rams

Dates/Control rams' ID	3	7	13	14	28	29	31	33	38	40
Nov. 16	160	80	320	40	320	320	320	40	80	40
Nov. 30	40	160	640	160	320	320	320	80	40	40
Dec. 7	160	160	160	160	160	160	160	160	320	160
Dec. 14	320	40	640	160	320	160	320	320	320	80

Table 4 Development of specific antibody titers against *M. haemolytica* over the four sampling dates in vaccinated rams

Dates/Vaccinated rams' ID	4	6	8	11	16	18	22	32	36	39
Nov. 16	1280	1280	640	640	640	320	320	640	320	320
Nov. 30	1280	1280	1280	1280	1280	1280	1280	1280	1280	1280
Dec. 7	640	40	40	40	40	40	40	40	40	40
Dec. 14	1280	1280	1280	1280	1280	320	1280	1280	1280	1280

Table 5 Development of specific antibody titers against *P. multocida* over the four sampling dates in control ewes

Dates/Control ewes' ID	1	2	5	9	10	12	15	19	24	35
Nov. 16	640	320	640	320	640	320	160	640	320	320
Nov. 30	160	160	320	160	160	320	320	160	640	320
Dec. 7	40	80	640	160	80	160	40	80	320	160
Dec. 14	80	320	320	160	160	320	320	640	320	320

Table 6 Development of specific antibody titers against *P. multocida* over the four sampling dates in vaccinated ewes

Dates/Vaccinated ewes' ID	17	20	21	23	25	26	27	30	34	37
Nov. 16	640	640	320	1280	320	1280	1280	640	640	640
Nov. 30	1280	1280	640	1280	640	1280	640	640	1280	1280
Dec. 7	40	160	160	320	160	320	80	160	80	160
Dec. 14	1280	1280	1280	1280	1280	640	1280	1280	1280	1280

Table 7 Development of specific antibody titers against *P. multocida* over the four sampling dates in control rams

Dates/Control rams' ID	3	7	13	14	28	29	31	33	38	40
Nov. 16	40	80	80	80	40	80	80	40	320	40
Nov. 30	160	320	640	320	320	640	160	160	160	80
Dec. 7	160	160	640	320	320	640	160	40	80	160
Dec. 14	640	160	640	160	640	320	320	320	320	320

Table 8 Development of specific antibody titers against *P. multocida* over the four sampling dates in vaccinated rams

Dates/Vaccinated rams' ID	4	6	8	11	16	18	22	32	36	39
Nov. 16	320	320	320	320	160	320	320	320	160	80
Nov. 30	320	320	320	320	640	160	160	320	320	320
Dec. 7	1280	80	320	80	160	80	40	160	80	40
Dec. 14	1280	160	320	160	160	160	320	80	320	160

4.2 Data analysis and discussion

ELISA titer values were analyzed by the Fit Model tests of JMP. We examined regression relationship of specific antibody titer with each of the three variables: treatment (control or vaccination), sex (ewes or rams) and dates of sampling (Nov. 16, Nov. 30, Dec. 7 and Dec. 24).

4.2.1 Vaccine efficacy against *M. haemolytica*

4.2.1.1 Factors affecting specific antibody titers against *M. haemolytica*

Models showed that the vaccine did induce significantly (P<.0001<.05) increased levels of specific antibodies against *M. haemolytica* in vaccinated lambs over unvaccinated animals. We also found that there was a significant regression relationship (P<0.0001<0.05) between the dates of sampling and specific antibody titers. However, there was no difference in specific antibody titers (P=0.9067>0.05) between ewe lambs and ram lambs.

4.2.1.2 Discussion of vaccine efficacy against *M. haemolytica*

(1) Interaction between treatment and dates of sampling in Ewes

According to Figure 1, after vaccination on Nov. 23, specific antibody titers peaked on Nov. 30, and were much higher than the titers on Nov. 16. However, the titer decreased sharply from Nov. 30 to Dec. 7, and then remained constant to Dec. 14. The specific antibody titer levels on Dec. 7 and Dec. 14 were still about 100 titer units higher than that of ewes on Nov. 16 before vaccination. For control ewes, there was a slight decrease in specific antibody titer from Nov. 16 to Nov. 30, and then the titer remained constant during the remaining three sampling periods.

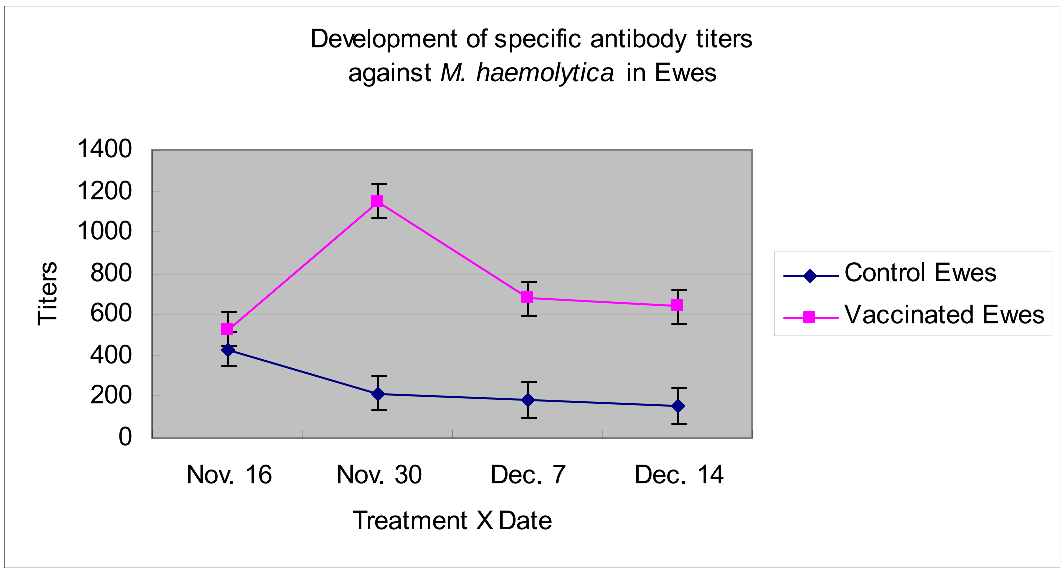

Figure 1 Development of specific antibody titers against *M. haemolytica* in ewes on Nov. 16, Nov. 30, Dec. 7 and Dec. 14.

To understand the trends in more detail, we also analyzed the specific antibody titer development for each ewe over the entire sampling period (Figure 2 and 3). In control ewes, specific antibody titers decreased in 7 out of the 10 animals from Nov. 16 to Nov. 30. Since antibodies from passive transfer of colostrum usually existed in lambs for only one to two weeks, it was not likely that the decrease was due to colostrum antibody decline. Instead, we proposed that the decline happened as a result of natural decrease of antibodies, which were probably induced by previous exposure or infection of *M. haemolytica*. Then from Nov. 30 to Dec. 7, 6 out of 10 control ewes' titers were again slightly decreased and remained constant on Dec. 14. In vaccinated ewes, specific antibody titers increased sharply in 8 out of 10 vaccinated ewes from Nov. 16 to Nov. 30. On Nov. 30, 8 out 10 animal's titers were 1280 while the remaining two were 640. Titers then declined sharply in 6 ewes and remained the same in the remaining 4 animals on Nov. 30 as on Dec. 7. Finally, from Dec. 7 to Dec. 14, titers decreased in 4 ewes, increased in 4 ewes and remained constant in the remaining 2 ewes.

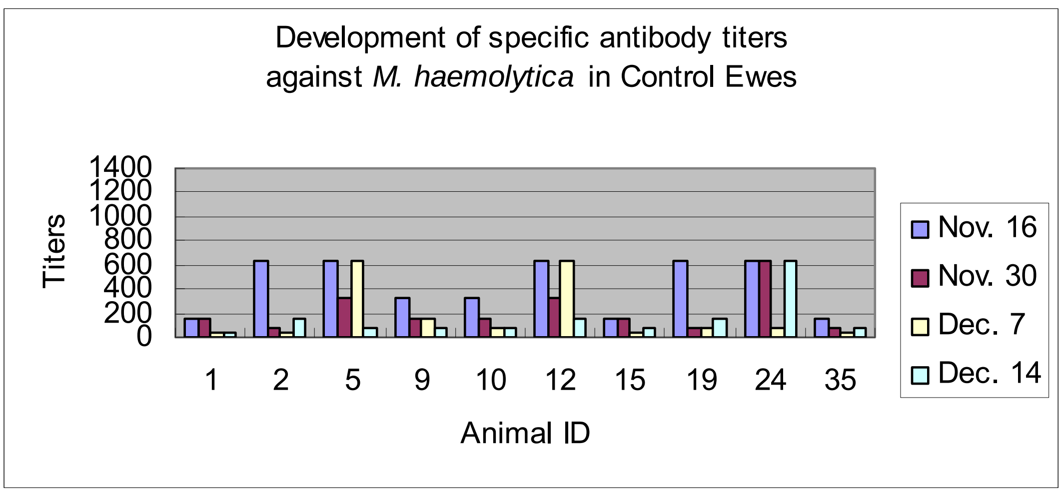

Figure 2 Specific antibody titers development against *M. haemolytica* over the four sampling times in control ewes. The X-axis denotes the ID of control ewes and Y-axis shows specific antibody titers.

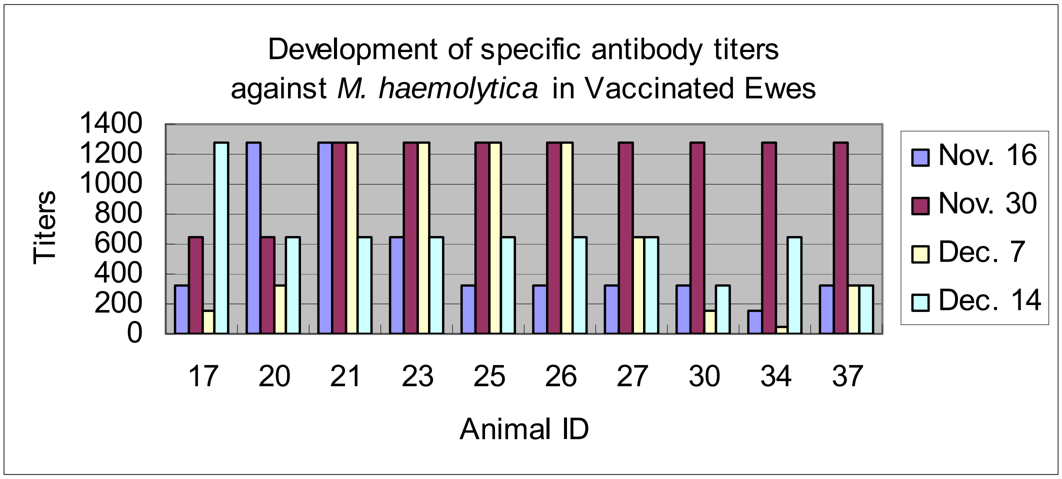

Figure 3 Specific antibody titers development against *M. haemolytica* over the four sampling dates in vaccinated ewes. The X-axis denotes the ID of vaccinated ewes and Y-axis shows specific antibody titers.

The results indicate that the autogenous vaccine did induce significant specific antibody production against *M. haemolytica* in the majority of the ewes immediately following vaccination.

(2) Interaction between treatment and dates of sampling in Rams

According to Figure 4, specific antibody titers in vaccinated rams were higher than that of control rams on all sampling dates except Dec. 7. For control rams, titers generally remained low and constant.

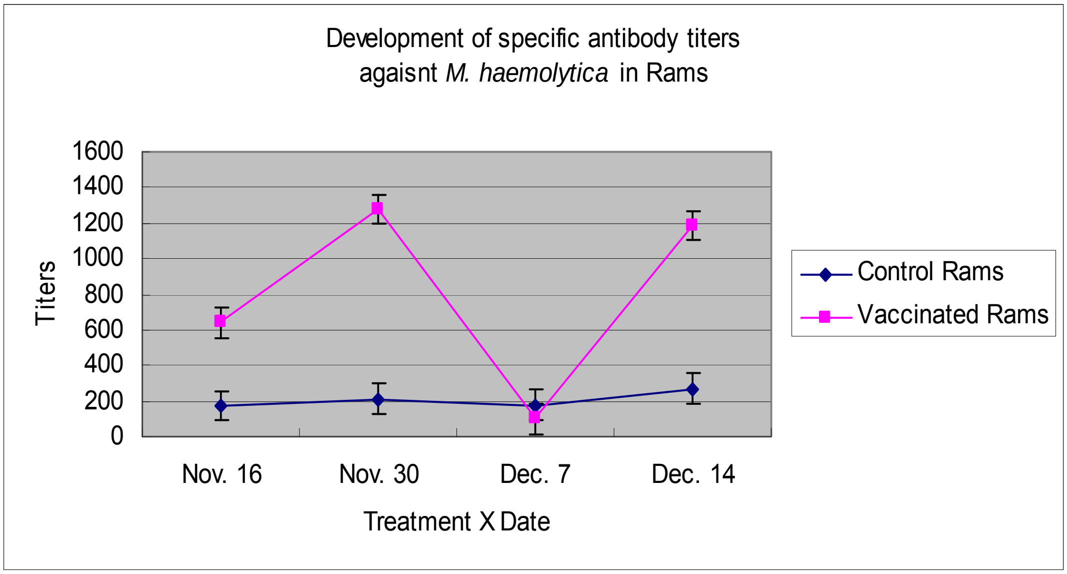

Figure 4 Development of specific antibody titers against *M. haemolytica* in rams on Nov. 16, Nov. 30, Dec. 7 and Dec. 14.

To understand the trends in more detail, we also analyzed specific antibody titer development in each ram over time (Figure 5 and 6). In the control group, specific antibody titer values in most of the rams were below 320 and there were no significant changes in titers over the four sampling times. In the vaccinated group, specific antibody titers in 8 out of 10 rams increased from Nov. 16 to Nov. 30; on Nov. 30, the titers of all vaccinated rams were 1280. But the titers of all the rams declined sharply from Nov. 30 to Dec. 7. On Dec. 7, 9 out of the 10 vaccinated rams' titers dropped to 40 but rose again to 1280 in 9 vaccinated rams.

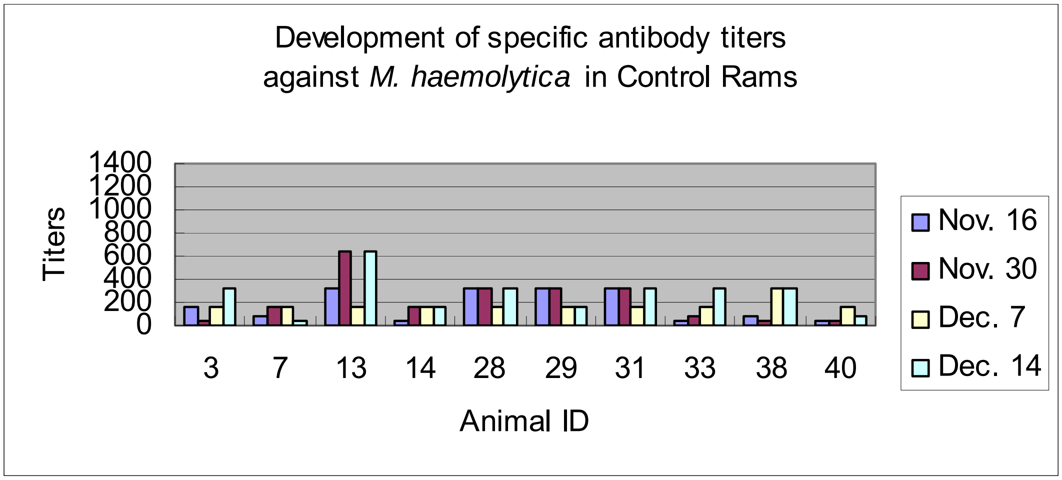

Figure 5 Specific antibody titers development against *M. haemolytica* over the four sampling dates in control rams. The X-axis denotes the ID of control rams and Y-axis shows specific antibody titers.

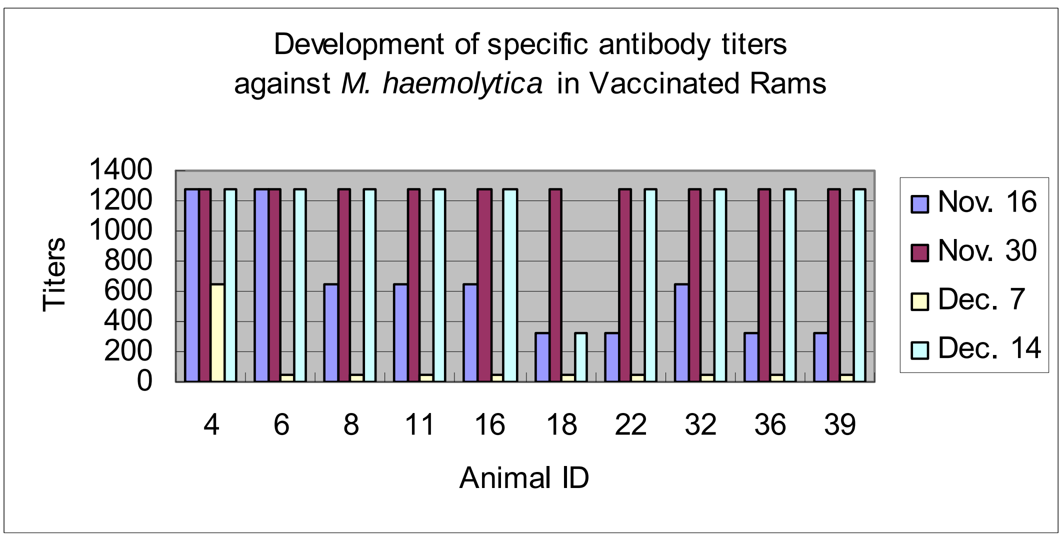

Figure 6 Specific antibody titers development against *M. haemolytica* over the four sampling dates in vaccinated rams. The X-axis denotes the ID of vaccinated rams and Y-axis shows specific antibody titers.

The results showed that the vaccine also induced significant antibody production against *M. haemolytica* in rams, which declined quickly, within 1-2 weeks.

(3) Vaccine efficacy against *M. haemolytica*

There are several previous studies examining vaccine efficacy against whole cell antigens of *M. haemolytica*. **Srinand et al. [10]** investigated antibody responses against *M. haemolytica* whole cell antigens in animals treated with four commercial vaccines and reported significant antibody level increases on day 28 post vaccination at P<0.05 level in animals vaccinated with Once PMH, One Shot, Presponse. **Mehmet et al. [9]** examined one vaccine's efficacy in inducing specific antibodies against whole cell antigens of *M. haemolytica* in lambs (including both ewes and rams) three weeks post vaccination using ELISA tests, and found ELISA titers differed from 80 and 320, and between 160 and 320 in lambs vaccinated once and twice, respectively, while titers in unvaccinated controls were negative. Lung lesion scores were also compared and significant decreases were observed in the trials compared with control groups at P<0.001.

In our study, we found that the overall production of specific antibody against *M. haemolytica* in both vaccinated ewe lambs and ram lambs was significantly higher than that of control lambs. This indicates that vaccination did induce specific antibody response in lambs, and agrees with the previous studies. In addition, in our study, vaccinated ewes had antibody titers at three weeks post vaccination ranging from 320 to 1280 (320 in 2 ewes, 640 in 7 ewes and 1280 in 1 ewe). Compared with titers of vaccinated groups in **Mehmet et al.** study, the titer level in our study was higher. In control ewes in our study, titers were from 40 to 640 (40 in 1 ewe, 80 in 5 ewes, 160 in 3 ewes and 640 in 1 ewe). Titers in 9 vaccinated rams were 1280; in control rams, titers ranged from 40 to 640 (40 in 1 ram, 80 in 1 ram, 160 in 2 rams, 320 in 5 rams, and 640 in 1 ram). The fact that we were able to detect some antibody in control animals may have been due to natural infection or exposure.

Interestingly, from Nov. 30 to Dec. 7, antibody titers in both vaccinated ewes and rams decreased dramatically. On Dec. 7, for vaccinated ewes, titers in all the other 6 ewes decreased except that 4 animals' titers remained at the titer level of 1280 as they were on Nov. 30. For vaccinated rams from Nov. 30 to Dec. 7, titers in 9 out of all 10 rams dropped from 1280 to 40. We may rule out the possibility of assay failure based on the fact that high antibody titers in sera taken on Dec. 7 were detected in 4 ewes. Since all tests for sera on Dec. 7 were performed at the same time, if the assay had failed, there would have been no antibody detected in every sera sample. On the other hand, **A.W. Confer et al. 2009 [16]** also reported decrease of specific antibody against whole cell *M. haemolytica* antigens in vaccinated animals between Day 14 and Day 21 after vaccination, which remained constant after Day 21, but the average response still remained higher than that of control animals; we did not see this in the present study. For the vaccinated ewes and rams in our experiment, we proposed that the titer decline from Nov. 30 to Dec. 7 may have also been due to natural antibody decrease, which usually declined to basal level within 14 days. It is possible that the titer increase from Dec. 7 to Dec. 14 in vaccinated rams may have been induced by exposure to *M. haemolytica* in the environment that would act as a booster, and induce a secondary immune response. This hypothesis is supported by the fact that the antibody titers against *M. haemolytica* in control rams, although slightly lower, also increased from Dec. 7 to Dec. 14, and again may have been due to exposure to the organism(s) in the environment.

Finally, the results suggest there were no gender differences in antibody response against *M. haemolytica* induced by vaccination.

4.2.2 Vaccine efficacy against *P. multocida*

4.2.2.1 Factors affecting specific antibody titers against *P. multocida*

The JMP model indicated that the vaccine did induce a significant increase ($P<0.0001<0.05$) in the production of specific antibody against *P. multocida* in vaccinated lambs over unvaccinated lambs. It also showed there was significant regression relationship ($P<0.0001<0.05$) between sex and specific antibody. In addition, titers of specific antibodies varied significantly ($P<0.0001<0.05$) across the four sampling dates.

4.2.2.2 Discussion of vaccine efficacy against *P. multocida*

(1) Interactions between treatment and dates of sampling in Ewes

In Figure 7, the specific antibody titers in the vaccinated group were higher than the corresponding titers in the control group over the experimental period except on Dec. 7. After vaccination on Nov. 23, there was an increase in specific antibody titer on Nov. 30, compared to Nov. 16, and also that of titers in the control group. However, there was a sudden titer decrease on Dec. 7, and a sharp increase followed on Dec. 14 for vaccinated sera. In the control group, the titer decreased from Nov. 16 to Dec. 7.

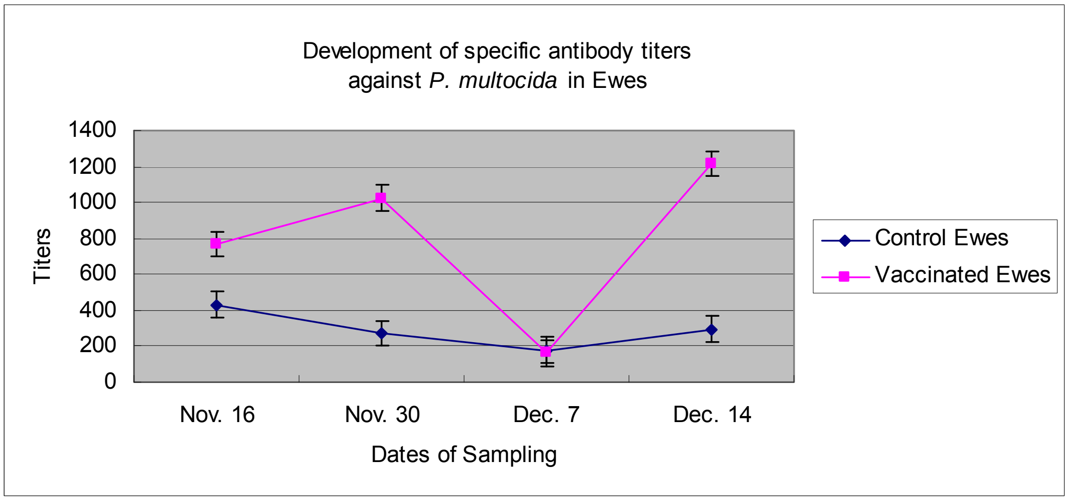

Figure 7 Development of specific antibody titers against *P. multocida* in ewes on Nov. 16, Nov. 30, Dec. 7 and Dec. 14.

To understand the trend in more detail, we analyzed the specific antibody titer in each ewe over time (Figures 8 and 9). In control ewes, from Nov. 16 to Nov. 30, titers in 6 out of 10 animals decreased, while titers for two animals increased and the remaining two were unchanged. Then from Nov. 30 to Dec. 7, titers decreased again in 8 animals. Finally, titers increased from Dec. 7 to Dec. 14 in 7 out of 10 animals. The titer on Dec. 14 was close to that on Nov. 30. Since antibodies from passive transfer of colostrum usually existed in lambs for only one to two weeks, it was not likely that the titer level decline in the majority of control ewes from Nov. 16 to Nov. 30 was due to colostrum antibody decrease. Instead, we proposed that the decline happened as a result of natural decrease of antibodies, which were probably induced by previous exposure or infection of *P. multocida*. In vaccinated ewes, titers increased from Nov. 16 to Nov. 30 in 6 out of 10 animals, while titers in three ewes remained the same and one decreased. On Nov. 30, titers for 6 ewes were 1280 and that of the remaining four were 640. Then titers decreased sharply from Nov. 30 to Dec. 7 in all 10 animals and titers on Dec. 7 ranged from 40 to 320. Finally, titers increased in all animals from Dec. 7 to Dec. 14, to 1280 in 9 animals on Dec. 14.

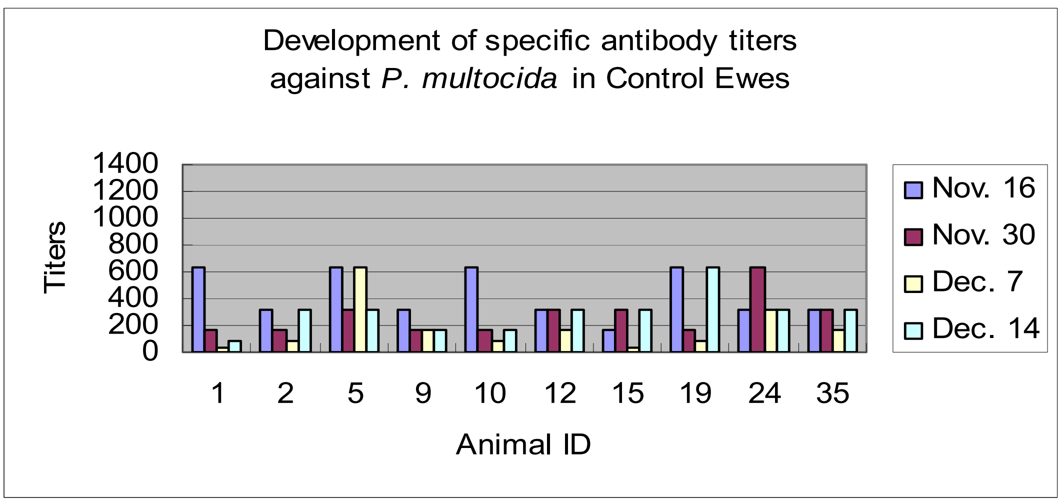

Figure 8 Development of specific antibody titers against *P. multocida* in control ewes over the four sampling dates. The X-axis denotes the ID of control ewes and the Y-axis shows the specific antibody titers.

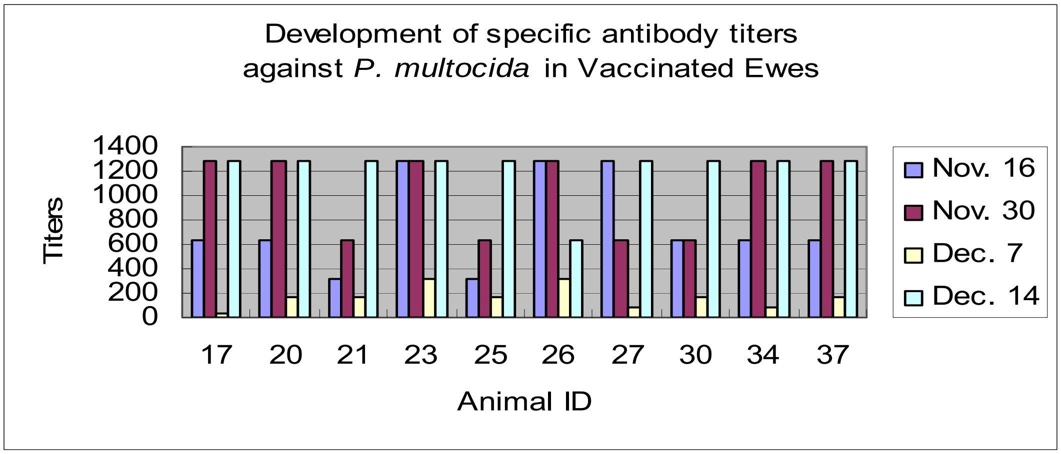

Figure 9 Development of specific antibody titers against *P. multocida* in vaccinated ewes over the four sampling dates. The X-axis denotes the ID of vaccinated ewes and the Y-axis shows specific antibody titers.

The results indicated that the vaccine did induce strong specific antibody production against *P. multocida* in ewes, which declined quickly in 1-2 weeks.

(2) Interactions between treatment and dates of sampling in Rams

In Figure 10, there was no significant difference in specific titers between vaccinated ram lambs and control ram lambs over the four sampling dates.

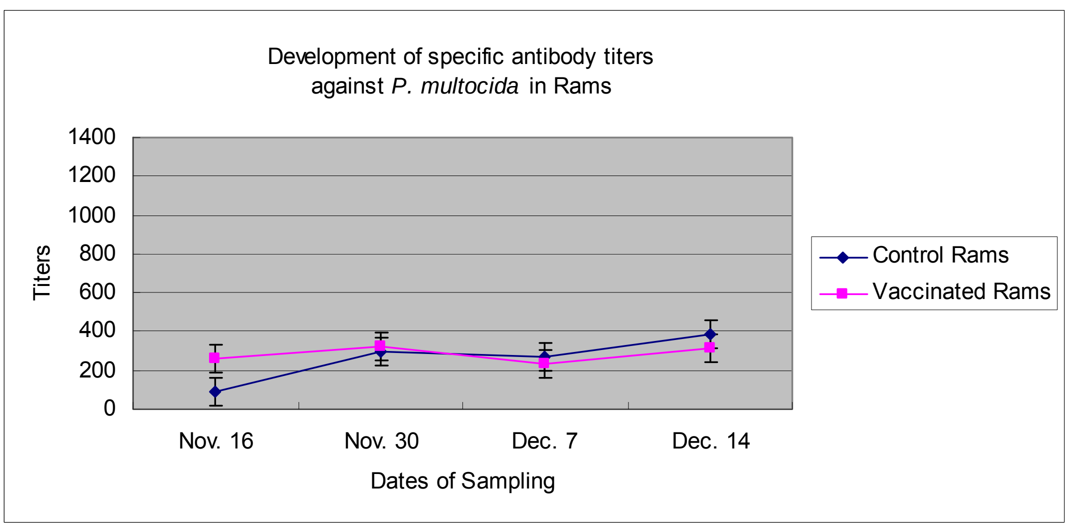

Figure 10 Development of specific antibody titers against *P. multocida* in rams on Nov. 16, Nov. 30, Dec. 7 and Dec. 14.

To understand the phenomenon in more details, we also analyzed the development of specific antibody titers in each ram over the four sampling dates (Figures 11 and 12). Specific antibody titers in almost all vaccinated animals across the four sampling times were below 400. Unexpectedly, more unvaccinated rams had titers greater than 400 compared to vaccinated rams. Also, after immediate vaccination titers were increased only in three vaccinated rams, from Nov. 16 to Nov. 30.

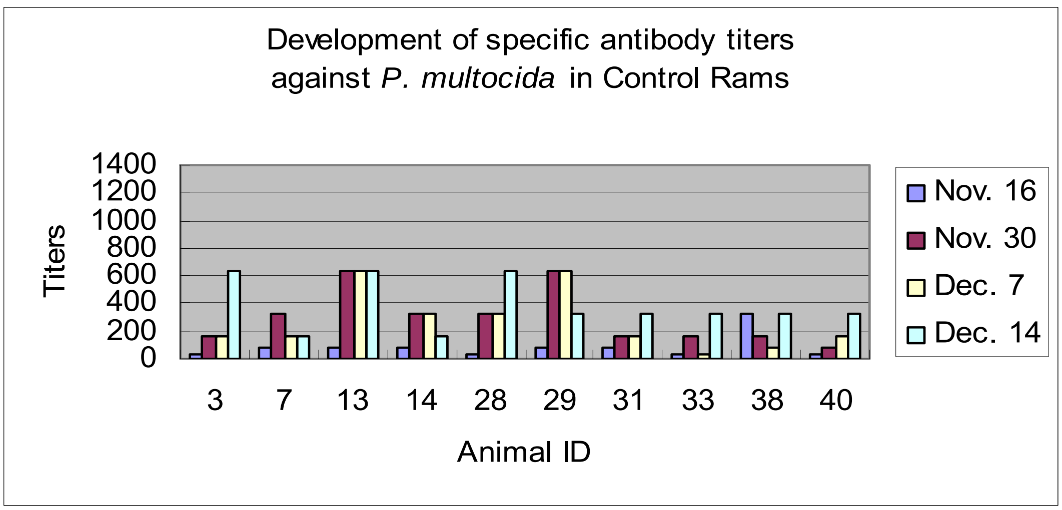

Figure 11 Development of specific antibody titers against *P. multocida* in control rams over the four sampling dates. The X-axis denotes the ID of control rams and the Y-axis shows specific antibody titers.

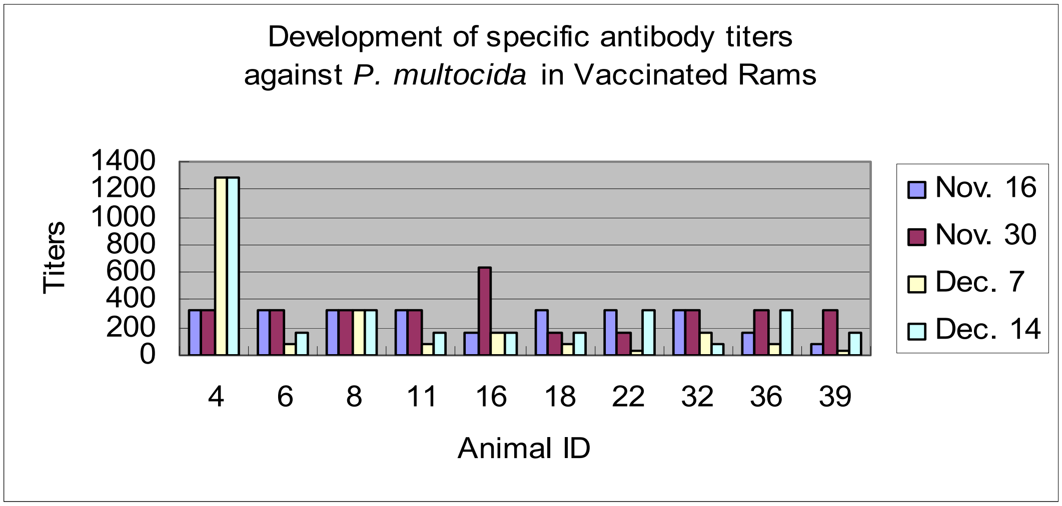

Figure 12 Development of specific antibody titers against *P. multocida* in vaccinated rams over the four sampling dates. The X-axis denotes the ID of vaccinated rams and the Y-axis shows the specific antibody titers.

The result indicated that the vaccine failed to induce significant specific antibody response in rams.

(3) Vaccine efficacy against *P. multocida*

Several previous studies investigated the efficacy of vaccination against *P. multocida* and found that vaccination against *P. multocida* was not as effective as against *M. haemolytica*. **Chandrasekaran S et al. 1991 [11]** tested an oil adjuvant vaccine incorporating antigens from both *M. haemolytica* and *P. multocida*, and reported significant pneumonic lesion reduction in vaccinated lambs after challenge with *M. haemolytica*, but not after challenge with *P. multocida*. **Carmen et al. 1983 [17]** argued that the limited protection of specific antibody against *P. multocida* via vaccination was probably because *P. multocida* strains did not share universal antigens, and they proposed that finding a new strain with wide spectrum of antigens would help solve this problem.

According to the data presented in section 4.2.2.2, we observed significant overall specific antibody response against *P. multocida* in vaccinated ewes compared with control ewes, while there were no significant differences between titers of control rams and vaccinated rams. The results indicated a gender difference in specific antibody response against *P. multocida* vaccines. The exact reason for gender difference in antibody response to vaccination is still uncertain, but several studies have shown that some immune responses in humans are higher in females compared to males, including numbers of CD4+ T cells **[18] [19] [20] [21]**. In particular, **Alberto Amadori et al. 1995 [19]** examined CD4 and CD8 T cell numbers in healthy donors and reported the CD4/CD8 T cells ratio was significantly higher in females than that in males, which, they proposed, was due to genetic regulation in humans. **Bret J. Rudy et al. 2002 [21]** performed flow cytometry analysis of lymphocyte subset markers on a group of sexually active, human immunodeficiency virus (HIV)-negative adolescents, and found that females had higher total CD4+ T cell and CD4+ memory T cell counts compared to males. These observations suggest, at least in part, why females may have responded better than males in terms of antibody production. Furthermore, the function of these T cells (TH1 or TH2) rather than number, may also be important **[22]**. In addition, considering the differences in the immune systems of humans and sheep, other factors may play a role in the gender differences in antibody response in lambs.

Interestingly, the antibody titer in vaccinated ewes declined sharply from Nov. 30 to Dec. 7. On Nov. 30, titers for 6 vaccinated ewes were 1280 and the other 4 ewe's titers were 640. On Dec.7, titers were as low as 40 to 320. The possibility that the ELISA assay failed was ruled out because other samples that were tested in the same assay showed significant antibody titers. It is possible that sampling or storage issues may have resulted in our inability to detect specific antibody in the sera of vaccinated ewes. It is also possible that the titer decline may have been due to a natural decrease in sera antibody levels, and the titer surge from Dec. 7 to Dec. 14 represented a secondary immune response induced by a natural *P. multocida* infection or exposure. This is supported by the fact that the vaccinated ewes' antibody titer on Dec. 24 was higher than that on Dec. 7. The slight titer increase that we noted in sera from Dec. 7 to Dec. 14 in both vaccinated and control rams, suggests that a natural infection or exposure to *P. multocida* may have occurred on the farm around Dec. 7.

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6. Appendix

6.1 Raw data from ELISA tests

Table 1 Absorbance of Control Ewes serum samples against *M. haemolytica* on Nov. 16

	Animal ID									
Dilutions	1	2	5	9	10	12	15	19	24	35
40	0.1534	0.2076	0.1975	0.2411	0.2124	0.1607	0.1466	0.1588	0.2172	0.1311
80	0.1246	0.1956	0.2143	0.1809	0.1739	0.1694	0.1373	0.1215	0.1427	0.1271
160	0.1239	0.1522	0.1526	0.1521	0.1438	0.0711	0.1144	0.1371	0.1432	0.1405
320	0.0913	0.1399	0.121	0.1434	0.1267	0.1511	0.0918	0.1349	0.1616	0.0899
640	0.0888	0.1096	0.1111	0.1014	0.0939	0.1272	0.072	0.1096	0.1346	0.1125
1280	0.0768	0.0987	0.0822	0.0885	0.0829	0.0993	0.0712	0.0789	0.0941	0.0744
blank	0.0662	0.0632	0.0642	0.0602	0.0589	0.0997	0.0631	0.0602	0.0548	0.0561

Table 2 Absorbance of Vaccinated Ewes serum samples against *M. haemolytica* on Nov. 16

	Animal ID									
Dilutions	19	20	21	23	25	26	27	30	34	37
40	0.1837	0.2083	0.2032	0.2074	0.2132	0.2304	0.2009	0.206	0.133	0.1498
80	0.1581	0.1847	0.176	0.1966	0.1401	0.1949	0.1583	0.151	0.1238	0.1569
160	0.1313	0.1666	0.1775	0.1687	0.1342	0.1353	0.1555	0.1364	0.113	0.1434
320	0.1762	0.1451	0.1232	0.138	0.1057	0.1051	0.1308	0.1147	0.0993	0.1044
640	0.1023	0.1778	0.1584	0.1428	0.0922	0.0979	0.1023	0.091	0.101	0.0924
1280	0.0946	0.1662	0.1397	0.0889	0.0805	0.0921	0.0922	0.0911	0.0752	0.0804
blank	0.1045	0.0807	0.0831	0.0679	0.0658	0.0724	0.0647	0.07	0.0627	0.0634

Table 3 Absorbance of Control Ewes serum samples against *P. multocida* on Nov. 16

	Animal ID									
Dilutions	1	2	5	9	10	12	15	19	24	35
40	0.3599	0.3809	0.5181	0.3229	0.2511	0.4124	0.5713	0.2885	0.4469	0.2626
80	0.2304	0.2652	0.3546	0.2811	0.3662	0.3526	0.3335	0.2497	0.2938	0.277
160	0.1566	0.1677	0.2832	0.246	0.2155	0.2207	0.2044	0.184	0.2397	0.2254
320	0.1244	0.148	0.2188	0.2361	0.2297	0.1748	0.1354	0.1604	0.1539	0.1499
640	0.1443	0.1255	0.152	0.1348	0.1507	0.1397	0.1332	0.1472	0.0976	0.1232
1280	0.0872	0.0949	0.1086	0.1149	0.1115	0.1063	0.0954	0.0962	0.1007	0.063
blank	0.0642	0.0759	0.0656	0.0703	0.0823	0.0652	0.0659	0.0679	0.0695	0.0695

Table 4 Absorbance of Vaccinated Ewe serum samples against *P. multocida* on Nov. 16

	Animal ID									
Dilutions	19	20	21	23	25	26	27	30	34	37
40	0.3718	0.4412	0.4122	0.3645	0.3798	0.4079	0.4261	0.3784	0.3081	0.2369
80	0.3283	0.2968	0.3264	0.382	0.3324	0.3328	0.34	0.2839	0.2394	0.2389
160	0.262	0.2472	0.2304	0.3401	0.245	0.2504	0.2475	0.2404	0.1953	0.2096
320	0.2368	0.2229	0.159	0.253	0.2548	0.2163	0.274	0.2306	0.2103	0.2115
640	0.1751	0.1695	0.126	0.2168	0.1342	0.1697	0.1793	0.1737	0.1556	0.1681
1280	0.122	0.1317	0.103	0.1544	0.1353	0.1517	0.1528	0.125	0.1159	0.1251
blank	0.0699	0.0686	0.0675	0.0689	0.0713	0.0713	0.0722	0.0729	0.0741	0.0708

Table 5 Absorbance of Control Ewe serum samples against *M. haemolytica* on Nov. 30

	Animal ID									
Dilution	1	2	5	9	10	12	15	19	24	35
40	0.1053	0.1132	0.1133	0.0954	0.0896	0.0872	0.0891	0.0861	0.1028	0.0877
80	0.0894	0.0997	0.1012	0.092	0.0833	0.0902	0.0837	0.0813	0.091	0.0883
160	0.0828	0.0811	0.0826	0.0874	0.0839	0.0877	0.0824	0.0772	0.0866	0.0795
320	0.0715	0.0794	0.0822	0.0777	0.0728	0.0819	0.0713	0.0739	0.0839	0.0759
640	0.0678	0.0741	0.0754	0.079	0.0726	0.0776	0.0699	0.0712	0.0853	0.0795
1280	0.0673	0.0681	0.0732	0.0753	0.0671	0.0755	0.0695	0.0731	0.0744	0.067
blank	0.0645	0.0657	0.0591	0.0609	0.0653	0.0579	0.0726	0.0681	0.0729	0.0736

Table 6 Absorbance of Vaccinated Ewe serum samples against *M. haemolytica* on Nov. 30

	Animal ID									
Dilution	17	20	21	23	25	26	27	30	34	37
40	0.3998	0.1339	0.1139	0.107	0.1084	0.111	0.1077	0.1417	0.1724	0.1843
80	0.1039	0.1391	0.1515	0.107	0.0937	0.1113	0.1052	0.2014	0.1323	0.1752
160	0.088	0.1044	0.107	0.1077	0.0942	0.0967	0.0973	0.1613	0.1297	0.1462
320	0.099	0.0967	0.1021	0.096	0.0938	0.0985	0.0858	0.1534	0.1097	0.1422
640	0.1112	0.1018	0.096	0.0957	0.0844	0.0862	0.0958	0.1079	0.0865	0.1093
1280	0.078	0.0788	0.0878	0.0904	0.0877	0.08	0.086	0.0985	0.0819	0.0989
blank	0.0607	0.0573	0.0587	0.0682	0.0687	0.0638	0.059	0.0676	0.0624	0.0618

Table 7 Absorbance of Control Ewe serum samples against *P. multocida* on Nov. 30

	Animal ID									
Dilution	1	2	5	9	10	12	15	19	24	35
40	0.191	0.1993	0.2943	0.1975	0.222	0.2917	0.2117	0.217	0.2525	0.2209
80	0.1628	0.1668	0.2524	0.1798	0.1773	0.2121	0.1874	0.2005	0.2334	0.1466
160	0.1434	0.1267	0.1805	0.1433	0.1386	0.1551	0.1664	0.1254	0.1749	0.1687
320	0.1108	0.1002	0.15	0.1081	0.1064	0.1425	0.1438	0.1327	0.17	0.1412
640	0.096	0.0842	0.1148	0.095	0.0889	0.1181	0.1201	0.1172	0.1471	0.109
1280	0.0861	0.0864	0.102	0.0882	0.088	0.1183	0.1123	0.0846	0.1153	0.0889
blank	0.0647	0.0756	0.077	0.0784	0.0743	0.085	0.069	0.0615	0.0624	0.0592

Table 8 Absorbance of Vaccinated Ewe serum samples against *P. multocida* on Nov. 30

	Animal ID									
Dilution	17	20	21	23	25	26	27	30	34	37
40	0.3251	0.3068	0.2824	0.3193	0.2613	0.2983	0.2829	0.2377	0.3471	0.2433
80	0.2759	0.3075	0.3027	0.3056	0.2357	0.2609	0.2459	0.25	0.2713	0.1869
160	0.2494	0.239	0.1882	0.2509	0.2025	0.2293	0.2166	0.2051	0.2188	0.2066
320	0.1698	0.2083	0.1684	0.1987	0.1775	0.1893	0.1618	0.1645	0.1804	0.1827
640	0.1714	0.1784	0.1472	0.1617	0.1523	0.1449	0.1415	0.1434	0.1446	0.1373
1280	0.1398	0.1307	0.121	0.141	0.1164	0.1229	0.1138	0.1208	0.1254	0.1301
blank	0.0669	0.0629	0.0647	0.0715	0.0674	0.0618	0.0628	0.0648	0.0671	0.0611

Table 9 Absorbance of Control Ewe serum samples against *M. haemolytica* on Dec. 7

	Animal ID									
Dilution	1	3	5	9	10	12	15	19	24	35
40	0.1049	0.1406	0.2397	0.1507	0.1679	0.1177	0.112	0.1322	0.1482	0.1418
80	0.0958	0.1118	0.1794	0.1215	0.1297	0.1683	0.1139	0.1352	0.1459	0.1066
160	0.0824	0.1171	0.1471	0.1245	0.108	0.1245	0.1088	0.1009	0.1104	0.0925
320	0.0811	0.1122	0.1524	0.1141	0.0956	0.1335	0.0945	0.0946	0.1168	0.0951
640	0.1035	0.1034	0.1211	0.1083	0.0933	0.1235	0.0904	0.1043	0.0995	0.0745
1280	0.0672	0.1027	0.1014	0.0911	0.0899	0.1072	0.0855	0.0904	0.1055	0.0766
blank	0.0625	0.0624	0.059	0.0577	0.056	0.0575	0.0634	0.0564	0.0575	0.0591

Table 10 Absorbance of Vaccinated Ewe serum samples against *M. haemolytica* on Dec. 7

	Animal ID									
Dilution	17	20	21	23	25	26	27	30	34	37
40	0.1469	0.1841	0.2077	0.2717	0.118	0.1356	0.1275	0.155	0.1207	0.1353
80	0.1311	0.1876	0.1811	0.1606	0.12	0.1315	0.1228	0.1128	0.1089	0.1407
160	0.1252	0.1248	0.1498	0.1578	0.1145	0.1198	0.1267	0.1219	0.0998	0.1317
320	0.099	0.1258	0.1634	0.1742	0.1193	0.1096	0.1175	0.098	0.0904	0.1202
640	0.1024	0.1084	0.1512	0.1373	0.1313	0.1531	0.19	0.1043	0.0806	0.0909
1280	0.12	0.1158	0.1418	0.1413	0.125	0.1206	0.1122	0.1239	0.0809	0.0871
blank	0.0629	0.0731	0.0669	0.0504	0.061	0.0597	0.0573	0.0585	0.0576	0.0622

Table 11 Absorbance of Control Ewe serum samples against *P. multocida* on Dec. 7

	Animal ID									
Dilution	1	2	5	9	10	12	15	19	24	35
40	0.1872	0.186	0.3527	0.1985	0.2294	0.2189	0.1966	0.2225	0.2386	0.219
80	0.1384	0.1754	0.3474	0.1944	0.184	0.1645	0.163	0.1781	0.2193	0.2114
160	0.1216	0.1634	0.3102	0.1706	0.1504	0.2049	0.159	0.1634	0.1851	0.1741
320	0.0999	0.1182	0.245	0.158	0.1354	0.1612	0.1365	0.1378	0.1672	0.1481
640	0.0908	0.1086	0.1878	0.1211	0.143	0.1408	0.1228	0.1208	0.1269	0.125
1280	0.0786	0.092	0.1467	0.1032	0.0903	0.128	0.107	0.113	0.1065	0.1171
blank	0.0615	0.0601	0.061	0.0703	0.0577	0.0566	0.0568	0.0561	0.059	0.058

Table 12 Absorbance of Vaccinated Ewe serum samples against *P. multocida* on Dec. 7

	Animal ID									
Dilution	17	20	21	23	25	26	27	30	34	37
40	0.1919	0.2737	0.2689	0.2454	0.1627	0.2083	0.1801	0.2103	0.2078	0.1812
80	0.1277	0.2065	0.2553	0.1968	0.2031	0.1823	0.1721	0.1786	0.2019	0.1664
160	0.1263	0.169	0.1962	0.2056	0.176	0.1887	0.1483	0.1899	0.1461	0.1646
320	0.1287	0.1824	0.1555	0.1725	0.1587	0.1814	0.1417	0.1635	0.1272	0.1252
640	0.1134	0.1335	0.1313	0.142	0.1389	0.1373	0.1321	0.1185	0.1233	0.143
1280	0.1113	0.1377	0.1055	0.132	0.1356	0.1146	0.1139	0.1412	0.1059	0.129
blank	0.0659	0.0592	0.0592	0.0631	0.0582	0.0594	0.0595	0.0628	0.0606	0.0615

Table 13 Absorbance of Control ewe serum samples against *M. haemolytica* on Dec. 14

	Animal ID									
Dilution	17	20	21	23	25	26	27	30	34	37
1:40	0.1855	0.2916	0.3401	0.248	0.215	0.2293	0.2756	0.286	0.4174	0.3026
1:80	0.155	0.231	0.4654	0.2192	0.232	0.2679	0.2327	0.2305	0.4113	0.247
1:160	0.1333	0.2183	0.1762	0.1719	0.1704	0.2423	0.1607	0.1929	0.3215	0.191
1:320	0.1056	0.1592	0.1422	0.1394	0.1513	0.1665	0.1567	0.1456	0.3517	0.1553
1:640	0.0903	0.149	0.1275	0.1123	0.122	0.1722	0.1259	0.1498	0.2355	0.1385
1:1280	0.0938	0.1127	0.0909	0.1038	0.0876	0.1518	0.1041	0.1117	0.1737	0.1119
Blank	0.0719	0.06	0.0636	0.06	0.0603	0.0603	0.0653	0.0644	0.0614	0.062

Table 14 Absorbance of Vaccinated Ewe serum samples against *M. haemolytica* on Dec. 14

	Animal ID									
Dilution	17	20	21	23	25	26	27	30	34	37
1:40	0.3932	0.4006	0.3977	0.5145	0.5558	0.4808	0.3239	0.34	0.5196	0.3196
1:80	0.3503	0.3336	0.3348	0.3472	0.3604	0.4006	0.3122	0.3952	0.4041	0.3119
1:160	0.2944	0.3388	0.3855	0.3307	0.3092	0.2705	0.2861	0.2386	0.3374	0.2308
1:320	0.2553	0.2326	0.2756	0.2502	0.2732	0.2296	0.2532	0.205	0.3516	0.2069
1:640	0.2207	0.21	0.2027	0.1975	0.2633	0.2211	0.1957	0.1476	0.213	0.1445
1:1280	0.2107	0.1671	0.1636	0.1679	0.1638	0.164	0.1265	0.1192	0.1605	0.1396
Blank	0.1182	0.0612	0.0626	0.063	0.0676	0.0646	0.0608	0.0604	0.0664	0.0694

Table 15 Absorbance of Control Ewe serum samples against *P. multocida* on Dec. 14

	Animal ID									
Dilution	1	2	5	9	10	12	15	19	24	35
1:40	0.2346	0.3487	0.5961	0.3806	0.3846	0.2895	0.3325	0.412	0.4374	0.341
1:80	0.1895	0.2771	0.4412	0.3081	0.3084	0.3145	0.3081	0.3465	0.2945	0.2606
1:160	0.1627	0.2413	0.2751	0.2205	0.225	0.2967	0.2316	0.2764	0.4374	0.231
1:320	0.1471	0.1962	0.1988	0.1719	0.1663	0.2516	0.2066	0.2747	0.2118	0.1845
1:640	0.0994	0.1474	0.1485	0.1581	0.1315	0.1345	0.1715	0.1904	0.1605	0.1783
1:1280	0.0834	0.1111	0.1184	0.1005	0.1114	0.1585	0.133	0.1361	0.139	0.1184
Blank	0.0638	0.0701	0.0635	0.0638	0.0637	0.0615	0.0641	0.062	0.0638	0.0618

Table 16 Absorbance of Vaccinated Ewe serum samples against *P. multocida* on Dec. 14

	Animal ID									
Dilution	17	20	21	23	25	26	27	30	34	37
1:40	0.6754	0.6545	0.6587	0.5687	0.5977	0.6212	0.4785	0.3853	0.6574	0.4599
1:80	0.6479	0.6446	0.5406	0.4582	0.6354	0.4617	0.479	0.414	0.7461	0.4423
1:160	0.6011	0.5775	0.53	0.4598	0.3777	0.4067	0.4712	0.4353	0.5303	0.3934
1:320	0.318	0.4415	0.3686	0.4234	0.4204	0.3156	0.3321	0.4212	0.4804	0.3753
1:640	0.2771	0.3918	0.2423	0.3329	0.2851	0.2374	0.2705	0.2843	0.3117	0.3066
1:1280	0.4044	0.2493	0.2261	0.2644	0.2023	0.1728	0.2094	0.2118	0.2042	0.2003
Blank	0.0768	0.0725	0.0739	0.0909	0.0642	0.0648	0.0722	0.0628	0.0682	0.0703

Table 17 Absorbance of Control Ram serum samples against *M. haemolytica* on Nov. 16

	Animal ID									
Dilution	3	7	13	14	28	29	31	33	38	40
40	0.1018	0.1158	0.1766	0.1006	0.1138	0.1408	0.1653	0.104	0.1014	0.0965
80	0.1063	0.1093	0.1393	0.0916	0.1167	0.1153	0.1574	0.0929	0.123	0.095
160	0.1058	0.0943	0.1189	0.0869	0.1059	0.1425	0.138	0.0863	0.0906	0.0802
320	0.0848	0.1077	0.1852	0.0727	0.0995	0.1322	0.1197	0.1792	0.113	0.0725
640	0.112	0.0901	0.0887	0.0757	0.0865	0.0965	0.0965	0.0758	0.0842	0.0758
1280	0.0647	0.0815	0.0748	0.0826	0.0809	0.0809	0.0868	0.0647	0.0689	0.07
blank	0.0594	0.0612	0.0572	0.0707	0.0603	0.066	0.0627	0.0561	0.059	0.058

Table 18 Absorbance of Vaccinated Ram serum samples against *M. haemolytica* on Nov. 16

	Animal ID									
Dilution	4	6	8	11	16	18	22	32	36	39
40	0.2134	0.1379	0.1337	0.308	0.1319	0.1372	0.1209	0.1437	0.1284	0.1257
80	0.1791	0.1742	0.1174	0.1304	0.1343	0.146	0.1298	0.1066	0.1182	0.1027
160	0.1672	0.1197	0.1402	0.1393	0.1075	0.1203	0.1311	0.1124	0.1426	0.0832
320	0.1279	0.109	0.1277	0.1366	0.1052	0.1151	0.1669	0.1091	0.125	0.0995
640	0.1295	0.1089	0.1139	0.1986	0.0986	0.0947	0.0899	0.1034	0.0902	0.0826
1280	0.1153	0.2168	0.0929	0.0822	0.0851	0.0867	0.124	0.0879	0.0833	0.0627
blank	0.3601	0.1207	0.0738	0.0613	0.0676	0.0671	0.0762	0.0656	0.0958	0.084

Table 19 Absorbance of Control Ram serum samples against *P. multocida* on Nov. 16

	Animal ID									
Dilution	3	7	13	14	28	29	31	33	38	40
40	0.1319	0.2144	0.1225	0.1681	0.1728	0.1897	0.157	0.1411	0.165	0.1594
80	0.1153	0.1409	0.1325	0.1305	0.12	0.1307	0.1426	0.1203	0.1288	0.1129
160	0.1122	0.1195	0.0997	0.1254	0.1279	0.1141	0.1257	0.1381	0.1331	0.0963
320	0.1234	0.0898	0.0892	0.0875	0.0922	0.1783	0.0881	0.0961	0.148	0.2139
640	0.0796	0.0827	0.0762	0.0804	0.0873	0.1011	0.0838	0.0818	0.1043	0.074
1280	0.0739	0.0837	0.0758	0.0723	0.0717	0.0785	0.0805	0.1275	0.0783	0.076
blank	0.0621	0.0611	0.0608	0.0617	0.0644	0.0722	0.0633	0.0625	0.1425	0.0614

Table 20 Absorbance of Vaccinated Ram serum samples against *P. multocida* on Nov. 16

	Animal ID									
Dilution	4	6	8	11	16	18	22	32	36	39
40	0.2624	0.2581	0.3064	0.2391	0.2047	0.259	0.2056	0.2551	0.161	0.2184
80	0.2295	0.2677	0.2212	0.1873	0.1779	0.1745	0.1847	0.1922	0.175	0.2397
160	0.1738	0.1716	0.1905	0.1897	0.142	0.1498	0.134	0.183	0.1883	0.1202
320	0.1455	0.141	0.1542	0.1301	0.109	0.1326	0.1707	0.1482	0.1147	0.1037
640	0.1202	0.1072	0.1037	0.104	0.0911	0.1248	0.0951	0.1216	0.0961	0.0963
1280	0.0918	0.0834	0.0856	0.1653	0.0992	0.084	0.0903	0.1101	0.0769	0.0739
blank	0.0766	0.0754	0.0599	0.0613	0.0708	0.062	0.063	0.1832	0.0665	0.0682

Table 21 Absorbance of Control Ram serum samples against *M. haemolytica* on Nov. 30

	Animal ID									
Dilution	3	7	13	14	28	29	31	33	38	40
40	0.1291	0.202	0.1704	0.1414	0.1418	0.1773	0.1938	0.1325	0.1019	0.1084
80	0.1112	0.1459	0.1804	0.1302	0.1491	0.181	0.1608	0.1244	0.0989	0.0951
160	0.113	0.1194	0.1542	0.1142	0.129	0.1212	0.1537	0.1009	0.0965	0.081
320	0.1017	0.1086	0.1419	0.105	0.1146	0.145	0.1271	0.0972	0.0827	0.081
640	0.0862	0.103	0.1266	0.09	0.0954	0.0999	0.1058	0.089	0.0888	0.0799
1280	0.0767	0.0878	0.0942	0.096	0.0903	0.1015	0.0962	0.0864	0.0773	0.0844
blank	0.0771	0.0877	0.0714	0.0657	0.0763	0.062	0.0585	0.06	0.0619	0.0672

Table 22 Absorbance of Vaccinated Ram serum samples against *M. haemolytica* on Nov. 30

	Animal ID									
Dilution	4	6	8	11	16	18	22	32	36	39
40	0.6438	0.4355	0.4177	0.3714	0.4047	0.4053	0.4556	0.4926	0.2853	0.3761
80	0.6532	0.392	0.3481	0.3739	0.3191	0.2592	0.2805	0.3393	0.2723	0.2284
160	0.4124	0.3691	0.2947	0.3123	0.2958	0.201	0.2203	0.2605	0.2837	0.229
320	0.3862	0.2796	0.3069	0.2768	0.2382	0.2325	0.204	0.2561	0.2236	0.205
640	0.269	0.2512	0.2441	0.2367	0.1812	0.1645	0.1993	0.1931	0.2006	0.1585
1280	0.2184	0.1808	0.1908	0.1682	0.1698	0.1187	0.1968	0.1637	0.1676	0.1684
blank	0.0661	0.0641	0.0646	0.0822	0.0653	0.0685	0.0635	0.0661	0.0669	0.0652

Table23 Absorbance of Control Ram serum samples against *P. multocida* on Nov. 30

	Animal ID									
Dilution	3	7	13	14	28	29	31	33	38	40
40	0.5554	0.8746	1.0379	0.6051	0.702	0.9075	0.6673	0.5821	0.5397	0.4351
80	0.6629	0.6412	0.697	0.574	0.6434	0.7202	0.6848	0.5693	0.5606	0.3784
160	0.3854	0.4902	0.6222	0.5301	0.5758	0.5722	0.4914	0.4206	0.3592	0.3151
320	0.2945	0.3402	0.5639	0.3572	0.4178	0.457	0.3019	0.2932	0.2837	0.2903
640	0.2191	0.2634	0.3192	0.2607	0.2676	0.3363	0.226	0.2169	0.155	0.2242
1280	0.2147	0.1978	0.2753	0.1993	0.2076	0.2479	0.1575	0.179	0.1467	0.1493
blank	0.1357	0.1216	0.1257	0.0936	0.0888	0.0726	0.0769	0.0692	0.0759	0.0916

Table24 Absorbance of Vaccinated Ram serum samples against *P. multocida* on Nov. 30

	Animal ID									
Dilution	4	6	8	11	16	18	22	32	36	39
40	0.8409	0.7265	0.585	0.6084	0.5308	0.5909	0.4915	0.5819	0.7728	0.5639
80	0.6303	0.5472	0.5404	0.5123	0.587	0.4444	0.4236	0.5252	0.5485	0.3906
160	0.5566	0.437	0.4033	0.4484	0.4844	0.3297	0.3309	0.386	0.4464	0.3302
320	0.4214	0.3496	0.34	0.3729	0.37	0.2919	0.2632	0.3429	0.3379	0.3464
640	0.3131	0.263	0.2658	0.284	0.3203	0.2417	0.1942	0.2765	0.2451	0.226
1280	0.2578	0.1963	0.1879	0.2283	0.2566	0.1826	0.1628	0.2407	0.1787	0.1859
blank	0.077	0.0714	0.0757	0.0855	0.0714	0.0774	0.0738	0.0728	0.0749	0.0704

Table 25 Absorbance of Control Ram serum samples against *M. haemolytica* on Dec. 7

	Animal ID									
Dilution	3	7	13	14	28	29	31	33	38	40
40	0.2631	0.4252	0.3605	0.7418	0.4705	0.5321	0.8336	0.5602	0.9114	0.6342
80	0.4775	0.4112	0.3412	0.3734	0.5365	0.6271	0.7153	0.6743	0.458	0.6177
160	0.5302	0.4909	0.3758	0.3426	0.5467	0.6066	0.7457	0.537	0.7123	0.9117
320	0.2064	0.1695	0.2059	0.2908	0.251	0.2642	0.1892	0.226	0.3398	0.2535
640	0.1307	0.1684	0.1546	0.1468	0.1718	0.5141	0.1876	0.5998	0.619	0.9749
1280	0.1912	0.1734	0.2677	0.1314	0.2746	0.1382	0.1815	0.1405	0.1648	0.156
blank	0.187	0.0739	0.0623	0.0677	0.0737	0.211	0.1374	0.5964	0.1842	0.2333

Table 26 Absorbance of Vaccinated Ram serum samples against *M. haemolytica* on Dec. 7

	Animal ID									
Dilution	4	6	8	11	16	18	22	32	36	39
40	0.4943	0.3597	0.1477	0.1935	0.1848	0.2121	0.1565	0.1524	0.1578	0.0917
80	0.7141	0.217	0.2144	0.232	0.1994	0.1905	0.1681	0.1729	0.1934	0.0929
160	0.6764	0.1702	0.2662	0.241	0.2359	0.1824	0.148	0.2147	0.2046	0.088
320	0.4527	0.2267	0.2261	0.2442	0.2007	0.1683	0.148	0.2042	0.1605	0.0863
640	0.4771	0.1782	0.1881	0.1935	0.1562	0.1597	0.1373	0.164	0.1372	0.082
1280	0.321	0.1604	0.1772	0.1604	0.1172	0.1282	0.0981	0.1219	0.1352	0.084
blank	0.068	0.0615	0.0625	0.0588	0.0638	0.0649	0.0598	0.0617	0.0582	0.0612

Table 27 Absorbance of Control Ram serum samples against *P. multocida* on Dec. 7

	Animal ID									
Dilution	3	7	13	14	28	29	31	33	38	40
40	0.336	0.3275	0.3935	0.3048	0.3031	0.3127	0.266	0.2034	0.249	0.2888
80	0.2562	0.3189	0.3981	0.3365	0.327	0.3865	0.2837	0.1557	0.1646	0.2484
160	0.2124	0.2387	0.2613	0.317	0.2601	0.277	0.2294	0.144	0.1228	0.1824
320	0.1514	0.1348	0.2094	0.2148	0.2045	0.2604	0.1562	0.092	0.0996	0.1574
640	0.1299	0.112	0.1676	0.1261	0.1421	0.1852	0.1006	0.0835	0.0816	0.1051
1280	0.1104	0.1011	0.1418	0.1183	0.1275	0.1506	0.0924	0.0716	0.0738	0.128
blank	0.0731	0.0676	0.0751	0.0681	0.0692	0.0681	0.0566	0.0589	0.0572	0.0607

Table 28 Absorbance of Vaccinated Ram serum samples against *P. multocida* on Dec. 7

	Animal ID									
Dilution	4	6	8	11	16	18	22	32	36	39
40	0.3959	0.1856	0.2281	0.2015	0.1944	0.2111	0.1693	0.1962	0.1941	0.1138
80	0.4005	0.1668	0.2062	0.1711	0.2083	0.1838	0.153	0.1777	0.1749	0.1206
160	0.3124	0.1437	0.1866	0.1392	0.169	0.1525	0.1308	0.1984	0.1467	0.1048
320	0.2632	0.1432	0.1661	0.1395	0.1391	0.1234	0.1131	0.1602	0.1246	0.0911
640	0.2471	0.0985	0.1247	0.1084	0.1178	0.1253	0.0975	0.1445	0.1056	0.0793
1280	0.1723	0.0932	0.086	0.1163	0.1055	0.0954	0.0738	0.1146	0.0907	0.0662
blank	0.0695	0.0875	0.0832	0.0701	0.0708	0.0743	0.06	0.0593	0.0575	0.0588

Table 29 Absorbance of Control Ram serum samples against *M. haemolytica* on Dec. 14

	Animal ID									
Dilution	3	7	13	14	28	29	31	33	38	40
40	0.7745	0.6469	1.0738	0.7327	0.9857	1.0421	1.1733	1.1153	0.824	0.7635
80	0.6013	0.4145	1.16	0.5485	0.8507	0.7376	1.4002	1.3259	0.6388	0.7001
160	0.6162	0.4185	0.869	0.4653	0.5901	0.5839	0.8041	0.6836	0.3719	0.3532
320	0.5148	0.3557	0.1812	0.3474	0.5967	0.4024	0.5642	0.4761	0.4436	0.2115
640	0.3897	0.2834	0.4486	0.2668	0.3187	0.335	0.3863	0.3776	0.3534	0.3702
1280	0.2902	0.2058	0.116	0.1807	0.3015	0.2699	0.3252	0.2649	0.2556	0.2549
Blank	0.1023	0.1204	0.1065	0.0767	0.1162	0.0838	0.0796	0.0828	0.103	0.1207

Table 30 Absorbance of Vaccinated Ram serum samples against *M. haemolytica* on Dec. 14

	Animal ID									
Dilution	4	6	8	11	16	18	22	32	36	39
40	1.964	1.508	1.5559	1.3463	1.2775	1.1108	1.3841	1.5459	1.7291	1.1893
80	2.3022	1.2801	1.4221	1.0602	1.1611	1.1108	1.0877	1.4232	1.6097	1.3296
160	2.1495	1.0539	0.4214	1.0157	0.8489	0.805	0.9618	1.1427	1.2002	0.9208
320	1.6586	1.0122	0.9566	0.8185	0.8034	0.6575	0.7932	0.988	0.8617	0.7701
640	1.4999	0.6983	0.6765	0.5257	0.7791	0.379	0.7385	0.6958	0.7935	0.5248
1280	1.5604	0.5619	0.5002	0.6005	0.4291	0.3334	0.4733	0.5153	0.6436	0.4478
Blank	0.2416	0.0885	0.0803	0.0896	0.1031	0.078	0.0736	0.0715	0.0852	0.1026

Table 31 Absorbance of Control Ram serum samples against *P. multocida* on Dec. 14

	Animal ID									
Dilution	3	7	13	14	28	29	31	33	38	40
40	1.3107	1.0641	1.7337	1.0809	1.9964	1.4608	1.4621	1.301	1.2415	1.0039
80	1.1777	0.924	1.4068	0.7102	1.1953	0.8609	1.0103	1.2667	2.2802	1.0328
160	0.6478	0.5935	1.0261	1.7613	0.9361	1.2545	1.1372	1.2805	0.9152	0.7598
320	0.6846	0.4133	0.959	0.5159	0.7422	0.6267	0.5758	0.6576	0.9117	0.5674
640	0.5421	0.3974	0.7146	0.3403	0.5581	0.3746	0.4716	0.4162	0.5144	0.3975
1280	0.4019	0.3047	0.5254	0.3326	0.4249	0.3841	0.3641	0.3338	0.5049	0.3718
Blank	0.6008	0.2237	0.2465	0.2371	0.1965	0.1281	0.1951	0.1946	0.2333	0.3524

Table 32 Absorbance of Control Ram serum samples against *P. multocida* on Dec. 14

	Animal ID									
Dilution	4	6	8	11	16	18	22	32	36	39
40	1.1153	0.9333	0.9181	0.8121	0.7806	0.7111	0.7316	0.8749	0.8543	0.7951
80	1.0434	0.7195	0.8123	0.7158	0.8229	0.6105	0.6877	0.7489	0.8541	0.6509
160	1.0614	0.6025	0.6992	0.5976	0.6236	0.5831	0.5761	0.5047	0.7307	0.5624
320	0.873	0.4861	0.5524	0.5128	0.4804	0.4535	0.5367	0.4574	0.5841	0.4848
640	0.765	0.4429	0.4033	0.4301	0.4453	0.3052	0.4382	0.3419	0.414	0.3938
1280	0.702	0.3596	0.3496	0.315	0.3652	0.3222	0.3356	0.3193	0.3312	0.3208
Blank	0.1174	0.1295	0.1058	0.1296	0.1054	0.1096	0.1098	0.1139	0.1136	0.1333

6.2 Specific antibody titers

Table 33 Summary of titers for all animals

Treatment	Animal ID	Sex	Dates of Sampling	Specific antibody titers against *M. haemolytica.*	Specific antibody titers again *P. multocida*
Control	1	Ewe	Nov. 16	160	640
Control	2	Ewe	Nov. 16	640	320
Control	5	Ewe	Nov. 16	640	640
Control	9	Ewe	Nov. 16	320	320
Control	10	Ewe	Nov. 16	320	640
Control	12	Ewe	Nov. 16	640	320
Control	15	Ewe	Nov. 16	160	160
Control	19	Ewe	Nov. 16	640	640
Control	24	Ewe	Nov. 16	640	320
Control	35	Ewe	Nov. 16	160	320
Vaccinated	17	Ewe	Nov. 16	320	640
Vaccinated	20	Ewe	Nov. 16	1280	640
Vaccinated	21	Ewe	Nov. 16	1280	320
Vaccinated	23	Ewe	Nov. 16	640	1280
Vaccinated	25	Ewe	Nov. 16	320	320
Vaccinated	26	Ewe	Nov. 16	320	1280
Vaccinated	27	Ewe	Nov. 16	320	1280
Vaccinated	30	Ewe	Nov. 16	320	640
Vaccinated	34	Ewe	Nov. 16	160	640
Vaccinated	37	Ewe	Nov. 16	320	640
Control	3	Ram	Nov. 16	160	40
Control	7	Ram	Nov. 16	80	80
Control	13	Ram	Nov. 16	320	80
Control	14	Ram	Nov. 16	40	80
Control	28	Ram	Nov. 16	320	40
Control	29	Ram	Nov. 16	320	80
Control	31	Ram	Nov. 16	320	80
Control	33	Ram	Nov. 16	40	40
Control	38	Ram	Nov. 16	80	320
Control	40	Ram	Nov. 16	40	40
Vaccinated	4	Ram	Nov. 16	1280	320
Vaccinated	6	Ram	Nov. 16	1280	320

Table 33

Continued

Vaccinated	8	Ram	Nov. 16	640	320
Vaccinated	11	Ram	Nov. 16	640	320
Vaccinated	16	Ram	Nov. 16	640	160
Vaccinated	18	Ram	Nov. 16	320	320
Vaccinated	22	Ram	Nov. 16	320	320
Vaccinated	32	Ram	Nov. 16	640	320
Vaccinated	36	Ram	Nov. 16	320	160
Vaccinated	39	Ram	Nov. 16	320	80
Control	1	Ewe	Nov. 30	160	160
Control	2	Ewe	Nov. 30	80	160
Control	5	Ewe	Nov. 30	320	320
Control	9	Ewe	Nov. 30	160	160
Control	10	Ewe	Nov. 30	160	160
Control	12	Ewe	Nov. 30	320	320
Control	15	Ewe	Nov. 30	160	320
Control	19	Ewe	Nov. 30	80	160
Control	24	Ewe	Nov. 30	640	640
Control	35	Ewe	Nov. 30	80	320
Vaccinated	17	Ewe	Nov. 30	640	1280
Vaccinated	20	Ewe	Nov. 30	640	1280
Vaccinated	21	Ewe	Nov. 30	1280	640
Vaccinated	23	Ewe	Nov. 30	1280	1280
Vaccinated	25	Ewe	Nov. 30	1280	640
Vaccinated	26	Ewe	Nov. 30	1280	1280
Vaccinated	27	Ewe	Nov. 30	1280	640
Vaccinated	30	Ewe	Nov. 30	1280	640
Vaccinated	34	Ewe	Nov. 30	1280	1280
Vaccinated	37	Ewe	Nov. 30	1280	1280
Control	3	Ram	Nov. 30	40	160
Control	7	Ram	Nov. 30	160	320
Control	13	Ram	Nov. 30	640	640
Control	14	Ram	Nov. 30	160	320
Control	28	Ram	Nov. 30	320	320
Control	29	Ram	Nov. 30	320	640
Control	31	Ram	Nov. 30	320	160
Control	33	Ram	Nov. 30	80	160
Control	38	Ram	Nov. 30	40	160
Control	40	Ram	Nov. 30	40	80
Vaccinated	4	Ram	Nov. 30	1280	320

Table 33

Continued

Vaccinated	6	Ram	Nov. 30	1280	320
Vaccinated	8	Ram	Nov. 30	1280	320
Vaccinated	11	Ram	Nov. 30	1280	320
Vaccinated	16	Ram	Nov. 30	1280	640
Vaccinated	18	Ram	Nov. 30	1280	160
Vaccinated	22	Ram	Nov. 30	1280	160
Vaccinated	32	Ram	Nov. 30	1280	320
Vaccinated	36	Ram	Nov. 30	1280	320
Vaccinated	39	Ram	Nov. 30	1280	320
Control	1	Ewe	Dec. 7	40	40
Control	2	Ewe	Dec. 7	40	80
Control	5	Ewe	Dec. 7	640	640
Control	9	Ewe	Dec. 7	160	160
Control	10	Ewe	Dec. 7	80	80
Control	12	Ewe	Dec. 7	640	160
Control	15	Ewe	Dec. 7	40	40
Control	19	Ewe	Dec. 7	80	80
Control	24	Ewe	Dec. 7	80	320
Control	35	Ewe	Dec. 7	40	160
Vaccinated	17	Ewe	Dec. 7	160	40
Vaccinated	20	Ewe	Dec. 7	320	160
Vaccinated	21	Ewe	Dec. 7	1280	160
Vaccinated	23	Ewe	Dec. 7	1280	320
Vaccinated	25	Ewe	Dec. 7	1280	160
Vaccinated	26	Ewe	Dec. 7	1280	320
Vaccinated	27	Ewe	Dec. 7	640	80
Vaccinated	30	Ewe	Dec. 7	160	160
Vaccinated	34	Ewe	Dec. 7	40	80
Vaccinated	37	Ewe	Dec. 7	320	160
Control	3	Ram	Dec. 7	160	160
Control	7	Ram	Dec. 7	160	160
Control	13	Ram	Dec. 7	160	640
Control	14	Ram	Dec. 7	160	320
Control	28	Ram	Dec. 7	160	320
Control	29	Ram	Dec. 7	160	640
Control	31	Ram	Dec. 7	160	160
Control	33	Ram	Dec. 7	160	40
Control	38	Ram	Dec. 7	320	80
Control	40	Ram	Dec. 7	160	160

Table 33

Continued

Vaccinated	4	Ram	Dec. 7	640	1280
Vaccinated	6	Ram	Dec. 7	40	80
Vaccinated	8	Ram	Dec. 7	40	320
Vaccinated	11	Ram	Dec. 7	40	80
Vaccinated	16	Ram	Dec. 7	40	160
Vaccinated	18	Ram	Dec. 7	40	80
Vaccinated	22	Ram	Dec. 7	40	40
Vaccinated	32	Ram	Dec. 7	40	160
Vaccinated	36	Ram	Dec. 7	40	80
Vaccinated	39	Ram	Dec. 7	40	40
Control	1	Ewe	Dec. 24	40	80
Control	2	Ewe	Dec. 24	160	320
Control	5	Ewe	Dec. 24	80	320
Control	9	Ewe	Dec. 24	80	160
Control	10	Ewe	Dec. 24	80	160
Control	12	Ewe	Dec. 24	160	320
Control	15	Ewe	Dec. 24	80	320
Control	19	Ewe	Dec. 24	160	640
Control	24	Ewe	Dec. 24	640	320
Control	35	Ewe	Dec. 24	80	320
Vaccinated	17	Ewe	Dec. 24	1280	1280
Vaccinated	20	Ewe	Dec. 24	640	1280
Vaccinated	21	Ewe	Dec. 24	640	1280
Vaccinated	23	Ewe	Dec. 24	640	1280
Vaccinated	25	Ewe	Dec. 24	640	1280
Vaccinated	26	Ewe	Dec. 24	640	640
Vaccinated	27	Ewe	Dec. 24	640	1280
Vaccinated	30	Ewe	Dec. 24	320	1280
Vaccinated	34	Ewe	Dec. 24	640	1280
Vaccinated	37	Ewe	Dec. 24	320	1280
Control	3	Ram	Dec. 24	320	640
Control	7	Ram	Dec. 24	40	160
Control	13	Ram	Dec. 24	640	640
Control	14	Ram	Dec. 24	160	160
Control	28	Ram	Dec. 24	320	640
Control	29	Ram	Dec. 24	160	320
Control	31	Ram	Dec. 24	320	320
Control	33	Ram	Dec. 24	320	320
Control	38	Ram	Dec. 24	320	320

Table 33

Continued

Control	40	Ram	Dec. 24	80	320
Vaccinated	4	Ram	Dec. 24	1280	1280
Vaccinated	6	Ram	Dec. 24	1280	160
Vaccinated	8	Ram	Dec. 24	1280	320
Vaccinated	11	Ram	Dec. 24	1280	160
Vaccinated	16	Ram	Dec. 24	1280	160
Vaccinated	18	Ram	Dec. 24	320	160
Vaccinated	22	Ram	Dec. 24	1280	320
Vaccinated	32	Ram	Dec. 24	1280	80
Vaccinated	36	Ram	Dec. 24	1280	320
Vaccinated	39	Ram	Dec. 24	1280	160