Enzootic pneumonia in sheep: ewe and lamb immune response after *Mannheimia haemolytica* vaccine administration under field condition in Italy

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SUMMARY

Mannheimia haemolytica related pneumonia is characterized by high morbidity and mortality in lambs and thus is a common cause of important economic losses in sheep industry due to reduction in lamb growing and decreased carcass value. Due to the frequency of the disease, vaccination is a very common practice in Italy but the extent and duration of colostral protection in Sarda lambs born from vaccinated dams is not known. In this study, the extent and length of colostral antibody protection in Sarda lambs born from *M. haemolytica*-vaccinated ewes was evaluated in field condition. The in-field trial took place in two different farms in Sardinia region (Italy). A total of forty-five adult healthy pregnant Sarda sheep were enrolled for the study (24 from the first flock and 21 from the second flock). Each flock in each farm was divided in two groups: unvaccinated control group (10 sheep from the first flock and 8 from the second flock) and vaccinated sheep (14 from the first flock and 13 from the second flock). A total of forty-five lambs born from both vaccinated (n=27) and unvaccinated (n=18) dams were included in the study. Colostrum was collected from all the dams within 6-12 hours after lambing. Blood samples were taken from all the lambs included in the study at 48 hours, 15, 30, 45, 60 days after parturition. Antibody responses following vaccinations were detected by ELISA test. In the trial, antibody titres both on serum of ewes and lambs and colostrum were comparatively examined and important differences were observed in vaccinated pregnant ewes where serum titres developed after vaccination resulted two times higher than in the unvaccinated group (p-value=0.0029); within the lamb groups, an increase of serum ELISA titres was found in lambs born from vaccinated ewes until 60 days after birth. Colostral titres didn't show any significant difference. In conclusion, when the control group and the trial group were compared, vaccinating the lambs belonged to vaccinated dams showed significant differences concerning antibody responses against *M. haemolytica*.

KEY WORDS

Vaccination; immunity, enzyme-linked immunosorbent assay; Sarda sheep.

INTRODUCTION

Sheep farming is a significant industry in Italy. According to 2018 data reported by the National Data Bank (BDN), more than a half of the total amount of sheep reared belongs to Sarda breed. Pneumonia is responsible for important economic losses in sheep farming worldwide[1]. *Mannheimia haemolytica* is a Gram-negative, facultative anaerobic, non-motile, opportunistic pathogen bacteria and is one of the most important respiratory pathogens in the family *Pasteurellaceae* able to cause serious outbreaks of acute pneumonia in sheep of all ages including in neonatal, weaned and growing lambs. *M. haemolytica* and the related pneumonia is common and is distributed worldwide although the prevalence of serotypes may vary by region and flock; it is a commensal of the upper respiratory tract of healthy sheep that can cause pneumonia either alone or in conjunction with other organisms. The interaction of the host with respiratory pathogens such as respiratory syncytial virus, adenovirus, parainfluenza virus type 3, and *Mycoplasma* spp. in particular, under the influence of environmental factors and stressors are thought to break down the mucosal barrier integrity of the lower respiratory tract, allowing to *M. haemolytica* to colonize, proliferate and induce significant tissue damage[2,3,4,5,6]. The disease is also called "shipping fever" because it appears to occur most often in animals that have undergone recent stress such as weaning, transportation, changes in diet or cohabitation with new animals from different farms. Clinical signs include an acute onset of hyperthermia (>40,5°C), dyspnea, anorexia, depression, inappetance, lethargy. In lambs pneumonia causes mortality, decreased growth at considerable cost to the industry, along with animal welfare implications. Death losses are high in severely affected animals within 12 hours of the onset of any clinical signs[7]. Gross lesions typically show a fibrinous, necrotic pneumonia. The introduction of an effective antibiotic therapy is necessary when the disease outbreaks into a flock. Treatment shall begin at the rise of the pathology be-

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cause of the rapid progression of lung damage and endotoxin release. Administering prophylactic antibiotics to at-risk lambs may be beneficial even if this would bring towards the development of antimicrobial resistance. Management measures to reduce known stressors such as overcrowding, poor ventilation, high and cold temperatures should also be considered. In severely affected flocks response to therapy is often disappointing because of the widely use of antimicrobials and the related resistance. For these reasons, for the economic costs of treatment and reduction of lamb weight in survivors, application of prophylactic measures would be desirable. Due to the frequency of the disease, vaccination is a very common practice in Italy but the extent and duration of colostral protection in Sarda lambs born from vaccinated dams is not known. Due to the lack of studies on immunities of *M. haemolytica* in Sarda sheep lambs, a key question is how long lambs born from *M. haemolytica*-vaccinated ewes under field condition are protected. The aim of this study was to measure the extent and length of colostral antibody protection in Sarda lambs born from *M. haemolytica*-vaccinated ewes. The immune response of both ewes and lambs with until 60 days of life was evaluated by measuring specific serum and colostral antibody titres produced against the bacterium by ELISA test.

MATERIALS AND METHODS

Study design, sample size and animals

The in-field trial took place in two different farms in Sardinia region (Italy). A total of forty-five adult healthy pregnant Sarda sheep were enrolled for the study (24 from the first flock and 21 from the second flock). Sheep selected were similar for body condition score, age (between 2 and 3 years) and at the same month of pregnancy. Each flock in each farm was divided in two groups: unvaccinated control group (10 sheep from the first flock and 8 from the second flock) and vaccinated sheep (14 from the first flock and 13 from the second flock). A total of eighteen unvaccinated sheep from the two flocks and the relative lambs were used as unvaccinated controls in the study. In total eighteen lambs were born from these ewes. Twenty-seven ewes from the two flocks were vaccinated for the first time six weeks prior to lambing. Vaccinated ewes were reinoculated at 2 week intervals. Serum was collected 2 weeks after the second vaccination (two weeks before lambing). In total twenty-seven lambs were born from these ewes. A total of forty-five lambs born from both vaccinated (n=27) and unvaccinated (n=18) dams were included in the study. The farmer checked carefully that all the lambs received colostrum. Colostrum was thus collected from all the dams within 6-12 hours after lambing. Serum samples were taken from all the lambs included in the study at 48 hours, 15, 30, 45, 60 days after parturition. A total of 5 lambs came to death during the study period (Table 1).
Colostrum samples and serum samples from both dams and lambs were tested for *M. haemolytica* antibodies by ELISA home made test.

Vaccination

The sheep were vaccinated subcutaneously with 2 ml per injection on the site around the neck. The vaccine was obtained from formalin killed cells of the epidemiologically most important serotypes of *M. haemolytica* (AI, A2, A6, A7 and A9), grown under iron restricted conditions: $5x10^8$ cells per strain in buffered physiological saline adsorbed onto aluminum hydroxide. Thiomersal was included as a preservative.

ELISA assay

Blood samples were taken lambs from the jugular vein using vacutainer tubes six times and sera were stored at −20°C until serological tests were performed.
The serum of a newborn lamb born from an unvaccinated ewe was used as negative control of the test. ELISA test was performed for the determination of antibody levels in lambs born from vaccinated and unvaccinated dams as follows: the vaccine was diluted in carbonate/bicarbonate buffer (0.05 M, pH 9.6) to a final concentration of $2.5x10^6$ cells/well and used to coat 96-well plate with 100 µl/well (MaxiSorp). To normalize the effect of the adjuvant two lane were coated with adjuvant diluted in carbonate/bicarbonate buffer to 1.25%. After incubation over night at +4°C, the plate was washed three times in PBS containing 0.05% Tween 20 (PBST) and blocked by adding 200 µl/well of buffer containing 1% of casein in PBST (saturating buffer) and incubated at room temperature under stirring (300 rpm) for 1 h. After decanting the saturating buffer, 2-fold serial diluted (1:100-1:12.800) in saturating buffer, of vaccinated and unvaccinated sera were added in duplicate to the plate at 100 µl/well. In each plate a negative control (serum taken from a lamb immediately after birth) was added. After 1 h of incubation at room temperature under stirring (300 rpm), the plate was washed with PBST and an antibody rabbit anti-sheep peroxidase-conjugated (Santa Cruz Biotechnology, Inc) was added at the dilution of 1: 5000 in saturating buffer for 1 h. The plate was washed with PBST and incubated in the dark, with a peroxidase substrate solution (1-step Ultra TMB-Elisa-Thermo Scientific) for 20 minutes at room temperature, and finally the reaction was blocked with a 0.5% sulfuric acid solution and the optical density was measured at 450 nm by spectrophotometer microplate reader (Sunrise™ Basic Absorbance Reader -Tecan Trading AG). The averages of the duplicated OD values subtracted from the adjuvant value were recorded.

Table 1 - Number of lambs sampled from 48 hours until 60 days after birth in both farms.

Farm	Group	48 hours	15 days	30 days	45 days	60 days
1	Unvaccinated dams	10	10	10	10	10
1	Vaccinated dams	14	14	14	14	14
2	Unvaccinated dams	8	8	8	8	8
2	Vaccinated dams	13	12	11	11	10
Total		45	44	43	43	42

Statistical analysis

The Pearson $\chi 2$ test was used to evaluate the difference between vaccinated and unvaccinated sheep and the presence of antibodies in dams, colostrum and lambs.
The non parametric Mann-Whitney test was used to assess at each time the quantitative difference between groups (vacci-

nated *vs.* unvaccinated). A p-value ≤ 0.05 was considered statistically significant. Calculations were carried out in Stata 11.2 and Miscrosoft Excel 2013.

RESULTS

Serum and colostrum antibodies titres on vaccinated and unvaccinated dams

To determine ELISA titres we considered that if the vaccine was effective in inducing specific antibody, the vaccinated animals would have higher specific antibody titres than control animals. In order of that, in this ELISA test, the cut off values were calculated similar to the methods described by Mehmet et al.[8] and on the unvaccinated control groups (sheep serum, colostrum, lambs at 48 hours, lambs at 15, 30, 60 days). The cut off values were calculated to the average values of the OD 450 nm obtained at the sample serum dilution 1:1600 adding 3 standard deviations. OD results were evaluated at the median serum diluition of 1:800 and each sample was considered "negative" when the value was below the cut-off and "positive" when was above.

Of the twenty-seven vaccinated ewes, 77.8% developed antibody titres >1: 800 detectable on serum by ELISA while of the eighteen unvaccinated ewes, 66.7% developed antibody titres ≤1: 800 (Table 2); a significant difference was found between these groups (p-value=0.0029).

Of the twenty-seven colostrum samples belonging to the vaccinated dams, only 18.5% developed antibody titres > 1:800 detectable by ELISA (Table 3). Colostrum samples did not showed any difference between the two groups (p-value=0.506).

Table 2 - Number of serum samples below and above the cut off value evaluated at the median serum diluition of 1:800 in vaccinated and no vaccinated ewes against *M. haemolytica* (% calculated on the total column).

Groups	Serum samples		
	≤ 1: 800	> 1: 800	Total
Vaccinated ewes	6 (33.3%)	21 (77.8%)	27 (60%)
Unvaccinated ewes	12 (66.7%)	6 (22.2%)	18 (40%)
Total	18 (100%)	27 (100%)	45 (100%)

Table 3 - Number of colostrum samples below and above the cut off value evaluated at the median serum diluition of 1:800 in vaccinated and no vaccinated dams against *M. haemolytica* (% calculated on the total column).

Groups	Colostrum		
	≤ 1: 800	> 1: 800	Total
Vaccinated dams	22 (57.9%)	5 (71.4%)	27 (60%)
Unvaccinated dams	16 (42.1%)	2 (28.6%)	18 (40%)
Total	38 (100%)	7 (100%)	45 (100%)

Serum antibodies titres on lambs born from vaccinated and unvaccinated dams

The results on the total of the serum samples (n=118) of the lambs born from vaccinated dams showed higher anti-*M. haemolytica* titres time by time until sixty days after birth in comparison to the samples from the lambs (n=90) born from the unvaccinated dams (Figure 1). In particular, significant differences were demonstrated at 15 days (p-value=0.04) after birth, whereas no significant differences were found at 2 days (p-value=0.501), 30 days (p-value=0.43), 45 days (p-value=0.74) and 60 days (p-value=0.246).

DISCUSSION

Infectious respiratory diseases of sheep and goats contribute to 5.6 percent of the total diseases of small ruminants[9]. *M. haemolytica* constitutes one of the most significant agents of

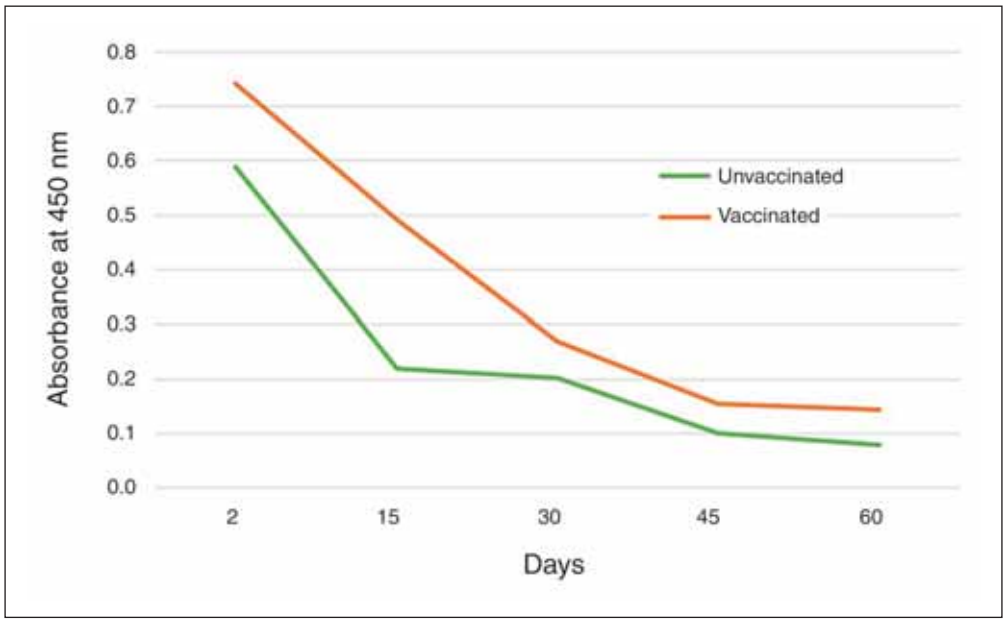

Figure 1
Mean antibody titres in serum against *M. haemolytica* in lambs from vaccinated and no vaccinated ewes at each sampling time.

respiratory infection, which is a major problem for sheep farmers. In a large Italian survey, 43.5% of the lambs between 25 and 35 days of life tested positive for *M. haemolytica* by PCR[3]. This result was in agreement with other reports in literature, which assess that lambs are most susceptible to the infection by this pathogen during the first months of life[7,10]. Until now, there are no published data on Sarda sheep supporting the knowledge about the extent and length of colostral antibody protection against *M. haemolytica* in lambs born from vaccinated ewes under field condition. This could lead to ensure that farmers can vaccinate their ewes at the optimal time prior to lambing to achieve maximum levels of colostral antibody transfer, maximizing the duration of colostral immunity, while also indicating when lambs should then be newly immunized without interfering with colostral immunity. Prevention of *M. haemolytica* infection by using a good vaccination program both on ewes and lambs would be expected to decrease pneumonia in lambs by developing good maternal and passive colostral immunity.

In this study, the length and efficacy of immunization with a vaccine against *M. haemolytica* infection in Sarda sheep were investigated. Serological determination of antibody titres in serum and colostrum in a group of vaccinated dams and relative lambs and that one of unvaccinated dams and relative lambs was tested. Antibodies were detected among the pregnant ewes and their lambs bled at 48 hours, 15, 30, 45, 60 days after birth by ELISA test. To the authors knowledge, the serum ELISA titres obtained in this study could not be compared because no similar studies were performed in Italy on Sarda sheep; on the contrary, for the determination of antibody titres obtained after *M. haemolytica* vaccinations, many foreign studies have been performed and different antigens and techniques have been used[11,12]. Amongst these techniques, ELISA is widely used[13]. Mosier et al.[14], showed a positive correlation between the ELISA titres and resistance to experimental pasteurellosis in cattle and reported that ELISA was a more feasible and faster technique considering the number of sera that could be tested readily. In this study, significant increase of serum ELISA titres following vaccination in pregnant ewes and its efficacy on immunity on lambs is shown. In vaccinated pregnant ewes, serum titres developed after vaccination resulted two times higher than in the unvaccinated group (Table 2), this difference was statistically significant (p-value=0.0029). On the contrary, colostral titres didn't show any significant difference between the two groups examined (Table 3). This result suggested that in many cases colostrum couldn't be sampled within the 6-12 hours after birth and it must be considered that this kind of matrix is highly degradable[15]. Within the lamb groups, an increase of serum ELISA titres was found in lambs born from vaccinated ewes until 60 days after birth (Figure 1). After this period, lamb passive immunity seems dangerously to decrease and this can lead to the development of pneumonia. The result is in agreement with the reports which assess that the major incidence of respiratory diseases (over 30%) is registered in sheep between 3 and 6 months of life[7,10]. The major differences regarding the concentration of antibody titres against *M. haemolytica* were found at 15 days (p-value=0.04). According to the results obtained in this investigation, *M. haemolytica* vaccination on pregnant ewes between six and four weeks prior to lambing seems to provide a good passive immunity to lambs until 60 days after birth.

CONCLUSIONS

Nowadays, the Italian sheep industry is facing many difficulties because of the minimal public funds given to the farmers and the lowest profits in the agricultural industry. Given the significant economic losses due to *M. haemolytica* infection and the increasing antibiotic resistance, it is necessary to adopt useful preventive tools for the benefits of the whole sheep industry. The results obtained from this investigation showed that immunization of ewes and lambs with a vaccine containing *M. haemolytica* AI, A2, A6, A7 and A9 bacterins, under field condition and between the sixth and the fourth week prior to lambing significantly increases serum antibody titres and provides good protection to lambs.

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