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Efficacy of vaccination with a *Klebsiella pneumoniae* siderophore receptor protein vaccine for reduction of *Klebsiella* mastitis in lactating cattle

P. J. Gorden,*[1] M. D. Kleinhenz,*[2] J. A. Ydstie,* T. A. Brick,* L. M. Slinden,† M. P. Peterson,† D. E. Straub,† and D. T. Burkhardt†
*Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames 50011
†Epitopix, LLC, Willmar, MN 56201

ABSTRACT

Clinical mastitis caused by *Klebsiella* spp. is an emerging problem in the US dairy industry and results in a high degree of financial losses to dairy workers. This study was conducted as a randomized, blinded, and placebo-controlled efficacy study of a *Klebsiella pneumoniae* siderophore receptor protein (SRP) vaccine (Kleb-SRP), with a total of 569 cows and heifers enrolled. The study was designed to look at vaccine effect on *Klebsiella* mastitis; however, the SRP in *Klebsiella* are highly conserved across coliform bacteria, which means that the vaccine has potential for cross-protection against all coliforms. Cows were paired based on parity, days in milk at enrollment, and somatic cell count. Within pairs, individuals were randomized to receive either Kleb-SRP or a placebo formulation. Following vaccination, the incidence of *Klebsiella* spp. and total coliform mastitis from natural exposure were compared to determine the efficacy of the vaccine. When analyzing all cows, the reduction of mastitis risk was not significant, though milk production increased 0.31 kg/d and somatic cell counts were reduced by 20.1%. When administered before calving, the vaccine reduced the risk of *Klebsiella* and total coliform mastitis by 76.9 and 47.5% respectively; however, we observed no significant effect when administered after calving. The vaccine, when administered before calving, also increased milk production by an average of 1.74 kg/d and reduced somatic cell counts by 64.8%. When administered after calving, we noted a slight decrease in daily milk production (0.39 kg) but no significant effect on somatic cell counts. All cows in the study (including vaccinates and placebo) received multiple doses of a commercially available licensed *Escherichia coli* bacterin. It should be noted that this herd was chosen because of the high number of clinical *Klebsiella* clinical mastitis cases this herd experienced before the trial and the extreme environmental challenge that was present from bedding with dried manure solids. The data from this study demonstrate efficacy of the Kleb-SRP vaccine against *Klebsiella* mastitis alone and coliform mastitis in general (including all coliforms) when administered before the initiation of a lactation cycle.
Key words: bovine mastitis, *Klebsiella* spp., siderophore receptors

INTRODUCTION

Mastitis is a disease of the mammary gland caused by bacterial infection and is the most common and costly health concern for dairy producers (Ruegg, 2003). Gram-negative clinical mastitis (**CM**) is more severe than gram-positive mastitis due to its effect on milk yield, discarded milk, treatment costs, death, and culling (Hertl et al., 2011). *Escherichia coli*, *Klebsiella* spp., and *Enterobacter* spp. are among the most common gram-negative bacteria associated with CM, accounting for as much as 40% of all cases of CM (Schukken et al., 2011; Oliveira et al., 2013).

Among coliform CM cases, *E. coli* tends to be the most prevalent, whereas CM caused by *Klebsiella* spp. tends to be the most severe (Gröhn et al., 2004; Pinzón-Sánchez et al., 2011). Severity of CM episodes, poor response to vaccination, and the lack of effective treatments make *Klebsiella* CM especially troublesome. The severity of CM due to *Klebsiella* is partially due to the animal's immune response to LPS, which is more severe than the reaction to *E. coli* (Schukken et al., 2012). Although this has not been studied in *Klebsiella* spp. isolates from bovine CM cases, it is speculated that the reason for the increased severity is due to an increased

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[1] Corresponding author: pgorden@iastate.edu
[2] Current address: Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, 226 Coles Hall, 1610 Denison Ave., Manhattan, KS 66506.

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number of acyl groups in the LPS molecule of *Klebsiella* spp. compared with other coliforms (Bardoel and van Strijp, 2011; Llobet et al., 2011). This increased severity leads to substantially longer loss of milk production and a greater risk of culling (Schukken et al., 2012). The experience, before the present study, of the dairy described here is that >50% of cows with *Klebsiella* CM were removed from the herd within that lactation despite aggressive therapy (M. D. Kleinhenz, unpublished data). Others have reported similar outcomes at commercial dairies from around the United States (Schukken et al., 2012; Munoz et al., 2007).

Due to disease severity and poor response to treatment, high-quality vaccines that could prevent or reduce the severity of coliform CM are a better solution for the dairy. Whereas core antigen (J5) vaccines are available, coliform mastitis, especially *Klebsiella* mastitis, continues to cause problems for dairy producers (Schukken et al., 2012). To provide a high-quality vaccine for coliform CM, new technologies must be applied that extend beyond traditional bacterins.

One potential approach is to target the iron-acquisition system of bacteria. All bacteria require iron to grow, but free iron is severely limited in all mammalian hosts (Hood and Skaar, 2012). To acquire sufficient iron, bacteria use special proteins, referred to as siderophores, which are released by bacteria to bind to iron and bring it back to the host cell for iron uptake through siderophore receptors (Miethke and Marahiel, 2007). The conservation of these iron-acquisition proteins among different species of bacteria, combined with their ubiquitous expression during infection, make them an extremely promising novel target for development of highly efficacious vaccines. Previously, siderophore receptor and porin protein (**SRP**) vaccines (Epitopix, Willmar, MN) have successfully been used for protection against *Salmonella* Newport (Emery et al., 2001) and *E. coli* O157 in cattle (Thomson et al., 2009). The SRP vaccine technology uses these iron-acquiring receptor proteins as antigens for vaccine formulation. To develop a *Klebsiella* SRP mastitis vaccine, dozens of typical field isolates of *Klebsiella pneumoniae* were screened and a specific strain was chosen as the donor organism based on its broad array of conserved siderophore receptor proteins.

The primary objective of our study was to determine whether vaccination with a *Klebsiella* SRP vaccine would reduce the incidence of CM caused by *Klebsiella* spp. and other coliform bacteria in a dairy herd following natural challenge as part of the regulatory vaccine-approval process. Secondary objectives were to determine the antibody response to vaccination, determine the effects of vaccination on daily milk production and monthly DHIA SCC, and to determine the effect of vaccination on severity of CM cases.

MATERIALS AND METHODS

Trial Herd

This study was conducted at the Iowa State University (**ISU**) Dairy Farm. The ISU lactating herd consisted of approximately 400 animals (approximately 90% Holstein and 10% Jersey), with a 365-d rolling herd average of 11,005 kg of milk, 399 kg of fat, and 342 kg of protein. The herd averaged 30 to 40 dry cows and raised all replacement heifers on site. The entire herd was used for this trial as described below. Throughout the trial, lactating cows were housed in a freestall barn bedded with recycled manure solids, which was standard practice for this dairy. Dry cows were housed in sand-bedded freestalls for the first portion of the dry period and on straw-bedded loose housing for the final 21 to 28 d of the dry period. Lactating and dry cows were fed TMR that were formulated to meet or exceed NRC requirements (NRC, 2001) and provided water ad libitum. Cows were milked 3 times daily at 0400, 1200, and 2000 h. Cow housing and management met or exceeded the recommendations listed in the *Guide for Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010). The ISU Institutional Animal Use and Care Committee approved the research protocol before commencement of trial procedures (protocol number 2–15–7943-B).

As part of the normal vaccination protocol before beginning the study and continuing throughout the study, the entire milking herd was vaccinated with a J5 core antigen vaccine (ENVIRACOR J-5, Zoetis Services LLC, Parsippany, NJ) approximately 42 and 28 d before calving and again approximately 25 and 90 d following calving. This herd was selected for the study because of an ongoing *Klebsiella* mastitis problem that was not being effectively controlled with the commercially available core antigen vaccine. In the year leading up to the study, the prevalence of *Klebsiella* in the herd was 14% of all the CM cases. As is typical with *Klebsiella* mastitis, 33 and 43% of the *Klebsiella* CM cases were moderate or severe, respectively, utilizing a clinical scoring scheme previously described by Wenz et al. (2001). Despite aggressive therapy, 38% of these CM cases were culled from the herd and 19% of the cases died as a result of the mastitis (M. D. Kleinhenz, unpublished data). With the exception of implementation of the trial vaccines for our study, all other vaccinations and treatments of the cows continued as per the dairy's normal protocols.

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Trial Vaccines

The test vaccine (**Kleb-SRP**; *Klebsiella pneumoniae* Bacterial Extract, Epitopix, Willmar, MN) was prepared by harvesting and purifying the siderophore receptor and porin proteins from fermentation cultures. *Klebsiella pneumoniae* was isolated from a milk sample of a cow with severe mastitis. The isolate was not sourced from the trial herd in our study, but rather was selected based on in vitro expression of siderophore receptor and porin proteins by screening isolates from several US herds. To prepare the vaccine, purified proteins were emulsified with an oil-in-water–based adjuvant. A placebo vaccine formulation was made identically, except the antigen fraction was replaced with sterile saline so the formulation had the same appearance but was lacking the critical antigenic components. To facilitate trial blinding, one vaccine formulation was assigned as vaccine A and the other as vaccine B at the vaccine production facility by random selection out of a hat. Visually, both products appeared identical, including the text on the bottles, with the exception of the words "Vaccine A" or "Vaccine B," and the color of the label. Production facility personnel were not involved with any other portion of the study.

Enrollment Criteria

All lactating and dry cows, as well as springing heifers, at the dairy were enrolled as subjects upon initiation of the study, with the exception of 3 specific groups of animals. The first group of cows not initially enrolled were $\leq$5 wk prepartum to 2 wk postpartum. Instead, these cows were enrolled and vaccinated once they were 2 wk postpartum. Enrollment of these cows was delayed because it is common practice in the dairy industry to not vaccinate near calving due to periparturient immune suppression (Kimura et al., 2002). The second group of cows not initially enrolled in the study were cows <21 d from scheduled dry off, which were cows >193 d carried calf (**DCC**). Instead, these cows were enrolled and vaccinated once they achieved 217 DCC, which was 1 wk before initiation of the dry-cow protocol. The final group of animals not enrolled in the study were animals that the farm had previously designated to be culled from the herd in the near future.

Cows were randomly assigned by the study monitor to treatment group based on lactation, SCC, and DIM. This information was retrieved from the most recent DHIA test date before enrollment. Cows were initially sorted by lactation number into 3 lactation groups (first lactation, second lactation, and 3 or more lactations). Cows were then sorted by their SCC to establish a high-, medium-, and low-SCC group within each lactation group. This yielded 9 groups of cows. Each group was then sorted by their DIM. At this point, the first 2 cows in the list comprised a pair, which were randomly assigned to receive either vaccine A or B based on a random number generated in a commercial computer spreadsheet program (Microsoft Excel 2013, Redmond, WA). This was continued for subsequent pairs until all cows were assigned to a treatment group.

Without pre-existing data, heifers were randomized with simple randomization using the randomization function in the commercial spreadsheet program (Microsoft Excel 2013). Briefly, heifers were listed by their ear tag identification and their anticipated calving date. Heifers were then sorted by their calving date from earliest to latest. A random number was assigned to each heifer. The first 2 heifers on the list were then sorted by their random number with the highest number being assigned to vaccine A and the lower number being assigned to vaccine B.

The vaccination crew and study monitor were aware of the vaccine assignments to perform weekly vaccinations and prepare the weekly vaccination lists. Both the vaccination crew and study monitor were blinded to the composition of vaccine A or B. Study personnel responsible for diagnosing mastitis and collecting data were double-blinded to vaccine assignment and vaccine content of each group. The study monitor was not involved in collection of data. The remaining study personnel remained blinded to vaccine composition until data collection was terminated.

Vaccinations

In early spring, all cows and heifers, with the exception of the 3 groups described above, were injected subcutaneously with 2 mL of the assigned vaccine treatment. A second dose was administered 3 wk later. Each week throughout the study, new vaccination lists were prepared by the study monitor and supplied to the vaccination crew. These included the excluded groups described above and cows and heifers that achieved 217 DCC during the previous week. For heifers this was their initial enrollment, whereas for cows that had been previously vaccinated at enrollment this was their first dry-off vaccination. Vaccinations were repeated 3 wk later. This vaccination schedule was maintained throughout the clinical observation period. Trial cows were monitored for any adverse events to the vaccine following each vaccination. Throughout the study, the first dose of vaccine was administered on the right side of the neck and the booster dose was administered on the left side. Subsequent booster vaccinations were giv-

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en on alternate sides of the neck. Local reactions were monitored after each vaccination visually throughout the study.

Antibody Response Following Vaccination

The last 20 cows from each group vaccinated at study initiation were selected to assess the serological response following vaccination with trial vaccines by an ELISA. Blood was collected in glass tubes with no additive (Becton, Dickinson and Co, Franklin Lakes, NJ) at the time of first vaccination, time of second vaccination, and 2 wk after the second vaccination. Following collection, blood was centrifuged at 1,000 × *g* for 10 min at 4°C, the sera was collected, and the sample was frozen at −70°C until shipment to the study sponsor for analysis.

The serological response to vaccination was measured by a proprietary ELISA (Epitopix, Willmar, MN). Ninety-six–well polystyrene plates were coated with *Klebsiella*-SRP vaccine antigen. Each serum sample was diluted 4 fold from 1:400 to 1:409,600 and tested in duplicate. Each plate contained 2 wells of a 1:400 target dilution of a known positive control sera, which served as an internal plate control to ensure a valid test, and used as a means of calculating serum titers. Titer was defined as the point at which a sample's dilution curve intercepted 50% of the mean optical density value of the positive control wells on the plate. A commercial computer spreadsheet program (Microsoft Excel 2013) was used to determine the intercept point to generate and report a calculated titer value for each serum sample tested on the plate.

Monitoring Phase

Cows were eligible for clinical monitoring 2 wk after their second vaccination, and the monitoring phase continued for 9 mo from study initiation. Cattle in this study were challenged via natural exposure to *Klebsiella pneumoniae* and other environmental pathogens naturally present on the dairy. We hypothesized that the level of natural challenge in our study was severe due to the recycled dried manure solids used at the dairy for bedding and the high incidence of coliform CM, especially *Klebsiella* CM.

In addition to analyzing all data together, we separately considered animals vaccinated before calving from those enrolled after calving. Cows that were already milking at study initiation were analyzed as vaccinated after calving for that lactation and as vaccinated before calving for any subsequent lactation. This separation was planned before data collection as part of the vaccine regulatory approval process.

Milk Culturing and Isolate Identification

Cows identified with CM by farm personnel were presented to study personnel responsible for tracking clinical cases. Cows underwent a full physical examination, which included the collection of 2 independent milk samples according to the recommended practices of the National Mastitis Council (1999). The first of the 2 milk samples from cows displaying signs of CM was submitted to the ISU Veterinary Diagnostic Laboratory for bacterial isolation and identification of the causative agent for the mastitis event, whereas the second sample was frozen at −20°C. If the first sample was determined to be contaminated by isolation of >2 distinct organisms, the duplicate sample was tested. Aerobic culture was completed by plating 100 μL of milk onto blood and MacConkey agar plates (Remel Microbiology Products, Lenexa, KS), utilizing guidelines described by the National Mastitis Council (1999). Confirmation of bacterial identification was performed using MALDI-TOF (MALDI Biotyper, Bruker Daltonics Inc., Billerica, MA) for all bacterial isolates. Any lactating cow that had signs of CM, such as abnormal milk (e. g., change in color or milk appearance, such as the presence of flakes, clumps), a swollen or painful udder, and had a milk sample that has been cultured and confirmed as positive for *Klebsiella* spp., was considered in epidemiological calculations. The same criteria were used for coliform epidemiological calculations, with the exception of culture results with cultures positive for any coliform (*E. coli*, *Enterobacter* spp., *Klebsiella* spp., and *Serratia* spp.) included in the calculations.

Incidence Calculations

Incident analysis counted the number of cases of *Klebsiella* and coliform mastitis and considered each day that an individual was clinically eligible as a day at risk. Within 14 d following each positive result (separately for *Klebsiella* and coliform mastitis), new positive culture results with the same pathogen (*Klebsiella* or all coliforms) were excluded, as they could not be distinguished from continuation of previous infection; these days were not counted as at risk. Because of these exclusions and slight differences in study enrollment, the number of days at risk differed for the 2 individuals in some pairs. This is accounted for in the analysis by including all days at risk in the denominator of calculations. Risk (and incidence) were assessed at the cow level, regardless of which quarter(s) were infected.

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Statistical Analysis

All statistical analyses were carried out in a commercially available software package (R, version 3.3.2, R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was determined if *P*-values were less than 0.05. Serology results were not normally distributed and were analyzed using Mann-Whitney analysis. The arbitrary selection of individuals for serology was done without regard to the study pairing; thus, these were not analyzed in a paired manner.

Risk of mastitis was analyzed using conditional logistic regression with each pair as a strata and no additional predictors using the survival package in the statistical software. The analysis returned qualitatively similar results when including DIM and date as predictors; however, these were not significant and resulted in higher Akaike information criterion values.

Milk production was analyzed by calculating the difference between the milk production of the vaccinate and placebo individual within each pair for each DIM. That is, if the vaccinate produced 50 kg of milk on DIM 50 and the placebo in its pair produced 45 kg on DIM 50, the difference of 5 kg was used for analyses. This means that only days in which both pair members produced milk are included in this analysis. Significance was tested with a *t*-test on this paired difference.

Somatic cell counts were analyzed both categorically and numerically. A value of 200,000 cells/mL was used as a threshold for subclinical infection (Dohoo and Leslie, 1991). Each measured SCC was counted as a separate event at risk of infection, and the paired odds ratio was calculated to compare risk of infection between groups. For numerical analyses, we conducted a *t*-test on the paired difference of the natural log-transformed values within each pair at each testing date.

Conclusion Criterion

An α of 0.05 was used to determine statistical significance for each test, and each significant result is presented with a 95% confidence interval.

RESULTS

At study initiation, 325 cows were enrolled into the study. This consisted of 165 cows vaccinated with the placebo vaccine and 160 cows vaccinated with Kleb-SRP. Throughout the study, cows were removed from the study due to normal culling protocols established at the dairy before study initiation. In addition, heifers were continually added as they calved. In all, 569 cattle were vaccinated to be enrolled in the study, from which 229 pairs were analyzed.

As a result, 67 pairs were only vaccinated before calving, 91 pairs were only vaccinated after calving, and 60 pairs were analyzed as vaccinated both before and after calving (each of the milking periods was analyzed separately in the appropriate group). In total, 127 pairs had both animals vaccinated before calving and 151 pairs had both vaccinated after calving. An additional 31 pairs were excluded because the 2 individuals failed to qualify for the same analysis (e.g., one was vaccinated before calving, the other was not vaccinated until after). An additional group of heifers (n = 71 animals) were vaccinated late in the study but were never eligible for analysis, as the study ended before they calved.

Table 1 shows the reasons why cows were culled and thus removed from the study. We found no significant difference in culling between the groups. Ninety-five trial animals were removed from the dairy for various reasons, most of which were unrelated to the study and typical of animal removals for a commercial dairy. Forty-eight were removed from the Kleb-SRP group and 47 animals removed from the placebo group. In total, 10 animals were removed due to *Klebsiella* spp. mastitis in the Kleb-SRP group, whereas 19 were removed from the placebo group. One Kleb-SRP animal was culled that had been diagnosed with both *Klebsiella* spp. and *E. coli* clinical mastitis simultaneously in different quarters. Six and 5 animals were culled for clinical coliform mastitis other than *Klebsiella* spp. from the Kleb-SRP and placebo groups, respectively. Study or dairy personnel reported no adverse systemic or local reactions following vaccination.

Table 1. Reasons for and numbers of removals from herd by treatment via established culling procedures in place at the dairy, which were followed throughout the study[1]

Item	Kleb-SRP	Placebo	Total
Klebsiella mastitis	10	19	29
Coliform mastitis (other than *Klebsiella*)	6	5	11
Klebsiella and *Escherichia coli* mastitis	1	0	1
Reproductive failure (sold)	9	6	15
Low production (sold)	7	5	12
Injury	6	6	12
Digestive disorder	3	2	5
Displaced abomasum	2	0	2
Heart failure/hardware	1	1	2
Lame (sold)	0	1	1
Metritis	1	0	1
Fatty liver	1	0	1
Milk fever	0	1	1
Exsanguination	0	1	1
Respiratory	1	0	1
Total	48	47	95

[1]Kleb-SRP = animals vaccinated with the trial vaccine; Placebo = animals treated with the placebo vaccine.

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Table 2. Bacterial isolates from clinical mastitis cases by treatment[1]

	Kleb-SRP after calving	Placebo after calving	Kleb-SRP before calving	Placebo before calving	Kleb-SRP all	Placebo all	Total
Klebsiella pneumoniae	21	12	6	20	31	38	69
Klebsiella oxytoca	0	1	0	0	0	1	1
Escherichia coli	15	11	9	11	27	31	58
Enterobacter spp.	2	3	1	5	5	8	13
Serratia spp.	1	2	6	2	7	6	13
Other gram-negative	0	1	2	1	2	2	4
Staphylococcus spp.	2	1	1	2	3	4	7
Streptococcus spp.	3	1	8	2	14	8	22
Enterococcus spp.	0	0	2	1	2	1	3
Lactococcus spp.	0	0	0	2	0	2	2
Other gram-positive	0	1	3	1	3	2	5
Prototheca spp.	0	1	1	0	2	1	3
No growth	12	15	6	19	20	41	61
Contaminated	1	1	1	0	3	2	5
Total	57	50	46	66	119	152	219

[1]Note that "all" includes incidents from when the pair members qualified for different subgroups (and thus were excluded from the subgroup analyses). Kleb-SRP = animals vaccinated with the trial vaccine; Placebo = animals treated with the placebo vaccine.

Table 2 shows the bacterial etiologies associated with the cases of CM that occurred during the study. Six *Klebsiella* spp. cases were detected in the Kleb-SRP group when vaccinated before calving compared with 20 in the placebo group. When vaccination occurred after calving, we observed 21 *Klebsiella* spp. cases in the Kleb-SRP group when vaccinated before calving compared with 13 in the placebo group. We found 22 coliform cases in the Kleb-SRP group when vaccinated before calving compared with 38 in the placebo group. When vaccination occurred after calving, 39 coliform cases were observed in the Kleb-SRP group when vaccinated before calving compared with 29 in the placebo group.

Table 3 shows the comparisons of risk between different vaccine times (all or before or after calving), bacterial pathogens, and treatment group (Kleb-SRP or placebo). When analyzing the data together, we found no significant difference in risk between groups for either *Klebsiella* or all coliforms ($P = 0.977$ and 0.387, respectively; conditional logistic regression). Within the pairs that were vaccinated before calving, the Kleb-SRP vaccine group had a significantly lower risk of mastitis caused by each *Klebsiella* and all coliforms, with 76.9

Table 3. Cases and risk for a measure (*Klebsiella* vs. all coliforms) and group (vaccinated before or after calving) for each treatment[1]

Measure	Group	Treatment[2]	Days at risk	Clinical mastitis cases[3] (no.)	Daily risk	Risk ratio	Conditional logistic effect (%)
Klebsiella	All	Placebo	46,267	38	0.00082	0.829	−0.7
Klebsiella	All	Kleb-SRP	45,510	31	0.00068		
Coliforms	All	Placebo	45,717	81	0.00177	0.814	−14.1
Coliforms	All	Kleb-SRP	45,052	65	0.00144		
Klebsiella	Before	Placebo	14,685	20	0.00136	0.282	−76.9*
Klebsiella	Before	Kleb-SRP	15,642	6	0.00038		
Coliforms	Before	Placebo	14,476	37	0.00256	0.532	−47.5*
Coliforms	Before	Kleb-SRP	15,449	21	0.00136		
Klebsiella	After	Placebo	25,051	13	0.00052	1.700	94.8
Klebsiella	After	Kleb-SRP	23,798	21	0.00088		
Coliforms	After	Placebo	24,835	28	0.00113	1.354	32.5
Coliforms	After	Kleb-SRP	23,589	36	0.00153		

[1]The days at risk include all milking days while eligible (except 14 d following a case, which were excluded to prevent double counting of a single infection). The daily risk gives the risk of mastitis from that measure on each milking day. The risk ratio is the ratio of the 2 risks, without accounting for the paired nature of the data. The conditional logistic percent effect gives the effect as percent change of risk from placebo to vaccinates when accounting for the paired nature of the data.

[2]Kleb-SRP = animals vaccinated with the trial vaccine; Placebo = animals treated with the placebo vaccine.

[3]The number of clinical cases in this table may be slightly different than in Table 2, as some clinical cases had more than 1 bacteria isolated on the same day.

*Indicates that the conditional logistic effect was significantly different ($P < 0.05$).

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and 47.5% reductions in risk respectively ($P = 0.004$ and 0.031, respectively; conditional logistic regression). However, the effect was not significant within the pairs vaccinated after calving ($P = 0.073$ and 0.285, respectively; conditional logistic regression).

Antibody response following vaccination is illustrated in Figure 1. Antibody response measured by ELISA showed a strong serological response following vaccination in cows vaccinated with Kleb-SRP compared with cows vaccinated with placebo. We observed a significant effect of treatment, time of testing, and an interaction between them (each $P < 0.0001$, ANOVA). Treated individuals did not significantly differ from placebo before vaccination ($P = 0.365$, Post-Hoc Mann-Whitney); however, a statistical difference between groups was present at the time of second vaccination ($P = 0.0283$) and 2 wk after the second vaccination ($P < 0.0001$).

Daily milk production by treatment are shown in Figure 2. In our study, Kleb-SRP–vaccinated cows averaged 0.31 kg more milk per day than paired placebo cows ($P < 0.0001$; 95% CI = 0.19 to 0.43 kg). In pairs vaccinated before calving, this effect was larger at 1.74 kg more milk per day ($P < 0.0001$, 95% CI = 1.54 to 1.94 kg per day, paired *t*-test). In pairs vaccinated after calving, the effect was reversed, with vaccinates producing an average of 0.39 kg less milk per day than placebos ($P < 0.0001$, 95% CI = 0.23 to 0.54 kg per day, paired *t*-test).

Tables 4, 5, and 6 show the compared risk of pairs for high SCC. The Kleb-SRP–vaccinated cows had 76.5% the risk for an SCC value above 200,000 SCC/mL, though this effect was not significant [Table 4; $P = 0.0581$, odds ratio (**OR**) = 0.765, 95% CI = 0.736 to 0.796]. When vaccinated before calving, vaccinates had 50% of the risk of having an SCC value above 200,000 SCC/mL (Table 5; $P = 0.0118$, OR = 0.5, 95% CI = 0.428 to 0.584, paired odds ratio). We found no significant difference in the risk of high SCC in pairs vaccinated after calving (Table 6; $P = 0.518$, OR = 0.887, 95% CI = 0.829 to 0.949, paired odds ratio).

Figure 3 shows the density distribution of individual SCC throughout the trial. The SCC value for individuals vaccinated with Kleb-SRP were 20.1% lower than its placebo match ($P = 0.003$, estimate difference = 0.183, 95% CI = 0.062 to 0.304). In pairs vaccinated before calving, the SCC value for vaccinates averaged 64.8% lower than the placebo in its pair ($P < 0.0001$, estimate difference = 0.499, 95% CI = 0.262 to 0.736, paired *t*-test of natural log values). The difference in SCC values was not significant in pairs vaccinated after calving ($P = 0.224$, estimate = 0.09, 95% CI = −0.055 to 0.236, paired *t*-test of natural log values).

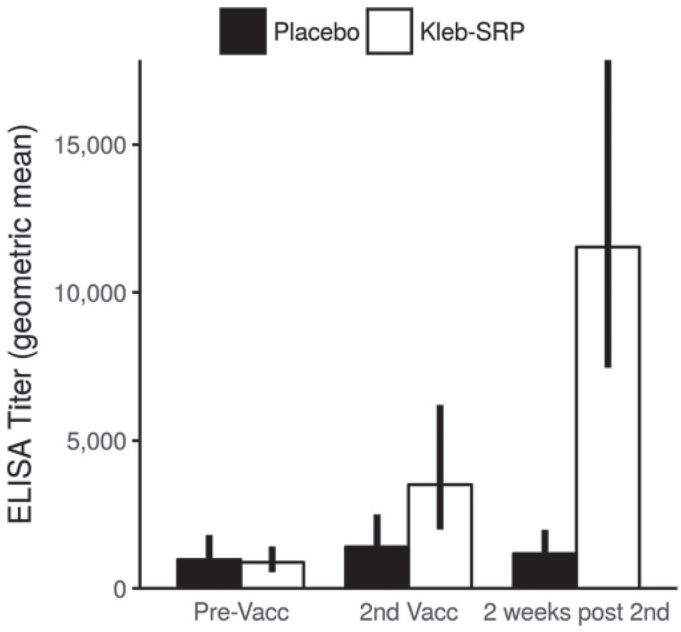

Figure 1. Mean serological response in 20 cows from each treatment group, measured by ELISA. Time points were before first vaccination (vacc), before second vaccination, and 2 wk following second vaccination with Kleb-SRP or placebo vaccine. Kleb-SRP = animals vaccinated with the trial vaccine; Placebo = animals treated with the placebo vaccine. Error bars represent the 95% CI.

DISCUSSION

Clinical mastitis caused by *Klebsiella* spp. produces clinical signs that are generally more severe than other mastitis pathogens (Schukken et al., 2012). In the herd in which our trial was conducted, 57% of cows with *Klebsiella* CM were culled or died as the result of the mastitis despite aggressive therapy during a 16-mo period before initiation of the current vaccine study. Preventative strategies to combat these cases through the use of a licensed J5 vaccine, which claims to aid in the control of clinical signs associated with *E. coli* mastitis, were a major focus before initiation of this vaccine trial. However, even with 4 doses of the licensed product per lactation, *Klebsiella* mastitis continued at the dairy (M. D. Kleinhenz, unpublished data). In a study to evaluate the efficacy of J5 vaccination against various etiologic agents, Wilson et al. (2007) demonstrated a reduced risk for culling (20% risk for non-vaccinates vs. 0% for vaccinates) due to *Klebsiella* mastitis. In Wilson et al. (2007), cows were vaccinated 2 times during the dry period with a commercial product. Interestingly, they did not show a difference in rates of coliform CM. As in Wilson et al. (2007), our clinical impression of J5 vaccination was that it was not significantly affecting rates of CM in this herd; however, we did not do a

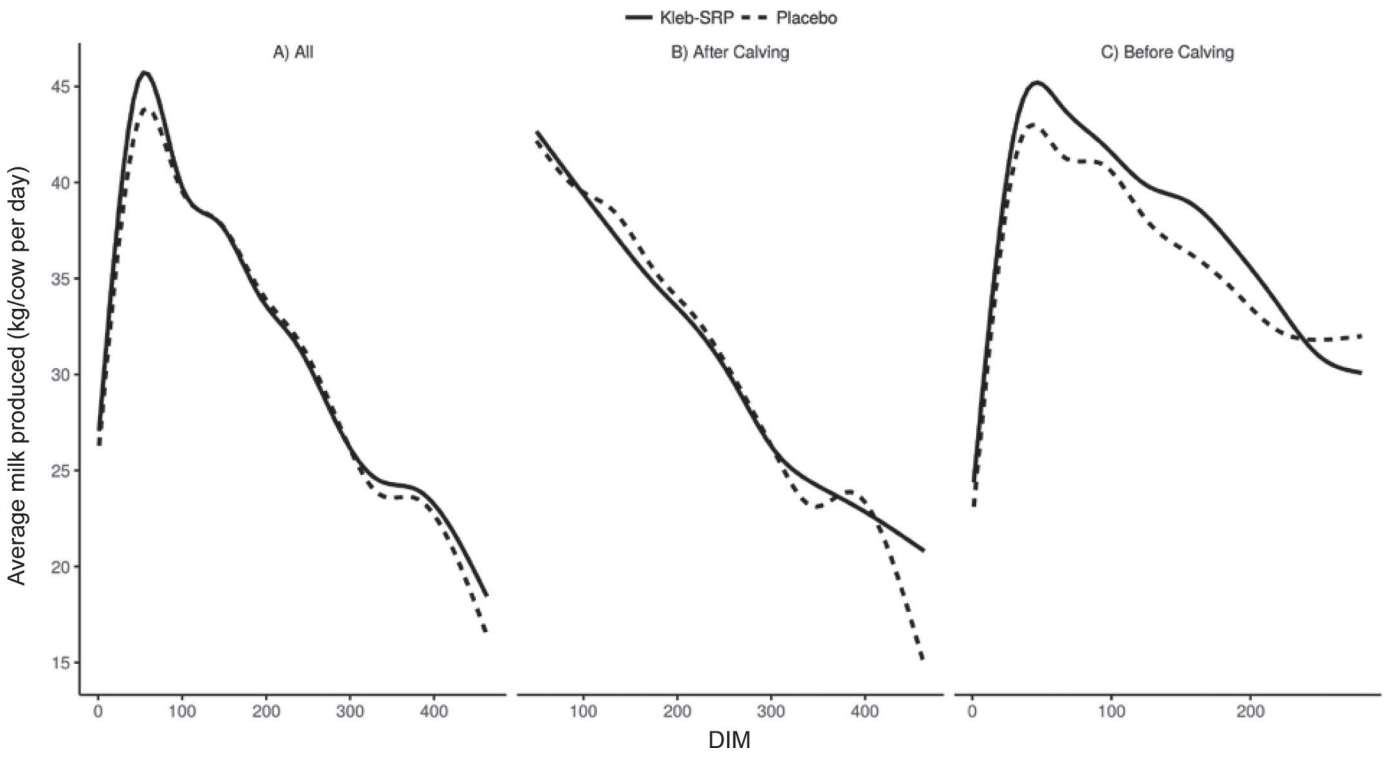

Figure 2. Plot of daily milk production per cow. Smoothed daily averages, using a generalized additive model, are shown for each treatment group. The lines represent the average for all individuals providing milk for the given DIM and do not account for the paired nature of the data. Kleb-SRP = animals vaccinated with the trial vaccine; Placebo = animals treated with the placebo vaccine.

controlled study on the efficacy of J5 versus *Klebsiella* CM. Additionally, bedding culture data (data not shown) indicated the bedding choice used in this herd did provide a severe environmental challenge against the farm's mastitis control program.

Due to the severe clinical nature of *Klebsiella* mastitis cases, dairies rely heavily on antibiotic therapies. However, even with aggressive therapy, losses due to culling or death are extremely high as a result of cases of *Klebsiella* CM (Schukken et al., 2012; M. D. Kleinhenz, unpublished data). Previous research has suggested that parenteral (Erskine et al., 2002) or intramammary (Schukken et al., 2011) administration of ceftiofur, a third-generation cephalosporin antimicrobial, may improve clinical outcome compared with no treatment. Ceftiofur is the most commonly used antimicrobial used in lactating dairy cattle (Zwald et al., 2004; Sawant et al., 2005; Schuler et al., 2017). Third-generation cephalosporins have been designated as critically important antimicrobials by the US Food and Drug Administration (**FDA**) due to heightened concerns about development of antimicrobial resistance (US FDA, 2003). Additionally, the US FDA issued extralabel prohibition for cephalosporins in 2012 in an attempt to limit the development of antimicrobial resistance (US FDA, 2012). With these new regulations, and potentially more to come, it is all the more important to provide the dairy industry with new, innovative, and effective vaccines to prevent diseases such as *Klebsiella* mastitis.

Cows vaccinated with Kleb-SRP showed a marked increase in antibody titer compared with the placebo group (Figure 1). Although it is not known what titer is sufficient for protection, the data shows that, upon second vaccination, a large anamnestic response was observed in Kleb-SRP vaccinates compared with pla-

Table 4. Pair results for all cows in the trial[1]

Item	Kleb-SRP negative	Kleb-SRP positive	Sum
Placebo negative	555	88	643
Placebo positive	115	30	145
Sum	670	118	788

[1]Values >200,000 SCC/mL were considered positive to capture subclinical infections. Kleb-SRP = animals vaccinated with the trial vaccine; Placebo = animals treated with the placebo vaccine. Each entry is the number of pairs in which the Kleb-SRP and placebo individual were either positive or negative on the SCC test. The paired odds ratio was calculated from pairs in which only one individual was positive.

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Table 5. Pair results when vaccinated before calving[1]

Item	Kleb-SRP negative	Kleb-SRP positive	Sum
Placebo negative	146	19	165
Placebo positive	38	7	45
Sum	184	26	210

[1]Values >200,000 SCC/mL were considered positive to capture subclinical infections. Each entry is the number of pairs in which the Kleb-SRP and placebo individual were either positive or negative on the SCC test. The paired odds ratio was calculated from pairs in which only one individual was positive.

Table 6. Pair results when vaccinated after calving[1]

Item	Kleb-SRP negative	Kleb-SRP positive	Sum
Placebo negative	354	55	409
Placebo positive	62	15	77
Sum	416	70	486

[1]Values >200,000 SCC/mL were considered positive to capture subclinical infections. Each entry is the number of pairs in which the Kleb-SRP and placebo individual were either positive or negative on the SCC test. The paired odds ratio was calculated from pairs in which only one individual was positive.

cebos. This demonstrates that the immune system was primed and a booster response is seen with subsequent exposure to the antigen.

The most common organism causing coliform CM in our study was *Klebsiella* spp., followed by *E. coli* (Table 2). It was surprising to see this much coliform mastitis in a herd where cows were vaccinated 4 times per lactation with a commercially available J5 vaccine. Bedding the cattle on recycled manure solids may partly explain the high incidence. However, even in the face of the high challenge, cows vaccinated before calving with Kleb-SRP had significant protection versus cows vaccinated with placebo.

The amount of milk produced by a cow can be a useful indicator of overall health. It is well known in the dairy industry that CM reduces milk production in affected cows (Gröhn et al., 2004; Pinzón-Sánchez et al., 2011). The statistically significant increase in milk production of 1.74 kg of milk per cow per day when vaccinated with Kleb-SRP before calving and decrease of 0.39 kg when vaccinated after calving is difficult to explain. Intuitively, the increase appears to be based solely on the differences in CM between the 2 groups, but there may have been some advantage to reducing subclinical infections on increased milk production. More work is needed to determine if protection against

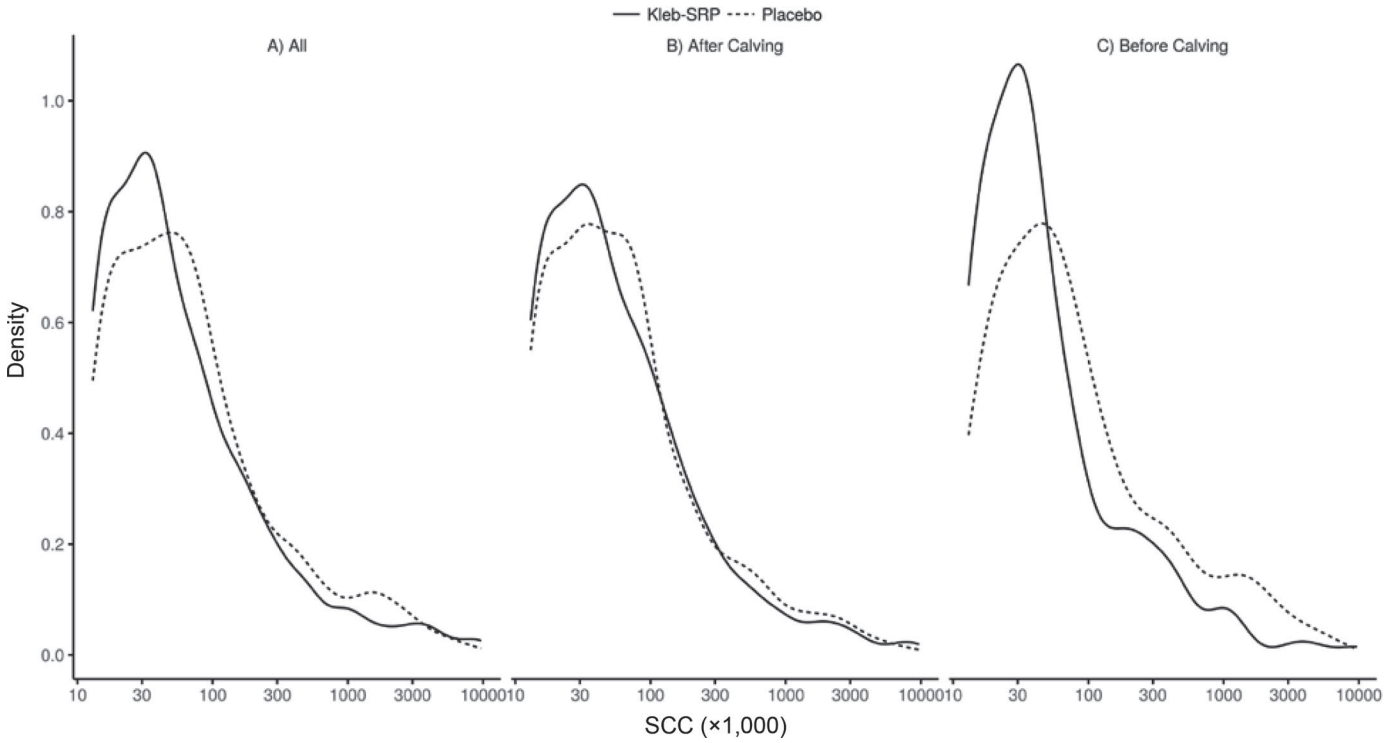

Figure 3. Distribution of SCC by treatment. The distribution of SCC values is shown as a density distribution for each treatment group. The area under each curve sums to 1 allowing a direct comparison of the portion of results that occurred in a particular range. Kleb-SRP = animals vaccinated with the trial vaccine; Placebo = animals treated with the placebo vaccine.

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subclinical infections may have caused these differences between groups.

Somatic cell counts are routinely used to monitor milk quality and typically increase during CM. In our study, we found a large reduction in SCC in the Kleb-SRP–vaccinated cows. Such a large reduction would not be expected if the vaccine only reduced SCC in CM cows. Somatic cell count is also a good indicator of subclinical mastitis. The reduced SCC and increased milk in the Kleb-SRP–vaccinated cows may be attributable not only to CM, but also to subclinical coliform infections. Further studies could evaluate the effect of such subclinical infections, as this finding suggests they are an important component of overall herd health and productivity.

The clear demarcation in protection against CM caused by *Klebsiella* spp. and other coliforms between animals vaccinated before versus after calving was unexpected and not an objective of the trial. This outcome, combined with the increased milk production and decreased risk of elevated SCC, would clearly suggest that vaccine protocols should be designed to administer the Kleb-SRP vaccine during the precalving period. Care must be taken when doing this to not overwhelm the immune system of the animal by administering too many vaccines simultaneously or too close to parturition. Additionally, protection against other coliforms besides *Klebsiella* spp. would suggest that the Kleb-SRP vaccine may be an alternative to J5 vaccines. Further research is needed to confirm the effect of timing of vaccination in relation to calving on protection and the effect of removal of J5 vaccine from a vaccine protocol when Kleb-SRP is inserted.

CONCLUSIONS

This study was independently conducted under field conditions by personnel at the ISU Dairy, which was managed similar to other US commercial dairy operations. Based on the results seen in this study, administering Kleb-SRP vaccine before calving can make a meaningful reduction of *Klebsiella* CM and CM attributed to coliform organisms. In addition, increased milk production and lower SCC in cows vaccinated before calving potentially provides a considerable advantage beyond the direct effect of controlling CM. It should be noted that this herd was chosen because of the high number of clinical *Klebsiella* CM cases experienced before the trial and the extreme environmental challenge that was present. Results in other herds may be substantially different from these findings. The Kleb-SRP vaccine should be considered, along with other on-farm interventions, to control these highly virulent pathogens in dairy cows.

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REFERENCES

Bardoel, B. W., and J. A. van Strijp. 2011. Molecular battle between host and bacterium: Recognition in innate immunity. J. Mol. Recognit. 24:1077–1086.

Dohoo, I. R., and K. E. Leslie. 1991. Evaluation of changes in somatic cell counts as indicators of new intramammary infections. Prev. Vet. Med. 10:225–237.

Emery, D. A., D. E. Straub, and L. Slinden. 2001. Evaluation of a novel vaccine consisting of siderophore receptor proteins and porins for controlling salmonellosis in a commercial dairy herd. Page 132 in Proc 34th Am. Assoc. Bovine Pract. Conf., Vancouver, BC, Canada, V. M. Publishing Co., Stillwater, OK.

Erskine, R. J., P. C. Bartlett, V. L. Van Lente, and C. R. Phipps. 2002. Efficacy of systemic ceftiofur as a therapy for severe clinical mastitis in dairy cattle. J. Dairy Sci. 85:2571–2575.

FASS. 2010. Dairy cattle. Pages 74–88 in Guide for the Care and Use of Agricultural Animals in Teaching and Research. 3rd ed. FASS Inc., Champaign, IL.

Gröhn, Y. T., D. J. Wilson, R. N. Gonzalez, J. A. Hertl, H. Schulte, G. Bennett, and Y. H. Schukkken. 2004. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. J. Dairy Sci. 87:3358–3374.

Hertl, J. A., Y. H. Schukken, D. Bar, G. J. Bennet, R. N. Gonzalez, B. J. Rauch, F. L. Welcome, L. W. Tauer, and Y. T. Gröhn. 2011. The effect of recurrent episodes of clinical mastitis caused by gram-positive and gram-negative bacteria and other organisms on mortality and culling in Holstein dairy cows. J. Dairy Sci. 94:4863–4877.

Hood, M. I., and E. P. Skaar. 2012. Nutritional immunity: Transition metals at the pathogen-host interface. Nat. Rev. Microbiol. 10:525–537.

Kimura, K., J. P. Goff, M. E. Kehrli Jr., J. A. Harp, and B. J. Nonnecke. 2002. Effects of mastectomy on composition of peripheral blood mononuclear cell populations in periparturient dairy cows. J. Dairy Sci. 85:1437–1444.

Llobet, E., M. A. Campos, P. Giménez, D. Moranta, and J. A. Bengoechea. 2011. Analysis of the networks controlling the antimicrobial-peptide-dependent induction of *Klebsiella pneumoniae* virulence factors. Infect. Immun. 79:3718–3732.

Miethke, M., and M. A. Marahiel. 2007. Siderophore-based iron acquisition and pathogen control. Microbiol. Mol. Biol. Rev. 71:413–451.

Munoz, M. A., F. L. Welcome, Y. H. Schukken, and R. N. Zadoks. 2007. Molecular epidemiology of two *Klebsiella pneumoniae* mastitis outbreaks on a dairy farm in New York State. J. Clin. Microbiol. 45:3964–3971.

National Mastitis Council. 1999. Laboratory and Field Handbook on Bovine Mastitis. W. D. Hoard and Sons, Fort Atkinson, WI.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Sci., Washington, DC.

Oliveira, L., C. Hulland, and P. L. Ruegg. 2013. Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin. J. Dairy Sci. 96:7538–7549.

Pinzón-Sánchez, C., V. E. Cabrera, and P. L. Ruegg. 2011. Decision tree analysis of treatment strategies for mild and moderate cases of clinical mastitis occurring in early lactation. J. Dairy Sci. 94:1873–1892.

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Ruegg, P. L. 2003. Investigation of mastitis problems on farms. Vet. Clin. North Am. Food Anim. Pract. 19:47–73.

Sawant, A. A., L. M. Sordillo, and B. M. Jayarao. 2005. A survey on antibiotic usage in dairy herds in Pennsylvania. J. Dairy Sci. 88:2991–2999.

Schukken, Y., M. Chuff, P. Moroni, A. Gurjar, C. Santisteban, F. Welcome, and R. Zadoks. 2012. The "other" gram-negative bacteria in mastitis *Klebsiella*, *Serratia*, and more. Vet. Clin. North Am. Food Anim. Pract. 28:239–256.

Schukken, Y. H., G. J. Bennett, M. J. Zurakowski, H. L. Sharkey, B. J. Rauch, M. J. Thomas, B. Ceglowski, R. L. Saltman, N. Belomestnykh, and R. N. Zadoks. 2011. Randomized clinical trial to evaluate the efficacy of a 5-day ceftiofur hydrochloride intramammary treatment on nonsevere gram-negative clinical mastitis. J. Dairy Sci. 94:6203–6215.

Schuler, A. M., C. Rice, and P. J. Gorden. 2017. Survey of treatment practices on Midwest dairy farms. Bov. Pract. 51. In press.

Thomson, D. U., G. H. Loneragan, A. B. Thornton, K. F. Lechtenberg, D. A. Emery, D. T. Burkhardt, and T. G. Nagaraja. 2009. Use of a siderophore receptor and porin proteins-based vaccine to control the burden of *Escherichia coli* O157:H7 in feedlot cattle. Foodborne Pathog. Dis. 6:871–877.

US FDA (United States Food and Drug Administration). 2003. Guidance for Industry #152: Evaluating the safety of antimicrobial new animal drugs with regard to their microbiological effects on bacteria of human health concern. Accessed Feb. 12, 2015. http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/ucm052519.pdf..

US FDA (United States Food and Drug Administration). 2012. New animal drugs; cephalosporin drugs; extralabel animal drug use; order of prohibition. Fed. Regist. 77:725–745.

Wenz, J. R., G. M. Barrington, F. B. Garry, K. D. McSweeney, R. P. Dinsmore, G. Goodell, and R. J. Callan. 2001. Bacteremia associated with naturally occurring acute coliform mastitis in dairy cows. J. Am. Vet. Med. Assoc. 219:976–981.

Wilson, D. J., B. A. Mallard, J. L. Burton, Y. H. Schukken, and Y. T. Gröhn. 2007. Milk and serum J5-specific antibody responses, milk production change, and clinical effects following intramammary *Escherichia coli* challenge for J5 vaccinate and control cows. Clin. Vaccine Immunol. 14:693–699.

Zwald, A. G., P. L. Ruegg, J. B. Kaneene, J. D. Warnick, S. J. Wells, C. Fossler, and L. W. Halbert. 2004. Management practices and reported antimicrobial usage on conventional and organic dairy farms. J. Dairy Sci. 87:191–201.