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Efficacy of Bovine Staphylococcal Mastitis Vaccine Composed of Alpha toxin, Capsular Polysaccharide and Fibronectin Binding Protein in Lactating Cows and Heifers

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비유우와 처녀우에서 황색포도상구균의 alpha toxin, capsular polysaccharide와 fibronectin binding protein으로 구성된 유방염 백신의 효능

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요 약 : capsular polysaccharide(CPS), alpha toxin과 재조합 fibronectin binding protein (FnBP)으로 구성된 유방염 백신을 개발하여 야외효능실험을 수행하였다. 이 subunit 백신은 비유우 23두와 처녀우 20두를 대상으로 10개월동안 각각 수행되었다. 비유우는 매 3주간격으로 2회 백신접종을 하였으며 대조군은 PBS를 상유방림프절 주위에 피하주사하였다. 처녀우를 대상으로 한 실험에서는 분만 8주전부터 시작하여 매 3주간격으로 2회 비유우 백신접종부위와 동일하게 접종하였다. 접종후 비유우에서는 황색포도상구균에 의한 총유선내 감염율이 대조군에 비해 유의성있게 감소하였으며($p<0.001$) 처녀우에서도 총유선내 감염이 백신접종군(1.6%)이 대조군(11.7%)에 비해 다음 비유기동안 유의성있게 낮았다($p<0.001$). 비유기동안 백신접종한 젖소의 체세포수는 변화가 없었으며 처녀우에서는 추가접종후 백신접종군에서 대조군의 체세포수에 비해 낮았지만 통계학적인 유의성은 없었다($p>0.05$). 본 실험결과 황색포도상구균에 대한 유방염 아단위 백신은 비유우와 처녀우에서 체세포수를 증가시키지 않고 총 유선내 감염율을 낮추어주었다. 하지만 본 실험은 1군데의 목장을 대상으로 하였기 때문에 향후 대규모 목장을 대상으로 하는 유방염 백신의 야외효능실험이 심도있게 이루어져야 한다고 생각된다.

Key words : alpha toxin, capsular polysaccharide, fibronectin binding protein, mastitis vaccine

Introduction

Bovine mastitis is the most important infectious disease. It causes the quality and the yield of milk to low. Thus it has been rocognized as the most economically important disease in dairy cows. All over the world, economical losses caused by this bovine mastitis amount to nearly $ 35 billion annually [2].

Despite several preventive programs have been used to minimize bovine mastitis, including hygienic milking and cleaning procedures, pre-and postmilking teat disinfection, antibiotic therapies and culling, prevalence of mastitis approaches 19 to 40% of dairy cows. According to the duration and severity of intramammary infection (IMI), the productive performance

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of infected cows may be dim- inished permanently.

A variety of studies on staphylococcal mastitis vaccine have been made, mainly on employing killed *Staphylococcus aureus* (*S. aureus*)[11,12], toxoids[18], protein A[16], capsular polysaccharide (CPS)[1,19] and fibronectin binding protein (FnBP)[8] or killed cells and toxoids[3,13,14,20]. Reportedly[10], these vaccines increased the spontaneous cure rate of infections and reduced the severity of the clinical signs, but did not prevent the occurrence of new infections during the study.

Recently several attempts have been made to prevent the major mastitis-causing pathogens. However, until now there were no efficient and completely preventive programs to reduce mastitis. Thus it is clear that new and efficient approaches to mastitis prevention are needed. Of all means to decrease and protect the impact of mastitis on the dairy industry, development of mastitis vaccine was focused on increasing the natural ability of the cows to resist infections because defense of the mammary gland against mastitis-causing pathogens is mediated by several protective factors. Of several virulence factors, alpha toxin, CPS and FnBP showed that these antigens had the protective ability in experimental animals and dairy cows.

This study was performed to evaluate the preventive effects of mastitis vaccine in the lactating cows and heifers of a commercial farm and to provide basic informations for development of a more efficacious and econo- mical mastitis vaccine against *S. aureus*.

Materials and Methods

Bacterial strains

The bacteria used were a highly encapsulated *S. aureus* Smith stram and alpha toxin and FnBP-producing *S. aureus* Wood 46, provided by Dr. Johnson (Sweden University, Uppsala, Sweden).

Confirmation of CPS, alpha toxin and FnBP

The presence of the capsule was confirmed by diffuse morphology in semisolid agar with rabbit serum and by transmissible electron microscopy. The presence of alpha toxin was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with alpha toxin antibody (Sigma). The recombinant FnBP was provided by a collaborator, Dr. Doo Kim (Kangwon National University).

Vaccine preparation

The vaccine was prepared by the modified methods of Watson[18], Nickerson *et al.*[10]. and Nordhaug *et al.*[13,14]. Briefly, cells of *S. aureus* were grown in brain heart infusion broth supplemented with 10% whey at 37°C with aeration to stationary phase (determined by optical density). The cells were inactivated with formalin (1%, v/v), and centrifuged (at 6,000 g min at 4 for 20 min), and resuspended in PBS (pH 7.2). Each 5-ml dose containing 1×10^{10} cfu/ml of *S. aureus* Smith strain and a crude extract of the capsule from about 1×10^{10} cells/ml of *S aureus*. The crude CPS was prepared by the methods of Poutrel *et al.*[17]. Live cells were centrifuged, resuspended in a small volume of phosphate buffered saline (PBS) (pH 7.2), and autoclaved twice for 20 min. This preparation was centrifuged under the same conditions, and crude CPS was added to the vaccine preparation at a concentration of approximately 5 mg of dry weight per dose. Four hundred of alpha toxin and 1 mg of FnBP per dose were also added.

Sodium azide were added as preservatives at final concentrations of 0.0015% (w/v). Dextran sulfate (50 mg/ml, MW : 500,000; Sigma) and mineral oil (d = 0.88 g/ml, Sigma) were added as a adjuvant.

Herd evaluation

This trial was carried out on a commercial dairy farm in Dae Kwan Ryung, Kangwondo during 10 months. The dairy farm kept 200 lactating cows and 100 dry cows. Calving from pregnant cows occurred throughout the entire year, but calving frequencies were slightly higher in spring than any other seasons. Automatic milking machine was used and pre-miliking mastitis test and post-milking teat dipping were practiced. Dry and lactating cows were routinely treated with susceptible antibiotics. The mean somatic cell count (SCC) in bulk tank was 300,000 cells/ml during the year prior to the start of the trial and varied between 220,000 and 370,000 cells/ml throughout the experimental period. Heifers then received the same feed and treatment as the other cows. Cases of clin-

ical mastitis were routinely defined with premilking by the dairy farmer or the veterinarian. After sampling, diagnosed cases were treated by intramammary administration of antibiotics. No quarters with clinical mastitis were detected in the heifers prior to calving.

Experimental design

Twenty three lactating cows were used, and vaccinated and control groups were allotted to 20 and 3 lactating cows respectively. Twenty heifers were allocated into vaccinated (n=17) and control (n=3) groups.

The heifer group received two injections of the vaccine at 7 and 4 weeks before calving, and the lactating group received two times every 3 weeks. The control group received two injections of PBS during the lactation period. The vaccine or the placebo was administered subcutaneously in the area of SMLN.

Premilking sampling and microbiological analysis

Milk samples from all quarters were collected every 2 weeks during the first 3 months and every 3-4 weeks during subsequent months of the trial in lactating cows. In heifers, samples were taken every 3-4 weeks following calving. Prior to taking samples, the teats were disinfected with 70% alcohol soaked with 1% iodine. The several streams of milk was discarded on the black-colored rubber plate, and 10-ml of the milk sample was collected in 15 ml sterile glass tubes by the laboratory staff. While the veterinarians were collecting samples, the milk and udder abnormalites (clotting, heat, swelling, discoloration) were recorded. The milk samples were kept at 4°C until processing in the laboratory and samples were processed within 6 hrs. For the bacterial isolation, 10 μl of milk sample was streaked on bovine blood agar (5%, v/v) and incubated at 37°C for 24 hrs. The number and type of the colonies and the pattern of hemolysis were recorded. Gram-positive cocci were selected and further investigated for production of catalase and coagulase. *S. aureus* was confirmed as the isolation method of the International Dairy Federation described previously[6]. The SCC was determined electronically with a Fossomatic coulter counter (Fossomatic Co., Ltd, Denmark) according to the technique recommended by the International Dairy Federation as described previously[7].

Definition of mastitis (clinical, subclinical and latent)

The determination of the clinical mastitis was based on the presence of a primary pathogen (*S. aureus*) in milk samples and clinical signs with heat, clotting, swelling of the udder. Teats with somatic cell count (SCC) of more than 250,000 cells/ml and absence of *S. aureus* were considered to be indicative of subclinical mastitis. The presence of *S. aureus* without a corresponding increase in SCC was included as a latent infection.

Statistical analysis

The chi-square test was used to compare the rates of mastitis. The Student's t test was applied to compare mean SCC. The SAS software (released 6.04; SAS institute, Cary NC, USA) was employed for these analyses. Significance was considered at $p<0.05$.

Results

Herd evaluation before experiment

No different parity was observed between vacci-

Table 1. Frequencies of bacteria isolated from milk prior to initiating the field trial

Organisms	Numbers*	Percentage (%)
Staphylococcus aureus	13/176	7.3
Staphylococcus spp#	17/176	9.7
Streptococcus spp**	10/176	6.0
Proteus spp	8/176	4.5
Pseudomonas spp	6/176	3.4
Fungus##	8/176	4.5
Contamination+	27/176	15 3
Negative+-	87/176	49.0

*Number of quarters inoculated.
#Coagulase positive and negative staphylococci were categorized in *Staphylococcus* spp.
***Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis* were categorized in *Streptococcus* spp.
+When premilk samples were taken, samples were contaminated with environmental microorganisms.
+-After premilk sample was streaked on 5% bovine blood agar plate at 37 for 24 hrs, no bacterial growth was observed.
*#Fungus included yeast and mold.

Table 2. Cumulative incidence of clinical, subclinical and latent infection in lactating cows immunized with mastitis vaccine composed of alpha toxin, capsular polysaccharide and fibronectin binding protein twice every 3 weeks

Diagnosis	Treatment group	
	Control (%)	Vaccinated (%)
Clinical mastitis*	12/104(11.5)[a]	33/584(5.7)[b]
Subclinical mastitis**	9/104(8.7)[c]	8/584(1.4)[d]
Latent infection***	12/104(11.5)[e]	21/584(3.6)[f]
Total intramammary infection	33/312(10.6)[e]	62/1752(3.5)[d]

[a,b](p<0.05); [c,d](p<0.001); [e,f](p<0.01) significant difference.
*Clinical mastitis was based on the presence of *S. aureus* in milk samples and clinical signs with heat, clotting, swelling of the udder.
*Subclinical mastitis was based on teats with somatic cell counts of more than 250,000 cells/ml and absence of primary pathogens.
***Latent infection was based on the the presence of a primary pathogen without a corresponding increase in SCC.

Table 3. Cumulative incidence of clinical, subclinical and latent infection in heifers immunized with mastitis vaccine composed of alpha toxin, capsular polysaccharide and fibronectin binding protein twice every 3 weeks

Diagnosis	Treatment group	
	Control (%)	Vaccinated (%)
Clinical mastitis*	5/40(12.5)[a]	6/168(3.6)[b]
Subclinical mastitis**	3/40(7.5)[a]	1/168(0 6)[b]
Latent infection***	6/40(15)[a]	2/168(1.2)[a]
Total intramammary infection	14/120(11.7)[c]	8/504(1.6)[d]

[a,b](p<0.05), [a,a](p>0 05); [c,d](p<0.001) significant difference.
*Clinical mastitis was based on the presence of *S. aureus* in milk samples and clinical signs with heat, clotting, swelling of the udder.
*Subclinical mastitis was based on teats with somatic cell counts of more than 250,000 cells/ml and absence of primary pathogens.
***Latent infection was based on the the presence of a primary pathogen without a corresponding increase in SCC.

nated and control group and total teats used in this study were 171 except 1 blind teat.

Mean SCC in herd was 300,000 cells/ml and non-infected Holstein-Fresian cows with *S. aureus* before initiating this experiment were selected and used. Non-infected cows were considered when pre-milking samples inoculated were negative in bacterial growth on 5% bovine blood agar plate incubated for 24 hrs.

Bacterial prevalence of the herd was shown in Table 1. The prevalence of *S. aureus* and Streptococci spp. are 7.3% (13/176) and 6.0% (10/176), respectively.

Clinical signs

Anaphylactic shock did not occur in the 37 vaccinated cows included both lactating cows and heifers after vaccination. However, a transitory swelling, erythema and heat around the vaccinated site were observed and swelling was disappeared spontaneously within 7-14 days following vaccination. Slight lameness was also appeared in hindlimb of vaccinated side. However, any other adverse reaction was not recorded in both lactating cows and heifers.

Incidence of mastitis

Table 2 shows the cumulative incidence of clinical mastitis caused by *S. aureus* in the quarters of the 23 lactating cows during 10 months. The clinical mastitis incidence following immunization was higher in the control group, compared with that in the vaccinates ($p<0.05$). The subclinical mastitis incidence following immunization was significantly higher in the controls than that of the vaccinated group ($p<0.001$). The cumulative incidence of latent infection in lactating cows showed no significant difference between groups after immunization ($p<0.05$). The frequencies of IMI caused by *S. aureus* in vaccinated group were lower than the control group following immunization in lactating cows ($p<0.001$). The frequencies of clinical and subclinical *S. aureus* mastitis (0.21) were lower in the quarters of lactating cows than those in the quarters of lactating cows in the control group. The frequencies of IMI caused by *S. aureus* in vaccinated group were lower than the control group following immunization in heifers ($p<0.001$).

Table 3 shows the cumulative incidence of clinical, subclinical and latent infection in heifers. The number of clinial, subclinical and latent infections were significantly lower in the vaccinated group compared with heifers in the control group ($p<0.05$, $p<0.05$, $p<0.001$). The frequencies of clinical and subclinical

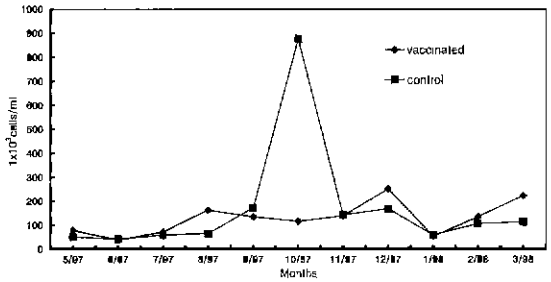

Fig 1. Mean SCC level of lactaing cows that were unvaccinated or vaccinated twice every 3 weeks in the area of supramammary lymph node with vaccine composed of alpha toxin, capsular polysaccharide and fibronectin binding protein.

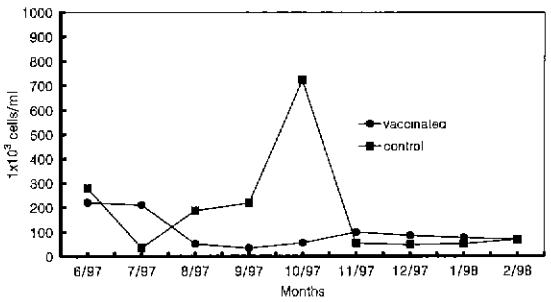

Fig 2. Mean SCC of heifers that were unvaccinated or vaccinated twice every 3 weeks in the area of supramammary lymph node with vaccine composed of alpha toxin, capsular polysaccharide and fibronectin binding protein.

S. aureus mastitis (0.35) were lower in the quarters of heifers than those in the quarters of heifers in the control group.

Somatic cell counts (SCC)

No significant differences were observed between the mean SCC from mammary quarters of the vaccinated and control groups. However, 3 months later, second postimmunization, the SCC in unvaccinated cows tended to be highest throughout the trial ($p<0.05$). The SCC among treatments prior to *S. aureus* immunization was not significantly different between the vaccinated and control groups (Fig 1). SCC of heifers throughout the trial tended to be decreased and lower than that of control group at 3 months postimmunization ($p<0.05$). The SCC prior to immunization was not significantly different between control and vaccinated groups (Fig 2).

Discussion

Mastitis vaccine developed by several researchers was mainly focused on reduction of the clinical signs, SCC and IMI rate. Thus, most of the vaccine were composed of major virulence factors, particularly protein A, CPS and toxins (alpha and beta toxoid). Of several virulence factors, FnBP was not tested extensively in the field although it showed high protective ability in mouse mastitis model.

Until now, many attempts were made to develop more efficient and economic mastitis vaccine, but all efforts was not successful yet.

The experimental mastitis vaccine of this study was developed on the basis of CPS, alpha toxin and recombinant FnBP. The results of the present trial revealed that the frequencies of clinical or subclinical mastitis in the vaccinated groups of heifers and lactating cows were significantly lower than those of the control groups. Similarly the frequency of latent *S. aureus* IMI were also reduced in the lactating cows of vaccinated group, compared with that of the control group.

Several studies[9,15] have shown advantages of administering a mastitis vaccine at drying off because of a better immune response and a higher susceptibility of the mammary gland during the first week after calving. A vaccine that was developed with a killed, encapsulated *S. aureus* was tested in heifers by Nordhaug *et al.*[13,14]. This vaccine showed a significant lower incidence of mastitis compared with the control. Similarly, Han *et al.*[4] developed a experimental mastitis subunit vaccine composed of three virulence factors (alpha toxin, CPS and FnBP). Although this vaccine was studied in rabbits, according to the results of Han *et al.*[4], the vaccinated groups showed the development of specific serum antibody against alpha toxin, CPS and FnBP specific antibody in serum when compared with PBS control group. The bacterial number also decreased until postchallenge 4 hrs and then trend of slight increase was observed 12 and 24 hrs postchallenge.

The vaccine evaluated in the present study was developed to determine its usefullness and protective ability against *S. aureus*. In the present trial, the

decreased incidence of clinical, subclinical and latent mastitis reveals that systemic protective ability in the vaccinated cows may be increased. And these clinical data show that the cows may have immune response to capsule, alpha toxin and FnBP that is opsonic for polymorphonuclear (PMN) cells after immunization. Therefore, it is estimated that antibody for alpha toxin may be responsible for reducing the clinical signs of cows infected with *S. aureus*, and antibody for CPS and FnBP could elicit a protective ability against colonization of *S. aureus* in the udder. Herbelin *et al.*[5] demonstrated that a bacterial virulence factor, alpha toxin, was able to induce immune recruitment of neutrophil for efficient bactericidal activity in milk when cows were immunized with alpha toxin. Therefore, in present trial it is likely that neutrophils recruitment from blood were activated after vaccination in response to particular antigens.

Effects of vaccination on SCC were shown in Figs. 1 and 2. In lactating cows, the level of SCC had no significant difference between groups except 6th sampling time when showed significantly higher SCC in the control group. Presumably, contagious mastitis caused by *Streptococci* spp. caused SCC to increase because *Streptococci* spp. were isolated more frequently than any other sampling time in the control group. Therefore, vaccination for cows during the lactation period had no effects of increasing SCC. In addition, although there was no significant difference between the groups, the level of SCC in heifers of vaccinated group showed lower than that of the control group following booster injection. Thus the vaccine might confer effective protection to cows against *S. aureus* strain. The results of the study showed that the bovine mastitis subunit vaccine had a protective effect on the incidence of *S. aureus* mastitis. When all this parameters included the udder health were considered together, the results revealed a potential protective ability of the vaccine during the entire lactation and dry period. Because only one herd was tested in this field trial, further study on a larger field was needed in near future.

Taken together, results of clinical parameters, microbiological assessments, and measuremetns of SCC suggested that vaccinated heifers and lactating cows had higher resistance to the naturally bacterial exposures.

Conclusion

Field trial of this study showed that this mastitis subunit vaccine had effects on decreasing the incidence of total IMI caused by *S. aureus* without increasing SCC in heifers and lactating cows. In addition, Since this vaccine showed positive effects on IMI and SCC, it is necessary that this experimental mastitis vaccine composed of three virulence factors would be further studied on a large scale of commercial farms.

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