A Field Trial with an Experimental Vaccine Against *Staphylococcus aureus* Mastitis in Cattle. 1. Clinical Parameters

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ABSTRACT

A total of 108 heifers were included in a placebo-controlled multicenter study on the use of an experimental *Staphylococcus aureus* mastitis vaccine containing whole, inactivated bacteria with pseudocapsule, α and β toxoids, and a mineral oil as adjuvant. The heifers were injected in the area of the supramammary lymph nodes twice before calving and were observed and sampled throughout the first lactation. None of the vaccinated cows suffered from clinical *Staph. aureus* mastitis, and only 8.6% suffered from subclinical *Staph. aureus* mastitis, but a total of 16.0% of the control cows suffered from clinical or subclinical *Staph. aureus* mastitis. Mean SCC in vaccinated and control cows were the same throughout the lactation. Local swellings at the injection site were palpable in a substantial proportion of the vaccinated cows. In the statistical analyses, when cow was used as the unit of concern, no significant differences occurred between groups. However, when all parameters on udder health were considered together, the results indicated a potential protective effect of this vaccine during the entire lactation.

(Key words: *Staphylococcus aureus*, vaccine, mastitis, field trial)

Abbreviation key: CMT = California mastitis test, CNS = coagulase-negative staphylococci.

INTRODUCTION

Immunization against *Staphylococcus aureus* mastitis in ruminants has been the subject of much research throughout this century, as reviewed by Anderson (1), Colditz and Watson (3), and Rainard and Poutrel (22). Different vaccines based on cellular or soluble antigens with and without adjuvants have been administered to dairy cows and other ruminants, but, until recently, the protection against infection and disease has been disappointing in most experiments (3). The lack of effective vaccines can be attributed to incomplete knowledge of the pathogenesis of staphylococcal mastitis and of the defense mechanisms of the ruminant udder (22, 29). Several soluble and cellular virulence factors may be involved in the pathogenesis of *Staph. aureus* mastitis. The hemolysins and leukocidin, which act on cell membranes, have received special emphasis, although the exact role of these toxins in pathogenesis has not been defined (15). *Staphylococcus aureus* expresses several components that are attached to the cell wall, such as protein A and fibronectin-binding proteins, which have been studied as possible cellular antigens in vaccines (5, 14). However, because of poor protection after immunization with traditionally prepared bacterin, attention recently has focused on the possible role of the surface polysaccharides of *Staph. aureus* in pathogenesis, as reviewed by Foster (5). Evidently, *Staph. aureus* surface polysaccharides increase resistance to phagocytosis (5, 22, 30). These exopolysaccharides are expressed during growth in vivo (5, 31). However, this production in vitro is influenced by the composition of the growth medium (9, 13, 17, 22, 23, 33). Cultivation methods to achieve optimal production of *Staph. aureus* exopolysaccharides are still the subject of debate, as is the exact structure of this pseudocapsule (5, 12, 17, 23, 33). How-
ever, several researchers (15, 22, 30) agree that this pseudocapsule should be included in a *Staph. aureus* mastitis vaccine.

Understanding of the specific defense mechanisms of the ruminant udder that are based on humoral and cellular factors has increased markedly during the last 20 yr, as reviewed by Colditz and Watson (3), Craven and Williams (4), and Norcross (15). In normal milk, the concentrations of antibodies and neutrophil granulocytes are low (3, 4, 11). The blood-milk barrier prevents all but a very small proportion of circulating IgG antibodies from reaching mammary secretion during lactation (3). However, after infection of the udder, antibodies and neutrophils can rapidly be recruited from blood (3, 8), and neutrophil phagocytosis supported by opsonic antibodies is considered to be the major defense mechanism directed at the elimination of pathogenic bacteria (4, 15, 22, 30). Neutralization of antibodies against staphylococcal toxins may contribute to a reduction in the severity and duration of the disease (1, 15, 30). Because adhesion of bacteria to the udder epithelium is important in the establishment of *Staph. aureus*, specific antibodies also may act as antiadhesives (5, 6, 14). The importance of the different Ig classes has been extensively studied (3, 15). The IgG2 isotype is probably the most important opsonic antibody (3, 26, 30), but neutralizing or antiadhesive activity may be performed by IgM, IgA, and both IgG isotypes (3, 15).

An ideal vaccine against *Staph. aureus* mastitis should prevent the establishment of an infection and the development of an inflammatory response. However, because of the high prevalence and subsequent large economic losses of mastitis, even the lesser achievement of reducing the severity of the symptoms of mastitis and obtaining a more rapid clearance of established infections would be of great value. Mastitis in heifers before calving, which is an increasing problem, should also be possible to prevent by vaccination. Reduction in mastitis severity or duration should lower the bulk SCC and milk loss. Ideally, the side effects associated with vaccination should not affect animal welfare or the SCC in milk (1).

Results of vaccine experiments (16, 21, 24, 29, 35) performed in experimental herds with or without challenge show promising effect on mastitis prevalence or incidence, milk yield, and SCC in milk. However, field trials in commercial dairy farms with no experimental challenge must be performed to allow complete evaluation of a *Staph. aureus* mastitis vaccine. Only a few recent reports describe field trials with such vaccines (32).

The aim of the present field trial with heifers was to evaluate the effect of an experimental *Staph. aureus* mastitis vaccine on naturally occurring infections and to measure specific antibodies against *Staph. aureus* antigens in serum and milk. The effect was assessed in relation to clinical and subclinical mastitis and SCC in milk. These results are presented with general and local adverse reactions. Antibody results are presented elsewhere (19).

**MATERIALS AND METHODS**

**Vaccine and Placebo**

The experimental vaccine consisted of equal amounts of *Staph. aureus* strains Smith and 2-8 (isolated from a case of clinical mastitis) (16). The bacteria were grown on modified *Staphylococcus* medium 110 containing 1.5% agar (34). The organisms were inactivated with .3% formalin. Both α and β toxoids were added to the vaccine. The antigens then were emulsified in a mixture of mineral oil and detergent [Montanide 103®; (Seppic Co., Paris, France)]. One dose of vaccine consisted of 25 × 10⁶ cfu of *Staph. aureus* (equal amounts of each strain) and .4 mg of N-toxoided α and β toxin.

A placebo was made from 20 g of skim milk powder and .6 g of chocolate powder/100 ml of saline containing .01% merthiolate.

**Farms and Cows**

Sixteen farms in four districts of Norway were selected on the basis of previous mastitis problems and the farmers' cooperation. During the year prior to the start of the trial, the mean incidence of clinical mastitis was 44.6%, and the mean SCC in bulk milk was 220,000 cells/ml. Results from quarter milk samples collected from all cows in the herds a few months prior to the first vaccination showed that 19.2% of the cows (minimum, 4.5%; maximum, 66.7%) were infected with *Staph. aureus*.  

Healthy, pregnant, nulliparous heifers (Norwegian Red Cattle) with no palpable udder abnormalities were selected. The heifers were out on pasture during the summer prior to calving and kept indoors from approximately 14 d before delivery until the next summer. Calving extended from July 12 to November 23.

Experimental Design

The trial was conducted as a placebo-controlled multicenter study. The heifers in each herd were randomized into two trial groups: one group received the vaccine, and the other group received the placebo. The heifers were injected deeply, using a 3.8-cm needle, in the area of the supramammary lymph nodes with a dose of 2.5 ml. The vaccine and the placebo were coded so that neither farmers nor veterinarians knew the treatment given to each individual cow. The veterinarians vaccinating the heifers were instructed to administer the two injections approximately 8 and 2 wk before calving, respectively. Most heifers received their first dose prior to going on pasture. The mean number of days from the first and second administrations of vaccine or placebo until calving were 72 (minimum, 25; maximum, 162) and 18 (minimum, 0; maximum, 77), respectively. Only heifers that received two injections and did not contract clinical mastitis prior to delivery were included in the trial; i.e., 58 vaccinated and 50 control heifers. The observation period was from the day of delivery until drop-out or drying off after the first lactation. Drop-out was defined as culling for reasons not related to mastitis or to a missing drying-off sample.

Sampling

Quarter foremilk samples were collected by the farmers just after calving (0 to 2 d), then by a technician at approximately 1 to 3 wk, 2 to 4 mo, 5 to 7 mo, and 8 to 10 mo after calving, and again by the farmers 0 to 3 d before drying off. When clinical mastitis occurred, veterinarians were asked to collect quarter milk samples prior to injection of antibiotics. Composite milk samples from the cows were collected every 2nd mo in most of the herds and every month in a few herds as part of the milk quality control program.

Laboratory Examination

Quarter milk samples were examined bacteriologically and cytologically, and the results were interpreted according to the Scandinavian recommendations presented by Klastrup (10) with the following modifications. The California mastitis test (CMT) was scored on a five-point scale. In the main part of lactation (mo 2 to 7 after calving), secretions with a CMT score ≥3 and a difference in CMT score ≥1 from the CMT score of at least one of the other quarters’ secretion were considered to be mastitic. Apart from this period, the difference in CMT score had to be ≥2 (7). If a secretion was judged to be a dry cow’s secretion, sediment after centrifugation of the samples for 10 min at 500 × g was assessed subjectively from 1 to 5 and used instead of CMT. Diagnosis was defined according to Bakken (2): infectious mastitis means mastitic secretion and isolation of udder pathogenic bacteria; non-specific mastitis means mastitic secretion without detection of bacteria in the sample. If the quarter milk samples were from a case of clinical mastitis, clinical was added to the diagnosis description, whereas all other samples with a mastitis diagnosis were assessed as subclinical. Demonstration of certain bacteria (Staph. aureus; streptococci groups B, C, G, or L; or Actinomyces pyogenes) without elevation of the SCC was assessed as latent infection caused by the bacteria in question.

A tablet containing 10 mg of bronopol and a pH indicator (C&F Control Systems, San Francisco, CA) was mixed with 40 ml of a composite milk sample, and SCC were determined by Fossomatic cell counter (A/S Foss Electric, Hillerød, Denmark).

Clinical Mastitis

Cases of clinical mastitis were registered by the farmer, diagnosed and treated by the local veterinary practitioner, and recorded in the Norwegian Health Card System (25). The clinical mastitis diagnosis was based on local symptoms (heat, pain, swelling, clots, or discoloration of milk) and, if present, general symptoms (body temperature >39.5°C or reduction in appetite). The mastitis was characterized as severe if general and local symptoms were both present and as mild if only local symptoms were registered. Because the farm-
ers continuously received feedback concerning the results from examination of all quarter milk sampled during the trial, some cases registered as clinical mastitis were probably detected and treated with antibiotics after the results of the laboratory examination had indicated their existence.

**Adverse Reactions**

General adverse reactions, such as loss of appetite or fever (>39.5°C) were registered by the farmer on the same afternoon as the vaccinations and on the morning and evening of the following day. Local adverse reactions at the injection site, such as swellings or pain on palpation, were recorded by the same trained technician in all cows on two occasions during the lactation.

**Statistical Analyses**

Mantel-Haenszel or Fischer exact test was used when the percentages of mastitis were compared, and log SCC were analyzed by a Students' t test, both of which were performed in Epi-Info version 5 (USD, Inc., Stone Mountain, GA). A log rank test was performed in Epidemiological Graphics, Estimation, and Testing (EGRET) package Analysis Module (PECAN) version 26.5 (Statistics and Epidemiology Research Corp., Seattle, WA) to account for the time from calving until the first occurrence of Staph. aureus mastitis. In all statistical analyses, cow, not quarter, was the unit of concern. Significance was declared at $P < .05$.

**RESULTS**

**Clinical Mastitis**

A total of 20.0% of the controls and 15.5% of the vaccinated cows were treated for clinical mastitis during lactation (Figure 1). One cow in each group was treated for clinical mastitis in the same quarter twice within 14 d. Clinical mastitis that was due to Staph. aureus was diagnosed in 3 cows (6.0%) in the control group (two severe and one mild case) compared with none in the vaccinated cows ($P = .096$; Table 1). Clinical infectious mastitis

**TABLE 1.** Cumulative incidence rates of cows and quarters with different Staphylococcus aureus diagnoses at one or several occasions during lactation.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Control Cows</th>
<th>Control Quarters</th>
<th>Vaccinated Cows</th>
<th>Vaccinated Quarters</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 50)</td>
<td>(n = 200)</td>
<td></td>
<td>(n = 58)</td>
<td>(n = 232)</td>
</tr>
<tr>
<td>Clinical <em>Staph. aureus</em> mastitis$1$</td>
<td>6.0</td>
<td>2.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Subclinical <em>Staph. aureus</em> mastitis$2$</td>
<td>14.0</td>
<td>4.0</td>
<td>8.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Clinical or subclinical <em>Staph. aureus</em> mastitis$3$</td>
<td>16.0</td>
<td>5.5</td>
<td>8.6</td>
<td>2.6</td>
</tr>
<tr>
<td><em>Staph. aureus</em> latent infection$4$</td>
<td>22.0</td>
<td>9.0</td>
<td>29.3</td>
<td>8.2</td>
</tr>
<tr>
<td>Total <em>Staph. aureus</em> infections</td>
<td>38.0</td>
<td>14.5</td>
<td>37.9</td>
<td>10.8</td>
</tr>
</tbody>
</table>

$1$Based on clinical data and results of laboratory examination of milk samples from clinical mastitis.

$2$Based on results of laboratory examination of quarter milk samples.

$3$The sums are lower than 1 + 2, because some cows suffered from subclinical and clinical mastitis during lactation.

$4$Based on results of laboratory examination of quarter milk samples, but cows suffering from subclinical *Staph. aureus* mastitis were not included.

TABLE 2. Cumulative incidence rates of cows and quarters with subclinical mastitis, except those caused by Staphylococcus aureus, at one or several occasions during lactation.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Control Cows</th>
<th>Quarters</th>
<th>Vaccinated Cows</th>
<th>Quarters</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 50)</td>
<td>(n = 200)</td>
<td></td>
<td>(n = 58)</td>
<td>(n = 232)</td>
</tr>
<tr>
<td>Streptococcus dysgalactiae mastitis</td>
<td>12.0</td>
<td>3.5</td>
<td>5.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Actinomyces pyogenes mastitis</td>
<td>2.0</td>
<td>.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mastitis caused by CNS, other streptococci, or Enterobacteriaceae</td>
<td>44.0</td>
<td>16.5</td>
<td>43.1</td>
<td>15.1</td>
</tr>
<tr>
<td>Nonspecific mastitis (no bacteria isolated)</td>
<td>60.0</td>
<td>22.5</td>
<td>70.7</td>
<td>29.3</td>
</tr>
</tbody>
</table>

1Based on results of laboratory examination of quarter milk samples.

Caused by other bacteria was diagnosed in 1 of the controls (coagulase-negative staphylococci [CNS]) and in 6 (10.3%) of the vaccinated cows (Streptococcus dysgalactiae, Streptococcus uberis, Escherichia coli, Streptococcus faecalis, and CNS). In addition, one control cow suffered from clinical mastitis, but no microorganisms could be isolated from the samples. No samples were collected prior to antibiotic treatment from 5 control cows and 3 vaccinated cows.

Most of the clinical episodes, including the clinical Staph. aureus mastitis, occurred during the first 3 wk after calving. No treatments were registered later than 200 d after calving (Figure 1).

Subclinical Mastitis

Subclinical Staph. aureus mastitis was diagnosed in 14.0% of the control cows compared with 8.6% of the vaccinated cows, whereas Staph. aureus latent infection was diagnosed in 29.3% of the vaccinated cows compared with 22.0% of the control cows (Table 1). In samples collected 0 to 2 d after calving, Staph. aureus mastitis was diagnosed in 8.0% of the control cows and in 5.2% of the vaccinated cows. Incidence rates for cows with subclinical infectious mastitis caused by other microorganisms are presented in Table 2. Subclinical Strep. dysgalactiae mastitis was diagnosed in samples from 12.0% of control cows compared with 5.2% of vaccinated cows. However, the percentage of cows with infectious mastitis caused by CNS, other streptococci or bacteria of the family Enterobacteriaceae, or non-specific mastitis was about the same in the experimental groups. No differences between experimental groups were significant.

In only 3 cows (2 controls and 1 vaccinated cow) was subclinical Staph. aureus mastitis followed by a further diagnosis of Staph. aureus in the same or another quarter after subsequent sampling. In as many as 94.1% of the control cows and 88.9% of the vaccinated cows with latent Staph. aureus infection, the affected quarter proved to be healthy on examination of the following sample (mean interval of 45 d). None of these quarters were subsequently affected by Staph. aureus subclinical mastitis or latent infection.

Total Staph. aureus Mastitis

A total of 16.0% of the control cows suffered from Staph. aureus mastitis (clinical or subclinical) compared with 8.6% of the vaccinated cows (only subclinical) (Table 1). Until the drying off period, the relative risk of Staph. aureus mastitis was .40 in vaccinated cows compared with control cows (P = .140).

The probability of not cows contracting Staph. aureus mastitis at various stages of lactation is shown in a Kaplan-Meier plot in Figure 2. The difference between the groups in probability of avoiding Staph. aureus mastitis increased during the first 40 d after calving, and the difference between the groups was constant from d 90 to 300 after calving. However, in both groups, the last cow to complete the study contracted Staph. aureus mastitis (subclinical) at drying off (control cow, 323 d after calving;
vaccinated cow, 373 d after calving), which further increased the difference in probability of avoiding Staph. aureus mastitis between the groups in late lactation. Neither cow was included in Figure 2.

SCC

The mean geometric SCC in composite milk samples were similar for the two groups throughout the trial (Figure 3). However, SCC were ≥500,000 cells/ml of milk for 14.0% of the control cows compared with 5.2% of the vaccinated cows (P = .107). The SCC were low (<100,000 cells/ml of milk in all samples tested during the lactation) for 51.7% of the cows in the vaccinated group compared with 40.0% in the control group (NS).

Adverse Reactions

General adverse reactions to immunization occurred in 2 vaccinated cows (3.5%) after the first and second vaccinations and in 1 control cow (2.0%) after the second injection (NS). Adverse reactions were slightly reduced appetite and lethargy during the afternoon of the day of vaccination.

On the day of vaccination, local swellings at the injection site were observed by the farmers in 11 vaccinated cows (19.0%) and 2 control cows (4.0%). Palpable swellings of about 5 cm (mean) in diameter at the injection site were detected in 32 of 46 vaccinated cows (69.6%) 72 to 216 d after vaccination and in 8 of 10 cows that were examined again 253 to 295 d after vaccination. No vaccinated cows showed signs of pain when the swellings were palpated. In the control group, local reactions were palpable in only 2 cows (98 and 126 d after injection of placebo). One control cow showed pain on palpation.

Culling

The culling rate during lactation and the reasons for culling were approximately the same in both groups (Table 3).

DISCUSSION

In the present study, 16.0% of the control cows suffered from clinical or subclinical Staph. aureus mastitis compared with 8.6% of the vaccinated cows suffering from subclinical Staph. aureus mastitis. Although these differences cannot be considered to be statistically significant, the results may indicate that the Staph. aureus vaccine had a potential protective effect. The difference especially concerned the severity of symptoms, because no vaccinated cows were diagnosed as having clinical Staph. aureus mastitis. These results agree with those of recently published studies (29) on Staph. aureus mastitis vaccines. Reduced severity of symptoms is probably mediated via antibodies neutralizing the Staph. aureus tox-
The vaccine did not seem to provide much protection against *Staph. aureus* mastitis at calving. However, the results indicated a time-dependent partial protection after calving, which increased during the first 40 d of lactation and continued until drying off. Previous experiments with this vaccine have demonstrated elevated antibody concentrations until 6 mo after vaccination (16), but antibody concentrations after injection of vaccines without oil adjuvants in sheep dropped 4 to 5 mo after vaccination (28). The inclusion of oil adjuvants in *Staph. aureus* mastitis vaccines seems to be necessary to sustain effect throughout lactation (29).

In the present study, SCC were equally low in both groups during lactation. Vaccination against *Staph. aureus* mastitis neither reduced nor increased mean SCC. The small reduction in the incidence of *Staph. aureus* mastitis did not influence the mean SCC in the vaccinated cows. In addition, the mean SCC is a general indicator of udder health status; the contribution from *Staph. aureus* mastitis is only a partial explanation. Because the somatic cells in milk constitute part of the udder defense, the risk of enhanced immunity after vaccination being mediated by a permanently higher SCC should be considered (1). When cows are vaccinated in the dry period, SCC should not be expected to increase in the following lactation; several reports (16, 35) with experimental *Staph. aureus* vaccines demonstrated a reduction in the mean SCC in vaccinated cows. However, vaccination of lactating ewes induced a short-term increase in the milk SCC (18).

This trial showed two conflicting results: nonspecific mastitis was less often diagnosed for control cows than for vaccinated cows, but the percentage of cows with SCC ≥500,000 cells/ml of milk at least once was higher for the control cows than for the vaccinated cows. A CMT score of 3 in only one quarter was sufficient to obtain a diagnosis of nonspecific mastitis, which explains the high frequency of this diagnosis in both groups and emphasizes the minor importance of this diagnosis. A SCC ≥500,000 cells/ml in a composite milk sample, which is disadvantageous for milk quality, is the result of a high SCC in one quarter or more moderate elevation in several quarters. Moreover, the differences in SCC between the groups

### TABLE 3. Drop-outs1 during lactation in each experimental group.

<table>
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<th>Controls (n = 50)</th>
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1Culling for reasons not related to mastitis or a missing drying off sample.

ins, and this effect may be the easiest to generate when *Staph. aureus* immunization is used (22). Although a reduction in the severity of mastitis is considered to be an important beneficial effect of vaccination by some researchers (30), others (1) point out that a mastitis vaccine should protect against mastitis per se, not only against the severity of the disease. A reduction in the severity of mastitis would be beneficial because of the great economic losses from mastitis but is probably not a satisfactory effect of immunization.

The overall percentage of cows infected with *Staph. aureus* was almost the same in both groups, but the *Staph. aureus* infections were diagnosed as mastitis more often in control cows than in vaccinated cows (Table 1). These findings, although not statistically significant, may indicate a higher specific immunity in the vaccinated cows and support the idea that vaccination against *Staph. aureus* mastitis cannot prevent infection but can slow the development of disease (1). Interestingly, none of the cows with *Staph. aureus* latent infection were given a new diagnosis on subsequent sampling, although *Staph. aureus* latent infection has previously been reported (2) to be followed by *Staph. aureus* mastitis in the same quarter in as many as 20% of the cows within 6 mo. Reliable information on the persistence or self cure rates of subclinical *Staph. aureus* mastitis was difficult to obtain from this study. The low incidence of clinical *Staph. aureus* mastitis, even in the control group, was probably mainly due to the fact that all cows were primiparous.

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were greater than that of nonspecific mastitis.

The slight difference in incidence of *Strep. dysgalactiae* mastitis between the experimental groups might be due to crossprotection, because several mastitis-causing streptococci also produce exopolysaccharides (22). However, no crossreactivity occurred between *Staph. aureus* and *Strep. agalactiae* in an experiment immunizing cows with those bacteria (20).

The general adverse reactions observed in a few vaccinated cows were moderate and considered to be acceptable side effects of the administration of a mineral oil vaccine during the dry period. However, local adverse reactions to vaccination occurred more frequently. More than two-thirds of the vaccinated cows developed palpable swellings at the injection site. Nevertheless, no signs of pain were observed in any of the vaccinated cows on palpation. The subcutaneous supramammary tissue probably constitutes a suitable site of injection for such vaccines, because the tissue is loose enough to permit the development of granulomas without causing pain. Furthermore, the tissue is not used for human consumption.

**CONCLUSIONS**

The results of the present study only indicated that the experimental *Staph. aureus* vaccine had a positive effect on the incidence of *Staph. aureus* mastitis and on the individual SCC; the differences were not statistically significant. However, when all parameters on udder health were considered together, the results indicated a potential protective immunity of the vaccine during the entire lactation. Because even a protective effect of a lower magnitude may be of economic importance in dairy cows, the results showed that the vaccine should be tested in a larger field trial.

**ACKNOWLEDGMENTS**

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**REFERENCES**

STAPHYLOCOCCUS AUREUS VACCINE FIELD TRIAL