EVALUATION OF A COMBINED VACCINE AGAINST STAPHYLOCOCCAL MASTITIS IN EWES

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Abstract

The effectiveness of combined staphylococcal vaccine (CSV), prepared from Staphylococcus aureus and coagulase negative staphylococci (CNS: Staphylococcus epidermidis, S. simulans, S. saprophyticus), on staphylococcal mastitis in sheep was determined. The group of pregnant sheep was vaccinated subcutaneously into supramammary lymph node twice at 3 week interval. Similarly, sterile saline was administered to controls. Blood and milk samples were collected before and at days 21 and 51 after the vaccination. The titre of S. aureus and CNS antibodies in serum and whey was determined using a modified ELISA. Levels of staphylococcal antibodies in serum and whey of the vaccinated animals were significantly higher compared with the controls. Intramammary infection rate by S. aureus was lower in the CSV group (7.70%) compared with the controls (30.76%). While intramammary infection rate for CNS was 19.23% in the controls, no isolate was demonstrated in the CSV group. Therefore, the effectiveness of CSV was found to be useful to decrease intramammary infection rate of S. aureus and CNS in ewes.

Key words: sheep, mastitis, vaccine, staphylococci

Mastitis is one of the most important health and economic problems in dairy farming (3, 16). While subclinical mastitis in sheep is a significant problem worldwide, sheep mastitis in the Mediterranean countries is particularly important because of extensive milk production (6, 11, 14).

Staphylococcus aureus is recognized as the most prevalent causative agent of contagious mastitis in dairy animals (3, 4, 16). Treatment and prevention of S. aureus mastitis with antimicrobials are difficult and expensive (21). S. aureus strains develop resistance against antimicrobial agents. This has constrained the scientists to seek alternative methods to prevent the disease (3). Immunization with vaccines has been advocated in dairy animals and encouraged since it is more friendly to the environment (5, 7). Coagulase negative staphylococci (CNS) were conventionally evaluated as apathogenic or weak pathogenic microorganisms (1, 13). Recently, it has been emphasized that CNS were also isolated from cases of clinical mastitis as primary agents of subclinical mastitis in sheep (4, 13). S. epidermidis, S. haemolyticus, S. simulans, S. xylosus, S. chromogenes, S. warneri and S. caprae were frequently isolated strains, in the decreasing order (4, 6, 14). However, the strains displayed different pathogenic properties (13). It was found that while S. epidermidis was mostly isolated from milk samples of mastitis affected animals, S. simulans was the most pathogenic microorganism among CNS (1, 19).

At least 30% of the staphylococcal subclinical mastitis do not respond to antibiotic therapy and/or control programmes (21). However, approximately 20% of the mastitis may recover spontaneously without antimicrobial therapy (20). The spontaneous recovery of intramammary infections (IMI) with no therapy has been explained by the development of humoral and cellular immunity in the udder resulting from the presence of bacteria (10). The development of local immunity in the udder by the vaccine causes increase in the spontaneous cure rate of IMI, lessens the severity of existing infections and decreases the milk yield loss (8, 12). Several field vaccination trials have revealed some degree of protection against intramammary infections (2, 5, 8-10, 12, 15, 17, 18).

The purpose of this study was to prepare a combined staphylococcal vaccine (CSV) containing local strains of S. aureus, S. epidermidis, S. simulans and S. saprophyticus isolated from clinical and subclinical mastitis cases, to measure the levels of antibodies against staphylococcal antigens in blood serum or whey and to determine the efficacy of CSV on the staphylococcal IMI under field conditions in ewes.

Material and Methods

Animals. Twenty pregnant Merinos crossbred ewes, 1.5-2 years of age, with no clinical udder abnormalities, were included in the study. Regarding the age, lactation number and expected time of lambing, the
ewes were divided into two equal groups: vaccinated and non-vaccinated.

**Bacterial strains.** *S. aureus*, *S. epidermidis*, *S. simulans* and *S. saprophyticus* strains were used as antigens. All the strains were isolated from clinical and subclinical ovine mastitis cases.

**Vaccine and vaccination.** The strains were streaked on plates of brain-heart infusion agar and incubated at 37°C for 18 h. Then, each strain was separately subcultured in brain-heart infusion at 37°C for 24 h. The bacteria were harvested by centrifugation (3000 g, 30 min), washed and resuspended in 0.15 mol/l PBS (pH 7.2). Bacterial concentrations were adjusted to 1.2 x 10^9 cells/ml. Formalin (0.3% v/v) was added to inactivate the bacteria. Equal volumes of each bacterial culture were mixed and then the mixed cultures were absorbed with 4% aluminium hydroxide (5).

One ml of the vaccine was administered subcutaneously in the draining region of the supramammary lymph node. The ewes were vaccinated twice at 21 d interval. The second dose was given 20 d before lambing. Controls were similarly treated with 1 ml of sterile normal saline (15).

**Sampling.** Blood samples were taken at the day of inoculation (day 0) and 21 and 51 d after vaccination. Milk samples were aseptically collected from all halves of vaccinated and non-vaccinated animals at the day of inoculation and 51 d after vaccination. In order to obtain whey, 0.2 ml of rennin and 0.1 ml of CaCl2 were added to 10 ml milk sample and the sample was incubated at 37°C for 2 h. Then, it was centrifuged at 3000 rpm for 15 min and the supernatant was removed as whey.

**Adverse reactions.** Adverse reactions after the vaccination were recorded by the observation of animal behaviour and palpating the injection site.

**Bacteriological examinations.** Quarter foremilk samples were taken aseptically and submitted to the laboratory within 1 h at 4°C. Bacteriological examinations were performed according to classical procedures (4).

**Detection of staphylococcal antibodies.** The serum and whey samples were analysed for the presence of IgG antibodies to killed *S. aureus*, *S. epidermidis*, *S. simulans* and *S. saprophyticus* antigens in a modified ELISA (7, 9). In brief, 96-well immunoplates were coated with 100 µl/well of the antigens and suspended in carbonate-bicarbonate buffer (pH 9.6) at 1.2x10^9 cells/ml. The plates were incubated at 37°C for 1 h and overnight at 4°C. Then, 100 µl of 3% bovine serum albumin was added to the wells and incubated for 60 min at room temperature. Then the plates were washed 3 times for 5 min with the solution of PBS-T; 0.15 mol/l, and 0.5 % Tween 20 (pH 7.2).

Blood serum samples were diluted 10, 20, ..., and 5120 times and 100 µl from each dilution was added to the wells and the plates were incubated at 37°C for 1 h. After washing, 100 µl of 0.4 mg/ml 0.05 M phosphate-citrate buffer, of δ-phenylenediamine dihydrochloride (δ-phenylenediamine tablets, Sigma P 8287, St Louis, USA) was added as substrate and the plates were reincubated for 20 min at room temperature. The plates were read in a microplate autoreader (Anthos Labtec Instruments, A 5022, Salzburg) at 450 nm. Positive and negative serum standards were added to each plate.

Whey samples were diluted 1:40 and the same procedures were applied.

Statistical analysis. Analysis of variance (ANOVA) was used to analyse the significance within the groups.

**Results**

The level of antibodies to staphylococcal antigens in blood sera of the vaccinated and non-vaccinated sheep were shown in Table 1. Although the mean pre-vaccination antibody levels increased on days 21 and 51 (P<0.05), the increase was especially notable in regard to *S. saprophyticus* and *S. simulans*. While the titres of antibodies to *S. aureus*, *S. saprophyticus* and *S. simulans* revealed to be significantly higher after the 2nd vaccination (P<0.05), no significant difference (P>0.05) towards *S. epidermidis* was observed. In the control group, the level of antibodies to staphylococcal antigens remained nearly constant during the study period.

Documentation of antibody level in whey is shown in Table 2. It is evident from the Table that the level of antibodies to staphylococcal antigens of vaccinated sheep outnumbered those of non-vaccinated sheep.

The results of microbiological examination of milk samples from vaccinated and non-vaccinated sheep were shown in Table 3. Lower rate of intramammary infections caused by *S. aureus* was recorded in vaccinated animals by comparison to controls. *S. aureus* was isolated from 8 (30.76%) and 2 (7.70%) udders with IMI in the controls and vaccinated group, respectively. While CNS were isolated from 5 (19.23%) udders of controls with IMI, no isolation was found in vaccinated sheep. In addition, some other microorganisms (*Corynebacterium* sp., *Bacillus* sp., *Proteus* sp., *Streptococcus* sp., *Citrobacter* sp.) were also isolated from milk samples.

**Discussion**

The aim of this study was to investigate CSV in the control of mastitis in ewes. Selected strains of *Staphylococcus* species (*S. aureus*, *S. epidermidis*, *S. simulans* and *S. saprophyticus*) isolated from cases of clinical and subclinical mastitis were used to prepare the CSV.
**Table 1**
The levels of antibodies to staphylococcal antigens in blood serum*

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Before vaccination</th>
<th>21 d after vaccination**</th>
<th>51 d after vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>1.70 ±0.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.90±0.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.80±0.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>1.70 ±0.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.40±0.34&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.70±0.21&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>1.80±0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.50±0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.70±0.37&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>1.80±0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.00±0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.70±0.21&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>S. simulans</td>
<td>1.20±0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.90±0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.00±0.57&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>1.20±0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.50±0.17&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.50±0.27&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>S. saprophyticus</td>
<td>1.80±0.39&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.80±0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.40±0.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>1.80±0.39&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.40±0.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.70±0.36&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Serum dilutions 1/10, 1/20, 1/40, and so forth, were expressed numerically as 1, 2, 3, and so forth, respectively. a, b, c: different numbers within the column mean statistically significant differences (P< 0.05)

**Table 2**
Antibodies to staphylococcal antigens in whey*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of halves</th>
<th>S. aureus</th>
<th>S. epidermidis</th>
<th>S. simulans</th>
<th>S. saprophyticus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>20</td>
<td>14</td>
<td>6</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Control</td>
<td>18**</td>
<td>5</td>
<td>13</td>
<td>5</td>
<td>13</td>
</tr>
</tbody>
</table>

* Whey was tested at the day of inoculation and 51 d after vaccination.  ** 1 half from 2 sheep was blind.

**Table 3**
The results of microbiological examinations of milk samples

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Groups</th>
<th>Vaccinated</th>
<th>Control</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>S. aureus</td>
<td>2</td>
<td>7.70</td>
<td>8</td>
<td>30.76</td>
</tr>
<tr>
<td>CNS</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>19.23</td>
</tr>
<tr>
<td>Corynebacterium sp.</td>
<td>3</td>
<td>11.53</td>
<td>2</td>
<td>7.70</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>7.70</td>
</tr>
<tr>
<td>Proteus sp.</td>
<td>-</td>
<td>1</td>
<td>3.84</td>
<td>1</td>
</tr>
<tr>
<td>Streptococcus sp.</td>
<td>2</td>
<td>7.70</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Citrobacter sp.</td>
<td>1</td>
<td>3.84</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

Administration of the vaccine to the draining area of the supramammary lymph node has been reported to stimulate the changes in the local lymph node and thus enhance the immunity response (10, 12, 20). However, it has been pronounced that swelling or sterile granuloma may be formed at the injection site as a result of adjuvants used (15). In our study, local adverse reactions were not observed after vaccination except limited swelling at the injection site. In addition to this, no adverse reactions were observed in mice used to check the vaccine safety and toxicity. Thus, CSV can be considered safe for use in animals.

Various experimental and field trials (2, 5, 8-10, 12, 15, 17, 18) showed that the antibodies against staphylococcal antigens in blood serum and whey of vaccinated animals were detected by two serological methods (ELISA and agglutination). It was also reported that the isolation ratio of *S. aureus* and CNS in microbiological examinations and challenge trials was lower in vaccinated animals than in controls (5, 8, 9, 15). Watson (17) reported that pregnant ewes immunized with dextran sulphate (DS) adjuvanted vaccine, containing staphylococcal β-haemolysin and killed *S. aureus* cells, showed significantly elevated *S. aureus* pseudocapsule and β haemolysin antibody titres.
and displayed lower incidence of various forms of mastitis after challenge. Watson (18) pointed out that the protection against staphylococcal mastitis was formed also in sheep vaccinated with attenuated S. aureus. Amerona et al. (2) reported that high serum antibody titres against whole cells of S. aureus, S. simulans, S. hyicus and S. epidermidis were obtained in sheep immunized with DS adjuvanted inactivated S. aureus and S. simulans vaccine. In addition, the prevalence of mastitis was lower. Tollersrud et al. (15) found that mineral-oil and water-soluble acid polymer resin (Carbopol) adjuvanted vaccines stimulated antibody production against the α- and β-haemolysins and the exopolysaccharide of S. aureus.

In this study, the higher titres of antibodies against staphylococcal antigens were demonstrated in serum and whey of vaccinated sheep than those of controls. The higher antibody titre to S. saprophyticus was observed after double vaccination. While the increase in antibody titres against S. aureus, S. simulans and S. saprophyticus was found statistically significant after double vaccination, no significantly different antibody titres to S. epidermidis were observed.

In our study, lower rate of IMI with S. aureus occurred in vaccinated animals than in controls. In addition, there was no isolate of CNS in the vaccinated sheep. Several authors (2, 10) reported that vaccines prepared from S. aureus strains did not prevent IMI caused by CNS. It has been found that CVS influenced the isolation of CNS from milk samples of the vaccinated animals. The findings of serological and microbiological examinations in our study are in agreement with the results reported by Watson (17, 18), Amerona et al. (2) and Tollersrud et al. (15).

In conclusion, results of our study showed that the CVS is very effective in the control of the incidence of IMI caused by Staphylococcus species. Therefore, staphylococcal vaccination programme will be recommended to be included in the control of mastitis together with hygienic methods in sheep herds. However, the vaccine should be checked earlier on bigger groups of vaccinated and control animals.

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References