

Effect of Re-17 Mutant *Salmonella typhimurium* Bacterin Toxoid on Clinical Coliform Mastitis

ALYN M. McCLURE and EDWARD E. CHRISTOPHER
Herd Health Management
Gilbert, AZ 85234-0809

W. A. WOLFF,¹ W. H. FALES,² GARY F. KRAUSE,³ and JOE MIRAMONTI¹
University of Missouri
Columbia 65211

ABSTRACT

The objective of this study was to test the hypothesis that the incidence and severity of clinical coliform mastitis could be decreased by Re-17 mutant *Salmonella typhimurium* bacterin toxoid. Holstein-Friesian cows from two Arizona dairies were selected for this study based on July through November projected calving dates; peak lactation occurred during the period of highest rainfall and peak environmental stress. The cows were randomly assigned to either a vaccinate or a control group, and 1292 cows were paired by herd, parity, calving date, and milk yield. The 646 vaccinates were injected twice during the third trimester of pregnancy with an Re-17 mutant *S. typhimurium* bacterin toxoid, and the 646 controls were not vaccinated. Vaccinated cows had significantly fewer clinical cases of coliform mastitis with positive coliform cultures and had lower culling rate from coliform mastitis than control cows during the first 5 mo of lactation. During the same period, the mortality rate from clinical coliform mastitis was 75% less in the vaccinated clinical coliform mastitic group than in the control group. Incidence of mastitis increased with advancing parity. The Re-17 mutant *Salmonella typhimurium* bacterin toxoid provided cross-protection against coliform mastitis; incidence and

severity of clinical coliform mastitis were significantly lowered during the first 5 mo of lactation.

(Key words: core antigen, coliform, cross-protection, mastitis)

Abbreviation key: CCM = clinical coliform mastitis, NSG = no significant growth.

INTRODUCTION

Clinical coliform mastitis (CCM) is a significant problem for dairy cows kept in modern conditions of intensive housing, especially for herds with low SCC in which the major contagious mastitis pathogens, *Streptococcus agalactiae* and *Staphylococcus aureus*, have been controlled (3, 4, 13). Increased treatment costs, discarded milk, lost quarters, premature culling, death rate, and decreased milk yield are all factors that contribute to substantial economic losses caused by CCM (1). The incidence of CCM is related to exposure of teat ends to coliform bacteria in the environment (1, 3, 7, 21). Increased incidence of CCM is strongly associated with high ambient temperatures, humidity, and moisture (3, 22). Environmental sources of coliform organisms include free stalls, loose housing, calving stalls, pastures, or any other areas where cows congregate between milkings (3, 15, 21). Moist organic bedding in free stalls and calving pens frequently harbors very high numbers of coliform organisms (3, 7, 21). Inadequate milking sanitation also contributes to the incidence of coliform mastitis because teat ends may be exposed to coliform organisms from fecal contamination during milking (16). Enhancement of the cow's immune defense may reduce the incidence of CCM from these high risk factors (12, 13).

The immunological control of CCM via antibodies against Gram-negative core antigens

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¹Department of Veterinary Medicine and Surgery.

²Veterinary Medical Diagnostic Laboratory.

³College of Agriculture.

has only recently become feasible with the development of mutant Gram-negative bacteria from which cross-protective vaccines can be made (2, 9, 22, 23). The Re-17 mutant *Salmonella typhimurium* bacterin toxoid is a USDA licensed vaccine (ENDOVAC-Bovi®; IMMVAC Inc., Columbia, MO) that stimulates the immune system to produce cross-protective antibodies against core antigens. The toxoid portion of the vaccine, E3, stimulates the lymphocytes to produce higher levels of antibodies against the Re-17 bacterin (23, 24). Previous studies have demonstrated that this vaccine provides cross-protection in horses against *Escherichia coli* and *S. typhimurium* endotoxemias (8, 22, 23) and in calves against *E. coli*, *Salmonella* spp., and *Pasteurella* spp. endotoxemias (22, 23). Because of the vaccine's demonstrated cross-protection against various Gram-negative endotoxins, the protection of cows against coliform mastitis with a two-injection immunization schedule appeared to be highly probable. Previous field trials using J-5 *E. coli* bacterin (Poultry Laboratories, Davis, CA) in a three-injection immunization schedule demonstrated significant reduction in the incidence of CCM in naturally challenged cows during the first 90 d of lactation (10).

The investigation was undertaken to determine whether double-injection immunization of nonlactating cows with Re-17 mutant *Salmonella typhimurium* bacterin toxoid would reduce incidence, clinical severity, mortality, and culling rate from CCM during the first 5 mo of lactation.

MATERIALS AND METHODS

Herd and Cow Selection

Two dairy herds near Phoenix, Arizona with histories of coliform mastitis were chosen for this study. The mean numbers of cows in these two herds during the trial period were 5603 (4787 in lactation) and 1323 (1104 in lactation). These dairies participated in programs of herd health and mastitis control supervised by the principal investigator and were enrolled in the Arizona DHIA monthly testing program. On-farm computerized record systems were backed up by the DHIA dairy records processing center in Provo, Utah and by the principal investigator's personal com-

puter system. These herds were housed in open, shaded drylot corrals, which are typical in the Southwest. The 365-d rolling herd averages during the 18-mo study period from July 1991 through December 1992 of the two herds ranged from 8947 to 8986 kg of milk, and the herds' weighted mean monthly SCC ranged from 232×10^3 to 235×10^3 /ml.

Holstein-Friesian cows from these herds were selected for this study on the basis of their predicted calving dates (August through October 1991) so they would be exposed during early lactation to the seasons of highest environmental risk, i.e., the late summer, fall, and early winter rains. The selected cows were assigned sequential numbers from a table of random numbers and randomly assigned to either the control group or the vaccinated group. Pluriparous cows in the vaccinated group were first injected twice intramuscularly with 2 ml of the Re-17 mutant *S. typhimurium* bacterin toxoid at or shortly after dry-off and again 2 to 3 wk prior to calving when the cows were moved to close-up pens. The vaccinated primiparous heifers were first injected with vaccine early during the last trimester of pregnancy and boosted when they were confined in the close-up pens approximately 3 wk before calving. Cows that did not receive both doses of vaccine before calving, cows that died or were culled before calving, and cows that had lost permanent identification were eliminated from the trial. There were 873 unvaccinated control cows and 752 cows vaccinated for the trial. Subsequently, 646 controls and 646 vaccinates (1292 total) were paired based on herd, parity, calving date, and projected 305-d mature equivalent milk yield for statistical analysis. The mean lactation number for the 646 pairs was 3.1. Calving dates for the cows in a pair differed by no more than 4 wk; mean difference was $.259 \pm .79$ wk ($\bar{X} \pm SD$). Each cow's extrapolated 305-d milk yield was measured between 15 and 45 d after calving and was within 10% of the mean for the pair. The other 333 cows were omitted from the paired analysis because they could not meet the stringent pairing criteria.

The vaccinates and controls in each herd were commingled and managed identically for this clinical trial. Milkers, caretakers, herdspersons, and supervising veterinary technicians did not know whether cows were vaccinates or controls.

Episode Identification

Milkers, caretakers, and herdspersons at both dairies were trained to recognize the signs of CCM when forestripping prior to attaching the milking unit. Cows with abnormal milk were separated and placed in a hospital pen without milking or treatment. Hospital attendants aseptically collected milk samples from involved quarters in labeled sterile vials and recorded clinical signs before any treatment was administered. The investigators monitored these procedures and verified clinical observations and records during farm visits three times a week.

A distinct episode of CCM was defined when the previously normal milk secretion from any single quarter or any combination of quarters was abnormal, i.e., the secretion contained garget, was watery, or was reddish, brownish, or yellowish, provided at least 8 d had elapsed since a prior episode. An episode was defined as a case of CCM when culture of the pretreatment milk sample from the affected quarter yielded significant growth of coliform bacteria. Multiple quarters affected simultaneously were considered to be one episode unless distinctly different bacteria were isolated.

Rainfall

Rainfall records for Phoenix, Arizona were obtained from the National Climatic Data Center (Asheville, NC). The average monthly rainfall for 30 yr prior to the year of this study were compared with the monthly values recorded during the study.

Parity Effects on CCM Incidence

Episodes of CCM were grouped and analyzed by parity group: lactations 1, 2, 3, and 4 and greater (20, 24). The incidence of CCM was compared between vaccinates and controls by parity group for the first 5 mo of lactation.

Therapy for CCM Episodes

All trial cows showing signs of CCM were treated with the routine regimens established as standard operating procedure for each herd, depending on clinical severity, history of most likely etiological agent, and response to treatment.

Bacteriological Culturing of Milk

Arizona Laboratory. Milk samples were frozen to -20°C immediately after collection and transported on ice within 12 to 72 h to the investigator's laboratory. Upon arrival at the investigator's laboratory, samples were thawed and cultured using standard techniques (10, 25) or stored at -20°C in the laboratory and then thawed and cultured within 24 to 72 h after arrival. Bacteria were isolated on 5% defibrinated bovine blood trypticase soy agar plates (Research Laboratory Media Room, University of California at Davis) and identified as *Strep. agalactiae*, streptococci other than *Strep. agalactiae*, coagulase-positive staphylococci, coagulase-negative staphylococci, lactose-fermenting Gram-negative rods (coliforms), other pathogens (*Corynebacterium bovis*, *Actinomyces pyogenes*, pseudomonads, and yeasts), and mycoplasma on mycoplasma agar plates (Research Laboratory Media Room, University of California at Davis) (10, 25). If more than three different organisms were isolated from any one milk sample, the sample was recorded as contaminated unless a specific contagious pathogen (e.g., *Strep. agalactiae* or *Staph. aureus*) was isolated, in which case the contagious pathogen or pathogens were recorded as the etiological agents. Samples that resulted in no significant growth (NSG) were recorded as NSG. Cases of CCM from which no milk samples were obtained were recorded as "unknown".

Missouri Laboratory. A majority of the coliforms isolated in Arizona were sent to the University of Missouri Veterinary Diagnostic Laboratory (Columbia) on trypticase-soy agar slants for definitive confirmation. Gram-negative organisms were identified using an automated commercial autoidentification fluorochrome substrate plate and the AP-80-Vet software program (Radiometer, Sensititre, Westlake, OH). Gram-positive organisms were identified using previously described methods (25).

Statistical Analyses

Statistical comparisons of vaccinated and control groups were based on rates per 100 cows and rates per 10,000 cow days based on the number of first episodes of CCM, the

TABLE 1. Analysis of clinical coliform mastitis (CCM) incidence rates for 646 pairs at 0 to 5 mo postcalving.

Category	First episodes		Repeat episodes		Total episodes	
	C ¹	V	C	V	C	V
Episodes, no.	78	49	12	4	90	53
CCM per 100 cows	12.1	7.6*	1.9	.6*	13.9	8.2*
CCM per 10,000 cow days	8.1	5**	1.3	.4**	9.3	5.4**
Reduction in vaccinates, %		37		67		42

¹C = Controls; V = vaccinates.

* Significantly different from control ($P < .05$) by chi-square analysis.

**Significantly different from control ($P < .05$) by Student's t test analysis.

number of repeat episodes of CCM, the total episodes of CCM, mortality rate, and removal rate caused by CCM for the first 5 mo of lactation (4). Statistical analysis was focused on the first 5 mo of lactation, the period of highest CCM incidence. The relationship of CCM to parity was calculated and statistically compared on the basis of rates per month per 100 cows (20, 21). Total episodes of CCM were defined as the number of episodes of CCM that cows experienced so long as the intervals between CCM episodes were 8 d or more. Cows with CCM were the number of cows with one or more culture-positive CCM episodes. Vaccinates and controls were statistically compared on the basis of 1) number of initial and total episodes of CCM, 2) number of cows that died within a month following the onset of an episode of CCM, 3) number of cows that died or were culled prematurely because of CCM, and 4) parity versus CCM incidence.

Thirty-year average monthly rainfall values were compared with monthly amounts recorded during the study.

Parameters were computed from the data according to previously described methods (14), and statistical comparisons of the control and vaccinated groups were made on the basis of rate per 100 cows and rate per 10,000 cow days using chi-square and Student's t test. Differences between groups were considered significant when $P \leq .05$.

RESULTS

Results of CCM initial, repeat, and total episode incidence, death, and culling were analyzed for the first 5 mo of lactation. Seventy-

eight (12.1%; CCM rate per 10,000 cow days of 8.1) control cows compared with 49 (7.6%; CCM rate per 10,000 cow days of 5.0) vaccinated cows experienced CCM, a 37% ($P < .05$) reduction from the rate for vaccinated cows or initial episodes (Table 1). Twelve (1.9%; CCM rate of 1.3) repeat episodes of CCM in the control group, compared with 4 (.6%, CCM rate of .4) in the vaccinated group, resulted in a 67% decrease in repeat episodes of CCM (Table 1). The CCM rates for total episodes of CCM were 9.34 (90 total episodes) and 5.43 (53 total episodes) per 10,000 cow days at risk for controls and vaccinates, respectively. This 42% episode decrease for CCM of vaccinated cows was significant ($P < .05$) (Table 1).

Group mortality rate per 100 cows and 10,000 cow days for cows with CCM was .6% and 1.1 for 4 dead controls and .2% and .4 for 1 dead vaccinate, respectively, a 67% decrease in death loss in favor of the vaccinated group (Table 2). The group removal rate (combined death and culling) per 100 cows (%) and 10,000 cow days from CCM was 2.3% and 1.6 for 15 controls and .9% and .3 for 6 vaccinates, a reduction of 61% in the vaccinates (Table 2).

The mortality rate per 100 cows for cows with CCM was 5.1% (4 of 78) for controls and 2% (1 of 49) for vaccinates, resulting in a 61% decrease ($P < .05$) in favor of the vaccinated group (Table 2). The removal rate (combined mortality and culling) per 100 cows was 19.2% (15 of 78) for controls compared with 12.2% (6 of 49) for vaccinates, a 61% reduction for the vaccinated group (Table 2).

Incidence of CCM during the first 5 mo of lactation increased with parity for both vaccinates and controls. Incidence of CCM for

TABLE 2. Analysis of group mortality and removal rates and case fatality and removal because of clinical coliform mastitis (CCM) at 0 to 5 mo postcalving.

Category	Dead or removed		Rate per 100 cows		Rate per 100 cows (% reduction)	Rate per 10,000 cow days	
	C ¹	V	C	V	C vs. V	C	V
Group mortality ²	4	1	.6	.2	67	1.1	.4
Group removal ²	15	6	2.3	.9	61	1.6	.3
Case mortality ³	4	1	5.1	2	61	NA ⁴	NA
Case removal ³	15	6	19.2	12.2	36	NA	NA

¹C = Controls; V = vaccinates.

²Population of 646 pairs and 1292 cows.

³Population of 78 controls and 49 vaccinates with CCM.

⁴Not applicable.

vaccinates was reduced for each parity grouping and was statistically significant for the group in fourth or greater lactation ($P < .05$) (Table 3).

One hundred ninety-two culture samples taken from cows in Arizona were positive for coliforms. One hundred forty-two, 74%, were submitted to the University of Missouri Veterinary Medical Diagnostic Laboratory for confirmation, and 129 were confirmed as positive for coliforms, resulting in a 91% agreement between the two laboratories. Any episode from which isolates submitted to the University of Missouri were not confirmed as coliform were not counted as CCM episodes. Of the 129 coliform isolates identified, 124 (96%) were *E. coli*.

Sixty-four percent of all cases of CCM during the 305-d lactations studied occurred during the first 5 mo of lactation versus 33% during the first 3 mo. Rainfall during the first 2 mo of this study, August and September, was less than the 30-yr average for this period. During the next 6 mo, October through March, rainfall was double the 30-yr average (Figure 1).

Mastitis was diagnosed in 217 (34%) of the control and 181 (28%) of the vaccinated cows during the first 5 mo of lactation. There were 250 (39 per 100 cows) mastitic episodes in the control group and 209 (32 per 100 cows) in the vaccinated group. During the 5-mo study, 78 control and 49 vaccinated cows experienced 90 and 53 episodes of CCM, respectively. Upon

TABLE 3. Analysis of interactions between parity groups and incidence of clinical coliform mastitis (CCM) in vaccinated and control cows during the first 5 mo of lactation.

Lactation	Treatment ¹	Monthly mean			Reduction in cases per 100 cows per mo
		CCM Cases	Lactating cows	Cases per 100 cows	
1	C	.8	58	1.37	1.03
	V	.2	59	.34	
2	C	4.4	215	2.04	.66
	V	3	217	1.38	
3	C	5.4	165	3.26	1.35
	V	3.2	168	1.91	
4+	C	7.4	195	3.8	1.7
	V	4.2	200	2.1*	

¹C = Controls; V = vaccinates.

*Different from control ($P < .05$).

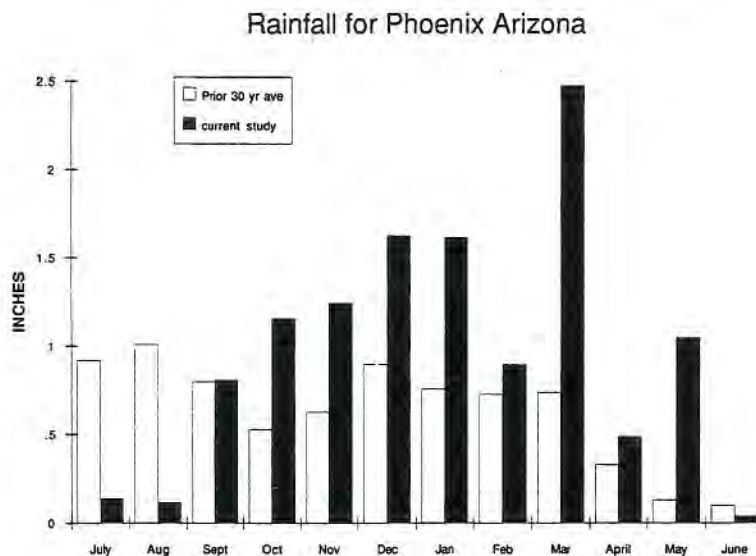


Figure 1. Previous 30-yr average monthly rainfall for Phoenix, Arizona compared with monthly rainfall during the study period, July 1991 through June 1992.

culture, NSG occurred on milk samples from 77 (12%) controls and 68 (11%) vaccinated cows, 95 and 85 episodes, respectively, that were diagnosed as exhibiting the signs of CCM. Culture results of the CCM cases caused by coliform and other bacteria were summarized (Tables 4 and 5). Mycoplasma organisms were not identified in any milk samples in this study.

None of the vaccinated cows developed noticeable adverse responses at injection site or systemically following vaccination.

DISCUSSION

In contrast to other studies in which the highest incidence of CCM occurred during the first 90 d of lactation (5, 6, 22), 64% of all cases of CCM during the 305-d lactations in this study occurred during the first 5 mo of lactation versus 33% during the first 3 mo. The extreme difference between monthly rainfall amounts during this study compared with those of the previous 30-yr averages probably accounted for the lower than expected CCM incidence during the initial 90 d of lactation and the higher than expected incidence during the first 152 d of lactation (Figure 1). In addition

to the unusually high rainfall during this study, the milking and housing facilities were overcrowded, which adversely effected milking hygiene and procedures. Although the impact of these adverse conditions was difficult to quantitate, they undoubtedly contributed to the extremely high incidence rate of CCM in the cows on the dairies in this study (21).

Incidence of CCM increased progressively as parity increased. Other investigators have reported increased incidence by parity in both dry and lactating cows (20, 21), which may be related to higher yield of older and higher yielding cows retained in the herd (20) and may also be associated with increased patency of the teat orifice that occurs as parity increases. During this study, the abnormally high rainfall and muddy pens probably increased exposure and susceptibility of some of the older cows (21). The significant reduction of CCM for older cows and the reduced incidence at all parities in vaccinates compared with that of controls is evidence of the value of vaccination and possibly extended herd life in cows that would otherwise die or be culled because of CCM (Table 3).

In this study, the significant decreases in the CCM incidence and mortality because of vac-

TABLE 4. Cows and mastitis incidence in treatment and mastitic groups at 0 to 5 mo postcalving.

Category	Cows ¹		Percentage of group		Percentage of mastitic cows	
	C	V	C	V	C	V
			(n = 646)	(n = 646)	(n = 217)	(n = 181)
Coliform						
<i>Streptococcus</i>	78	49	12.1	7.6	35.9	27.1
non- <i>Strep. agalactiae</i>	21	30	3.3	4.6	9.7	16.6
<i>Strep. agalactiae</i>	4	3	.6	.5	1.8	1.7
<i>Staphylococcus</i>						
Coagulase-negative	8	9	1.2	1.4	3.7	5
Coagulase-positive	4	3	.6	.5	1.8	1.7
Miscellaneous	10	7	1.5	1.1	4.6	3.9
NSG ²	77	68	11.9	10.5	35.5	37.6
Contaminated or unknown	15	12	2.3	1.9	6.9	6.6
Total	217	181	33.6	28.1	100	100

¹C = Controls; V = vaccinates.

²No significant growth.

cination were similar to those observed in other studies utilizing similar but not identical technology (2, 9). Previous studies have suggested that leukocyte chemotaxis and other immune mechanisms were related to protection of udders from *E. coli* bacteria (11, 17). The mechanism for this response in the vaccinated cows was probably related to enhanced antigen-antibody complexing, opsonization, or phagocytosis within the udder. In this study, the higher percentage of cases with NSG cul-

tures versus coliform-positive cultures in the vaccinated group than in the control group suggested that vaccination caused more rapid and more complete elimination of coliform bacteria from the udder. The fewer repeat coliform positive episodes in the vaccinates than in the controls suggested extended protection and more complete elimination of coliform bacteria from the udder.

It has been previously confirmed that 70% of the cultures resulting in NSG from cows

TABLE 5. Mastitis episodes and incidence in treatment and mastitic groups, 0 to 5 mo postcalving.

Category	Episodes ¹		Percentage of group		Percentage of episodes	
	C	V	C	V	C	V
			(n = 646)	(n = 646)	(n = 250)	(n = 209)
Coliform						
<i>Streptococcus</i>	90	53	13.9	8.2	36	25.3
non- <i>Strep. agalactiae</i>	22	34	3.4	5.3	8.8	16.3
<i>Strep. agalactiae</i>	5	3	.8	.5	2	1.4
<i>Staphylococcus</i>						
Coagulase-negative	8	9	1.2	1.4	3.2	4.3
Coagulase-positive	4	3	.6	.5	1.6	1.4
Miscellaneous	10	8	1.6	1.2	4	3.8
NSG ²	95	85	14.7	13.2	38	41
Contaminated or unknown	16	14	2.5	2.2	6.4	6.7
Total	250	209	39	32	100	100

¹C = Controls; V = vaccinates.

²No significant growth.

with the concomitant clinical signs of CCM actually did have CCM (26). Therefore, the high rate of NSG cultures for cows that had been diagnosed as CCM was not unusual. Coliform mastitis is usually self-limiting, and bacterial numbers in the udder are often greatly reduced by the time clinical signs appear (26). The rapid influx of neutrophils into the udder in response to acute coliform infection is accompanied by rapid elimination of the bacteria (19). Numbers of bacteria in milk from coliform-infected quarters are frequently below levels detectable by routine culturing methods (11, 18, 24). In the experience of the authors, it is not unusual for 30 to 40% of cultures from cows with CCM to be culture-negative in herds free from or with a very low incidence of intramammary infections caused by the contagious pathogens, *Strep. agalactiae* or *Staph. aureus*.

The high degree of agreement between bacteriological cultures at the principal investigator's laboratory and at the University of Missouri Veterinary Medical Diagnostic Laboratory indicated that laboratory diagnosis of CCM in Arizona was accurate; probability was high that bacteriological techniques were not missing coliform-infected cows or over-diagnosing cultures positive for coliforms.

CONCLUSIONS

This study demonstrated that two injections of the Re-17 mutant *S. typhimurium* bacterin toxoid administered to cows during the dry period significantly reduced the incidence, repeat episodes, mortality, and culling rate from CCM during the first 5 mo of lactation, the time of highest incidence in the herds studied.

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