Evaluation of an alternative dosing regimen of a J-5 mastitis vaccine against intramammary *Escherichia coli* challenge in nonlactating late-gestation dairy cows

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ABSTRACT

The objective of the study was to evaluate the efficacy of an alternative vaccination regimen of a J-5 bacterin against intramammary *Escherichia coli* challenge in nonlactating late-gestation dairy cows. The parameters analyzed to assess the effect of vaccination were milk yield, milk conductivity, somatic cell count, J-5-specific serum IgG titers, and clinical signs. Twenty multiparous Holstein cows from the Cornell teaching and research dairy herd were paired by days in milk and were randomly selected to receive either the alternative off-label regimen of commercial J-5 bacterin or act as nonvaccinated controls. Cows received a first dose of bacterin 15 d before dry off, a second dose with the same product at the day of dry off, and the third dose 2 wk after dry off. The cows in both groups were challenged 10 d before the expected calving date. Serum IgG (total, IgG1 and IgG2) levels were higher in vaccinates compared with control cows. Eighty-five percent of challenged quarters became infected between both groups of animals. Eight of the 10 vaccinated and 9 of the 10 control cows showed signs of clinical mastitis postfreshening. A non-severe clinical mastitis was observed 24 to 48 h postparturition, characterized by flakes or clots in milk and mild swelling or pain. Off-label vaccination did reduce the clinical severity of clinical mastitis in the vaccinated group of cows as evidenced by reduced California mastitis test score, fewer flakes and lower overall clinical mastitis score. No significant differences between vaccinated and control groups were detected for rectal temperature. In conclusion, the alternative off-label vaccination scheme used in our study and evaluated in a novel *E. coli* challenge model did not prevent new intramammary infections but reduced clinical severity of experimentally induced *E. coli* mastitis.

Key words: *Escherichia coli*, intramammary infection, J-5 bacterin, late gestation

INTRODUCTION

Bovine mastitis caused by environmental pathogens such as *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., and *Citrobacter* spp. is usually high during the early postpartum period in dairy cows (Olde Riekerink et al., 2008). Coliform mastitis is often associated with systemic symptoms and may lead to severe illness, substantial milk loss, premature culling, or even death (Scott et al., 1998; Gröhn et al., 2004, 2005; Cha et al., 2011). Clinical mastitis is characterized by visible changes in milk, including the presence of clots, flakes, serum, or even blood inclusion. The mammary gland may show heat, pain, or swelling and in severe cases systemic signs such as fever, anorexia, dehydration, and depression are present. Several preventive strategies have been applied to minimize the incidence of bovine mastitis, including optimization of milking procedures and milking hygiene, antibiotic therapies, vaccinations, and culling of persistently infected cows (Zecconi et al., 2003). However, mastitis remains an important disease on dairy farms and because of high costs of clinical mastitis, reduction in the severity of the symptoms of mastitis and obtaining a more rapid clearance of established infections is of great value to dairy farmers.

Vaccines using core cell wall antigens have been developed to provide protection against a variety of gram-negative pathogens and have been registered for the reduction of severity in mastitis caused by the coliform organisms (Hogan et al., 1992, 1995). The severity of clinical symptoms of coliform mastitis has been shown to be reduced by immunization with commercially available J-5 bacterin (Hogan et al., 1995). The efficacy of this vaccine for the prevention of mastitis caused by *E. coli* has been investigated in experimental challenge studies (Hill, 1991; Scott et al., 1998; Tomita et al., 2000; Wilson et al., 2007b). All these studies implied that immunization with J-5 bacterin reduced the sever-

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ity of local and systemic signs of clinical mastitis following intramammary challenge. Vaccination with the J-5 bacterin has been reported in some early studies to reduce the incidence of naturally occurring clinical coliform mastitis during early lactation (González et al., 1989). However, in subsequent field studies, when J-5 vaccination was administered at drying off, 4 wk later, and 1 to 7 DIM, a reduction in culling due to clinical mastitis was observed (Wilson et al., 2007b) but no reduction in the incidence of clinical coliform mastitis.

Clinical coliform mastitis that occurs immediately postpartum is likely the result of an IMI in late lactation (Green et al., 2002). Infections with coliform bacteria approximately 2 wk before calving in late gestation result in an established IMI without clinical signs as long as pregnancy lasts but will show clinical signs shortly after parturition (Quesnell et al., 2012). Prevention of such late-gestation IMI would be important and a true preventative intervention. However, the current vaccination scheme of the registered J-5 bacterins may not reach its peak protection until after parturition. For that reason, an alternative vaccination scheme with all 3 vaccinations occurring before a late-gestation IMI may be hypothesized to provide benefit. The objectives of this study, therefore, were to evaluate the efficacy of an alternative vaccination scheme with an *E. coli* J-5 bacterin on the development of antibody titers before parturition, risk of IMI, severity of clinical mastitis, SCC, conductivity, and milk yield in a novel challenge model using a late-gestation intramammary challenge with *E. coli*.

MATERIALS AND METHODS

Experimental Animals

Twenty adult Holstein cows were selected from the Cornell University Teaching and Research Dairy herd (Ithaca, NY). Selection was based on the following criteria: cows should have completed at least 1 lactation and have a predicted dry period of approximately 42 d (6 wk). Also, cows with any signs of clinical illness, including but not limited to clinical mastitis at time of dry off or within the last 6 mo, were excluded. Cows were also required to have individual SCC in the 3 mo before dry off below 250,000 cells/mL. Finally, cows with any major traumatic injury to the teat ends were excluded.

Vaccination Scheme

The selected cows were paired by lactation number and DIM and randomized to be vaccinated or serve as untreated controls. The J-5 bacterin (*E. coli* bacterin,

J-5 strain; Pfizer Animal Health, Kalamazoo, MI) was administered by the investigators (5 mL) subcutaneously on the upper part of the rib cage just posterior of the scapula. The cows in the control group were not vaccinated and did not receive any sham injection. The vaccination scheme consisted of a first dose of J-5 bacterin 15 d before dry off, followed by a second dose at dry off and a third dose 15 d after dry off.

Experimental Animal Housing and Management

Cows in both groups were dried off by abrupt cessation of milking and all 4 quarters were treated with dry-cow antibiotics but received no internal teat sealant. Cows in both groups were housed, fed, and managed identically throughout the dry period and immediately postcalving. Three weeks before the anticipated calving, cows were transferred from freestalls to the Cornell University Large Animal Research and Training Unit facility. In this facility, cows were housed in individual maternity pens and were trained daily to go into tie-stalls. All animal procedures such as milking, intramammary challenge, and blood and milk sampling took place in tie-stalls. Cows remained in the study from the first vaccination, throughout the dry period, calving, and first 10 d of the ensuing lactation. In the postcalving period, the animals were milked twice per day in the tie-stalls using a quarter milker that was connected to 4 Lactocorders (WMB AG, Balgach, Switzerland). The use of the quarter milker and Lactocorders enabled determining milk production and conductivity from individual quarters. After the first 10 d of lactation, cows were moved back to the lactating cow pens in freestalls at the Teaching and Research Dairy herd but were monitored for clinical mastitis by the milking staff throughout lactation. The study protocol was approved by the Cornell University Animal Care and Use and Committee.

Intramammary Bacterial Challenge

Escherichia coli strain C1 (Dogan et al., 2006), a strain originally isolated from a cow with persistent mild clinical mastitis, was used as the intramammary challenge strain. The challenge inoculum was prepared by inoculating a frozen stock culture of the C1 strain into Luria Bertani (LB) broth. The LB broth was incubated for 18 h at 37°C. A total of 100 µL of this culture was inoculated into fresh LB broth and incubated for approximately 2.5 h at 37°C. The log-phase LB broth culture was used for inoculation. A 1:10 dilution of the log-phase culture was made in PBS and adjusted to 100 cfu of inoculation dose. The colony-forming units per milliliter of the challenge bacteria was determined by

plating 100 μ L in duplicate on LB agar plates. Quarters had to be culture negative with a quarter cell count below 200,000 cells/mL to be inoculated. Two quarters of each cow in the study were challenged by infusion of approximately 100 cfu of *E. coli* C1 strain suspended in PBS. A third quarter received a similar infusion of vehicle (PBS) only, whereas the fourth quarter remained unchallenged. Preferably, both hind quarters were challenged, with the front quarters serving as within-cow controls. The cows were challenged approximately 10 d before the expected calving date.

Mammary Gland Sampling and Blood Sample Collection

Milk or dry cow mammary gland secretions (MGS) were aseptically collected in sterile vials for microbiological culture and SCC analysis at each vaccination time point, 7 d and 4 d before intramammary challenge, at the time just before challenge and every 12 h postchallenge for the first 3 d and every 24 h for the next 4 d. Throughout the trial, MGS were macroscopically checked for color and consistency at every sampling time point. All 4 quarters were sampled individually and the samples were transported on ice to the microbiology laboratory for culture. An aliquot of each milk sample was sent to Dairy One (Ithaca, NY) for SCC determination using a Fossomatic somatic cell counter (Foss Electric A/S, Hillerød, Denmark). Data were expressed as the logarithm (base 10) SCC per milliliter.

Blood samples from the tail vein were collected in Vacutainer glass tubes (Becton Dickinson Corp., Franklin Lakes, NJ) from all cows at the same time points as indicated above. Blood was allowed to clot at room temperature (20–22°C) and centrifuged at $1,500 \times g$ for 15 min, and the clear serum supernatant was collected, aliquoted, and stored at -80°C .

Serum Antibody Titers to J-5 *E. coli*

Serum was used to determine anti-J-5 *E. coli* total IgG, IgG1, and IgG2 concentrations using a previously described ELISA (Wilson et al., 2009). Briefly, flat-bottom 96-well ELISA plates (Falcon; BD, Bedford, MA) were coated with 100 μ L of J-5 *E. coli* (1×10^9 cfu/mL in sterile saline, killed with 1% phenol). Negative control wells with no *E. coli* were added on each plate to assess nonspecific binding of antibodies to plastic. Plates were washed and various control and test samples were added to appropriate wells. Four wells of each plate received a 1:400 dilution of low-immunoglobulin fetal bovine serum (Life Technologies Corp., Rockville, MD) as a negative control. An additional 4 wells received

a 1:400 dilution of serum from a J-5-hyperimmunized dairy cow as a positive control. Finally, additional control wells included 2 blanks (no *E. coli* or other test reagents) against which the plate reader was blanked, 2 wells with no *E. coli* or test serum but with all other test reagents added, 2 wells with *E. coli* but no other test reagents except serum, and 2 wells with *E. coli* plus all other test reagents except serum, leaving 80 wells of test samples of sera per plate. Once all samples were delivered to appropriate wells, ELISA plates were sealed and incubated at 37°C for 45 min. Following incubation, the plates were washed and 100 μ L/well of detection antibody (1:25,000 in sample diluent; horseradish peroxidase-conjugated sheep anti-bovine IgG, IgG1, or IgG2; Bethyl Laboratories, Montgomery, TX) was added to wells of the appropriate plates. Plates were again sealed and incubated and washed, and then 125 μ L/well of substrate (hydrogen peroxide-azino-bis-3-ethyl-benzthiazoline sulfonic acid; Sigma, St. Louis, MO) was added. The levels of isotype-specific antibodies in the variously diluted samples were recorded as optical density (OD) following spectrometric analysis at dual wavelength (405 nm normalized against 450 nm) using an ELISA plate reader (Benchmark; Bio-Rad Laboratories Inc., Hercules, CA). The plate reader was calibrated against the blank wells of each plate.

Bacteriology and Random Amplification of Polymorphic DNA Strain Typing

Milk and MGS samples were cultured according to the protocols recommended by the National Mastitis Council (NMC, 2004). Briefly, 100 μ L of milk or MGS were plated on Columbia sheep blood agar (Oxoid, Waltham, MA) and MacConkey agar plate. Plates were incubated for 24 to 48 h at 37°C and were examined daily for bacterial growth. The results of the bacteriological analysis were expressed as logarithm (base 10) colony-forming units per milliliter. Individual colonies were isolated and stored at -70°C for molecular strain typing by random amplification of polymorphic DNA (RAPD) analysis. Individual isolates from bacterial culture plates were grown in LB broth at 37°C for 12 h. The DNA was isolated from samples using a QIAquick DNeasy isolation kit (Qiagen Inc., Valencia, CA). The RAPD primers designed specifically for RAPD typing of gram-negative bacteria were as follows: forward 5'-AGTAAGTGACTGGGGTGAGCG-3' and reverse 5'-TACATTTCGAGGACCCCTAAGTG-3'. These primers have previously been shown to provide discernment between mastitis *E. coli* bacterial strains (Dogan et al., 2006). The PCR products were evaluated using gel electrophoresis in a 1.5% agarose gel at 60 V for 1.5 h.

Clinical Observation for Local and Systemic Signs

Systemic and local symptoms of inflammation were assessed throughout the trial period as described earlier by Quesnell et al., (2012). For systemic signs, rectal temperature, appetite, dehydration, and general attitude were evaluated. Cutoffs were for rectal temperature $\geq 39.5^{\circ}\text{C}$ (103°F), for appetite a 50% decrease in intake in DM, for hydration score a moderate to marked enophthalmos, and for attitude score showing signs of marked depression. Systemic signs were scored on a 4-point scale: 1 = no signs to 4 = severe systemic signs, with a 1-point score for each of the 4 criteria. The udder was palpated for soreness, swelling, temperature, and hardness. The udder was scored on a 1 to 4 scale, with 1 being normal and 4 indicating that the gland was swollen, warm, sore, and firm, again with a point for each criterion. The milk appearance, consistency, and color were scored daily in foremilk before milking at an approximate 12-h interval. The milk appearance was scored on a 4-point scale, with 1 being normal white homogenous milk and 4 being dark yellowish secretion with blood. The score for milk appearance was 1 for normal milk, 2 for flakes or clots, 3 for watery, and 4 for serum or blood. Clots and flakes were also detected on the Lactocorder filter. All quarters were tested with the California mastitis test (CMT) in the foremilk and scored on a 1 to 4 scale, with 1 being normal and 4 being highly abnormal with elevated clumps present even after swirling stopped. Clinical score was the sum of the above 4 scores and ranged between 4 and 16. Cows with overall clinical score of 4 were recorded as having no mastitis, those with scores between 5 to 12 as having mild to moderate mastitis, and those with scores of >12 as having severe mastitis.

Data Analysis

All data was analyzed using the SAS v 9.3 statistical analysis program (SAS Institute Inc., Cary, NC). Initially, data were graphed and evaluated for outliers and unlikely observations. Outliers were studied in detail and reanalyzed to confirm where necessary. Presence or absence of IMI in challenged quarters between control and vaccinates was statistically evaluated using chi-squared analysis. Differences in SCC dynamics, serum IgG, systemic, and local scores for clinical mastitis between groups were compared using mixed models (SAS, PROC MIXED), adjusting for random cow effects and repeated measures within the same quarter over time. A Bonferroni correction for post hoc tests was used. The generalized linear mixed model had the following generic format:

$$Y_{ijk} = \text{intercept} + \text{vaccination}_i \\ + \text{time point}_j + \text{cow}_k + \text{Re}_{ijk},$$

where Y_{ijk} is the outcome, vaccination_i is a binary variable indicating whether a cow was vaccinated or control ($i = 1$ or 0), time point_j is a set of indicator variables for each measurement time j , cow_k is a random cow effect for cow k , and Re_{ijk} is a complex error term with a repeated measures within quarter correlation term (typically AR-1) and a random error term. Relevant interactions were tested in the model, particularly vaccination $\times$ time point to evaluate consistency of any vaccine effect over time. Differences were considered statistically significant when the probability of a type I error was <0.05 .

RESULTS

Characteristics of the cows included in the study are shown in Tables 1 and 2. All cows completed the study and all cows went back to the lactating herd without major remaining issues.

Vaccination and J-5-Specific IgG1, IgG2, and Total Antibodies

No adverse reactions to the bacterin including swelling at the injection site were observed in the J-5-vaccinated animals. In blood samples taken before the first vaccination, no significant difference was observed in the J-5-specific total IgG, IgG1, and IgG2 OD titers in study cows randomly allocated to be vaccinated or serve as controls (Figure 1). The total IgG values were higher in subsequent blood samples in vaccinated cows compared with control unvaccinated cows (Figure 1a, 1b, and 1c). The largest difference in the J-5-specific total IgG serum OD titer in vaccinated cows compared with unvaccinated cows at *E. coli* was 1.04 OD units and occurred at the time of the bacterial challenge (Figure 1c). The J-5-specific IgG1 values were significantly higher in vaccinates compared with controls starting at the time of the second vaccination ($P < 0.01$; Figure 1a). A significant difference was observed in the IgG2 serum values in vaccinates and controls at the 7-d prechallenge and challenge time points ($P < 0.05$; Figure 1b). All these data indicated that at the time of the bacterial challenge, the humoral immune response was expected to be better in vaccinated compared with control animals.

IMI

The MGS from all challenged quarters of both groups of animals had tested negative for any major

Table 1. Results of *Escherichia coli* C1 challenge in late gestation and early lactation in 10 vaccinated cows¹

Cow ID	Challenge			Calving relative to due date (d)	Postpartum			
	Lactation number	Challenge location	Quarters infected at 24 h		Quarters infected	Clinical signs	Severity score	Mastitis at 100 DIM
8004	3	F	LF	7	—	LF	1	Y
8288	2	R	LR, RR	−6	LR,RR	LR, RR	4	Y
8439	1	F	LF, RF	−2	LF	LR, RR	3	N
8313	1	R	LR	−3	—	—	—	Y
8487	1	R	LR, RR	−7	LR	LR, RR	3	N
8544	1	R	LR, RR	2	RR	—	—	N
8600	1	R	LR, RR	7	LR,RR	RR	2	N
8386	2	R	LR, RR	−2	LR,RR	RR	1	N
8612	2	R	LR, RR	−3	LR,RR	LR, RR	3	N
8657	2	R	LR, RR	−2	LR,RR	LR, RR	3	Y

¹Presented in this table is the cow identification (ID), lactation number, location of quarters challenged (F = front quarters; R = rear quarters), quarters infected at 24 h postchallenge, calving relative to due date (d), quarters infected postpartum, presence of clinical signs postpartum, severity score of clinical signs, and the occurrence of clinical mastitis in the first 100 DIM after the return of the cow to the dairy herd (Y = yes; N = no). Quarter coding is left rear (LR), right rear (RR), left front (LF), and right front (RF).

mastitis pathogens before the coliform challenge. The bacteriological status of quarters immediately postchallenge and postfreshening is shown in Tables 1 and 2. In vaccinated cows, 90% (18 out of 20) of the challenged quarters were infected, as indicated by the recovery of the inoculated strain C1 at least once at or at 24 h postinoculation (Table 1), whereas 80% (16 out of 20) of the inoculated quarters in control cows showed the presence of the C1 strain postchallenge (Table 2). This difference in IMI was not statistically significant (chi-squared, $P > 0.05$). Similarly, no significant difference (linear model, $P > 0.05$) in the bacterial counts in MGS or milk was observed between the vaccinated and control group of animals (Figure 2). Bacteria were consistently recovered from the challenged quarters from both groups of animals up to 7 d postchallenge and up to 6 d postparturition. No significant difference was observed between bacterial recovery from challenged quarters in the vaccinated group and the control group

($P > 0.05$). Virtually all *E. coli* organisms that were recovered from the challenged quarters were shown to be the genotype of the challenge organism. Isolates were strain typed using RAPD and a typical result of the strain typing is shown in Figure 3. This figure has isolates over time from cows 8004, 8136, and 8288; all isolates showed the identical genotype as the challenge strain C1 (far right lane). In the postcalving samples (7 d postcalving), 65% (13 out of 20) of inoculated quarters showed presence of the challenged strain in vaccinated cows compared with 45% (9 out of 20) in control cows. This difference in IMI postpartum was also not statistically significant (chi-squared, $P = 0.21$).

Clinical Signs

Intramammary inoculation of 100 cfu/mL of *E. coli* during late gestation did not result in any significant systemic symptoms immediate postchallenge in any

Table 2. Results of *Escherichia coli* C1 challenge in late gestation and early lactation in 10 control cows¹

Cow ID	Challenge			Calving relative to due date (d)	Postpartum			
	Lactation number	Challenge location	Quarters infected at 24 h		Quarters infected	Clinical signs	Severity score	Mastitis at 100 DIM
8136	3	F	LF, RF	1	—	—	—	N
8349	2	R	RR	5	—	RR	2	N
8398	2	F	LF, RF	−11	LF, RF	LF, RF	4	Y
8440	1	R	LR	3	—	LR, RR	4	N
8488	1	R	LR, RR	−6	LR, RR	RR	1	N
8466	1	R	RR	1	RR	LR, RR	1	N
8500	1	R	LR	5	—	LR	3	N
8362	2	R	LR, RR	2	—	LR, RR	3	N
8681	2	R	LR, RR	−2	LR, RR	LR, RR	2	N
8699	2	R	LR, RR	−1	LR, RR	LR, RR	3	N

¹Presented in this table is the cow identification (ID), lactation number, location of quarters challenged (F = front quarters; R = rear quarters), quarters infected at 24 h postchallenge, calving relative to due date (d), quarters infected postpartum, presence of clinical signs postpartum, severity score of clinical signs, and the occurrence of clinical mastitis in the first 100 DIM after the return of the cow to the dairy herd (N = no; Y = yes). Quarter coding is left rear (LR), right rear (RR), left front (LF), and right front (RF).

Figure 1. Mean $\pm$ SEM serum IgG1 (a), IgG2 (b), and total IgG (c) titers reported as optical density (OD) in J-5 vaccinated and unvaccinated control cows at the time of first vaccination (V1), second vaccination (V2), third vaccination (V3), 7 d before *Escherichia coli* C1 challenge (C-7d), day of challenge (C), 7 d postchallenge (C+7d), parturition (P), and 7 d postparturition (P+7d).

of the challenged cows. A moderate increase of approximately 0.5°C was observed in the average rectal temperature in cows 24 to 36 h postchallenge (Figure 4); this was similar in vaccinated and control cows ($P > 0.5$). Three out of 10 vaccinated cows showed a rectal temperature increase to a value $>40^{\circ}\text{C}$, and only 1 out of 10 control cows showed this increase in rectal temperature. The rectal temperature returned to normal in all cows without any apparent clinical signs within 12 h after peaking in both groups.

In 80% of the vaccinated cows, a non-severe clinical mastitis was observed 24 to 48 h postparturition and this was the case for 90% of control cows (Tables 1 and 2). Mastitis was clinically characterized by flakes or clots in the milk; clots were also observed on the

Lactocorder filter. Cows were followed up to 100 d in lactation and in this period no significant difference (chi-squared, $P > 0.5$) in the incidence of clinical mastitis was detected between vaccinated and control cows (Tables 1 and 2).

SCC and Conductivity

Somatic cell count was overall not significantly different ($P > 0.05$) between J-5-vaccinated and control cows (Figure 5a). The mean quarter SCC in vaccinated cows was not significantly different from controls at the first 2 time points postcalving, but was significantly higher at 7 d postcalving ($P < 0.05$). Electrical conductivity was not different between vaccinates and controls ($P > 0.2$; Figure 5b).

Milk Production

No significant difference in milk production was shown between J-5-vaccinated and control cows when the actual kilograms of milk were analyzed. However, vaccinated cows had a lower baseline milk production but increased production faster than control cows. When correcting for the baseline milk production on d 1 of lactation, the increase in milk production was significantly higher in vaccinated cows compared with control cows ($P < 0.05$; Figure 6).

Clinical Severity of Mastitis

Vaccination with the J-5 bacterin had a significant effect on the CMT scores in the first milking postcalving, whereby vaccinated animals had a significantly lower CMT score ($P < 0.05$; Figure 7, top). After 2 d, this effect disappeared. A significant difference ($P < 0.05$) was also observed in the presence or absence of flakes or clots in the milk of vaccinate and control cows (Figure 7, middle), wherein the vaccinated group showed reduced flakes compared with controls in the first 48 h postparturition. No significant difference ($P > 0.05$) was noted between the observed swelling of the challenged quarters between vaccinates and control cows. Vaccination with J-5 bacterin did reduce the overall clinical severity of coliform mastitis compared with the unvaccinated controls ($P < 0.05$; Figure 7, bottom) in the first 48 h postparturition.

DISCUSSION

In this study, vaccinating cows with *E. coli* J-5 vaccine with an off-label alternative dosing regimen in late lactation and the early dry period increased the antibody titers relative to controls. Challenging cows

Figure 2. Mean $\pm$ SEM bacteria counts expressed as 10 logarithm of *Escherichia coli* C1-positive quarters in vaccinated (●) and nonvaccinated control (---) cows challenged by intramammary infusion with *E. coli* C1 in late gestation. The indicators on the x-axis show the time since challenge (C) or parturition (P) in hours or days (days indicated by d).

Figure 3. Random amplification of polymorphic DNA (RAPD) patterns of *Escherichia coli* C1 isolates obtained before and after intramammary challenge. The indicators above the lanes show the cow number, quarter (LR = left rear; RR = right rear; LF = left front; RF = right front), and time since challenge (C) or parturition (P) in hours. *Escherichia coli* C1 is a pure culture of the challenge strain.

Figure 4. Mean $\pm$ SEM body temperature relative to a late-gestation *Escherichia coli* C1 intramammary challenge. Values for J-5-vaccinated cows (●) and control cows (---) are shown. The indicators on the x-axis show the time since challenge (C) or parturition (P) in hours or days (hours indicated by h).

in late gestation turned out to be a reliable model for introducing *E. coli* IMI both in late gestation and persisting into early lactation. Although vaccination did not prevent against postchallenge and postfreshening IMI, the clinical severity of the IMI was lower in vaccinates compared with controls.

Both controls and vaccinates had J-5-specific IgG1, IgG2, and total IgG antibodies in MGS and milk before the first vaccination. These results indicate that all the cows included in the study had some previous exposure to coliform pathogens, sufficient to elicit some humoral immune response. The levels of IgG1, IgG2, and total IgG were significantly higher ($P < 0.05$) in the J-5 vaccinates before the challenge compared with controls, as expected from previous studies (i.e., Wilson et al., 2007b). The alternative dosing scheme turned out to be effective when judged by the presence of antibody titers at the chosen time of intramammary *E. coli* C1 challenge. At the time of the challenge, the difference in antibody titer between vaccinated and control cows peaked (Figure 1). The increase in the antibody titers against *E. coli* J-5 agrees with the results reported in previous trials on either a similar alternative vaccination scheme or the on-label J-5 vaccination scheme (Hogan et al., 1992, 1995; Wilson et al., 2007b). As expected, antibody titers peaked earlier with the use of the alternative vaccination scheme. Several studies

in mice and other species, including cows (Stevens et al., 1988; Brown et al., 1998; Estes and Brown, 2002), have shown that increased production of IgG1 antibody is indicative of T helper (Th)2 response, whereas an increase in IgG2 levels is considered indicative of Th1 response. Our results showed that the antibody response was mainly due to high levels of J-5-specific IgG1 and, to a lesser extent, IgG2 in serum of vaccinated cows. Given the Th2 bias that was shown to exist in late-gestation pregnant dairy cows (Quesnell et al., 2012), this IgG1 predominance may be expected. These results were similar to those observed in other studies (Wilson et al., 2007b).

Immunization with J-5, resulting in increased antibody titers against J-5, did not confer protection against *E. coli* IMI (Tables 1 and 2; Figure 2). The percentage of quarters infected postparturition in vaccinates was 65% compared with 45% in controls (45%). In our study, vaccination with J-5 bacterin with an alternative vaccination schedule was associated with peaking J-5-specific IgG, IgG1, and IgG2 antibodies in blood at the time of challenge. Still, virtually all challenged quarters (18 out of 20 in the vaccinated cows) showed an established IMI with the challenge strain at 24 h postinoculation. Although titers in vaccinated animals were higher in serum, we did not measure the concentration of opsonizing antibodies at the site of the

Figure 5. Changes in the milk SCC (top) and conductivity (bottom) in early lactation in vaccinated cows (●) and control cows (---) following intramammary *Escherichia coli* C1 infection. Results are presented as mean $\pm$ SEM.

challenge infection. The presence of specific antibodies in the mammary gland would be essential to aid the immune system in phagocytosis and killing of *E. coli* bacteria by macrophages and neutrophils.

Vaccinating cows with J-5 bacterin using the off-label schedule decreased the severity of clinical mastitis following experimental challenge with the mastitis-causing *E. coli* C1. Significant difference was observed in CMT, SCC, the presence of flakes or clots in milk, and overall clinical score in vaccinated cows compared with control cows (Figure 6). The measured improvement in the severity of mastitis was particularly present immediately postcalving. The overall clinical mastitis score, which was mainly composed of milk scores, of severe systemic signs were virtually absent in all cows. We also observed a significant difference ($P < 0.05$) in the increase in milk production immediately following calving in vaccinated animals compared with control animals. The reduction in the overall severity of clinical mastitis based on CMT scores and flakes could not be attributed to enhanced bacterial clearance from challenged quarters relative to controls. Bacteria counts remained virtually identical in vaccinated compared with control cows (Figure 1). These results are not in agreement with other studies (Tomita et al., 1998) where immunization with J-5 bacterin enhanced bacterial clearance from challenged quarters.

These results and the results of many J-5 efficacy trials before this (Hill, 1991; Hogan et al., 1995; Wilson et al., 2007a) suggest that a change in J-5 immunization

Figure 6. Changes in quarter milk production relative to milk production at the day of calving in vaccinated cows (●) and control cows (---) following intramammary *Escherichia coli* C1 challenge in late gestation. Results are presented as mean $\pm$ SEM. P = parturition.

Figure 7. Changes in clinical score over time in vaccinated cows (●) and control cows (---) following intramammary *Escherichia coli* experimental challenge in late gestation. Top: milk California mastitis test (CMT) scores; middle: milk flakes scores; bottom: overall clinical severity. Results are presented as mean $\pm$ SEM. P = parturition.

schedules may be beneficial to attain optimum late-gestation antibody protection. The alternative vaccination scheme was successful in shifting peak antibody titers to coincide more closely with the incidence of naturally occurring late-gestation IMI. However, despite the presence of peak antibody titers, no significant change in the risk of IMI was observed between vaccinates and controls (Tables 1 and 2). It may be speculated that a parenteral vaccination alone will not be able to truly prevent IMI and that contemporary immune stimulation of the mucosal surfaces in the mammary gland may prove to be essential. Previous studies that boosted mucosal immunity have shown significant higher titers in MGS and milk (Hogan et al., 1997), but also failed to protect the gland from IMI (Smith et al., 1999). However, recent enhancement in our understanding of mucosal immunity may provide further opportunities to study this mucosal immune protection route (Pavot et al., 2012).

With this study, we have further established an *E. coli* intramammary challenge model, developed to mimic IMI in late gestation. As we showed earlier with small numbers of animals (Quesnell et al., 2012), late-gestation inoculation with 100 cfu of *E. coli* C1 results in a predictable establishment of an IMI during the last weeks of gestation. A high proportion of the initially established IMI (65%) were still present in early lactation and generally presented as mild clinical mastitis. The value of such an infection model lies in the ability to study the pathobiology and immune response patterns of infections in late gestation and early lactation. Late-gestation IMI often result in clinical mastitis in early lactation and form one of the most important challenges to reduce clinical mastitis on well-managed dairy farms. One of the limitations of the *E. coli* C1 challenge model may be the selection of a challenge strain that was identified as causing persistent mild clinical symptoms. Although this strain successfully colonized the late-gestation mammary gland, the resulting IMI were never associated with severe clinical mastitis. The efficacy of the alternative vaccination schedule might have been more pronounced using an *E. coli* strain that causes more severe disease.

CONCLUSIONS

An alternative vaccination scheme for a J-5 bacterin was evaluated in a novel late-gestation *E. coli* intramammary infection challenge model. Vaccination resulted in significantly increased IgG titers, particularly of the IgG1 isotype. Intramammary challenge resulted in IMI establishment in 85% of quarters. Vaccination did not prevent new IMI, but generally reduced clinical severity of resulting clinical mastitis cases. The impact of these findings and possible directions for future research in mastitis vaccines were discussed.

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