Efficacy and Pharmacokinetics of Bacteriophage Therapy in Treatment of Subclinical *Staphylococcus aureus* Mastitis in Lactating Dairy Cattle†

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Bovine mastitis is an inflammation of the udder caused by microbial infection. Mastitis caused by *Staphylococcus aureus* is a major concern to the dairy industry due to its resistance to antibiotic treatment and its propensity to recur chronically. Growing concerns surrounding antibiotic resistance have spurred research into alternative treatment methods. The ability of lytic *S. aureus* bacteriophage K to eliminate bovine *S. aureus* intramammary infection during lactation was evaluated in a placebo-controlled, multisite trial. Twenty-four lactating Holstein cows with preexisting subclinical *S. aureus* mastitis were treated. Treatment consisted of 10-ml intramammary infusions of either 1.25×10^{11} PFU of phage K or saline, administered once per day for 5 days. The cure rate was established by the assessment of four serial samples collected following treatment. The cure rate was 3 of 18 quarters (16.7%) in the phage-treated group, while none of the 20 saline-treated quarters were cured. This difference was not statistically significant. The effects of phage intramammary infusion on the bovine mammary gland were also studied. In healthy lactating cows, a single infusion of either filter-sterilized broth lysate or a CsCl gradient-purified phage preparation elicited a large increase in the milk somatic cell count. This response was not observed when phage was infused into quarters which were already infected with *S. aureus*. Phage-infused healthy quarters continued to shed viable bacteriophage into the milk for up to 36 h postinfusion. The phage concentration in the milk suggested that there was significant degradation or inactivation of the infused phage within the gland.

Bovine mastitis is the inflammation of the bovine mammary gland caused by pathogen infection. This disease is one of the largest production concerns in the dairy industry worldwide. The bovine udder consists of four mammary glands, or quarters; each quarter may contract mastitis independently of the others. The overall frequencies of intramammary infection (IMI) among cows were estimated to be 48.5% in a survey conducted in the United States (35) and 30.6% in a Finnish survey (27). A Canadian survey found that 19.8% of cows experienced clinical mastitis during lactation (30). Among the mastitis pathogens, *Staphylococcus aureus* is considered an agent of major concern due to the low cure rate of *S. aureus* infections by antibiotic treatment and its ability to persist in a herd in the form of undetected, subclinical infections. In studies involving short-duration lacational therapy against established *S. aureus* IMIs, bacteriological cure rates of 9% (9) to 35% (26) have been reported. Cure rates of up to 80% have been reported with therapy of *S. aureus* IMI at drying off (12).

Antibiotic resistance in *S. aureus* is also a growing concern, with the overall rates of antimicrobial resistance in bovine *S. aureus* isolates varying widely by region (21, 27, 28). Human clinical isolates of *S. aureus* are also becoming increasingly resistant to antibiotics. The continued emergence of nosocomial and community-acquired strains of methicillin-resistant *S.*

aureus (MRSA) in humans (11, 13) and MRSA in agricultural animals (17) signals a need for the development of novel antimicrobial therapies targeting this pathogen. The treatment of bacterial infections with bacteriophages, termed phage therapy, is one such option.

The theoretical benefits and history of phage therapy have been reviewed extensively (see, for instance, references 2 and 34). Recent interest in phage therapy was sparked by some early successes in the treatment of *Escherichia coli* infections in both mice and calves (32, 33). More recent experiments have demonstrated the ability of bacteriophages to treat various bacterial infections in mouse models, including infections caused by *Enterococcus faecium* (5), *Vibrio vulnificus* (7), and *S. aureus* (22). Previous work by Lerondelle and Poutrel (18) have shown that single and double doses of bacteriophage K, which is a known, strictly lytic bacteriophage capable of lysing a broad variety of *S. aureus* strains (23), were ineffective in treating experimentally induced *S. aureus* mastitis in lactating dairy cows.

The objective of this study was to evaluate the effectiveness of phage therapy in controlling naturally occurring cases of subclinical bovine mastitis caused by *S. aureus* during lactation. This study was designed to treat established *S. aureus* IMIs in cows housed in a commercial production environment, which imparts clinical relevancy to the trial outcome. Infected cows were treated by a 5-day course of intramammary infusions with either the lytic *S. aureus* bacteriophage K or a saline placebo. In the treatment of *S. aureus* IMI with conventional antibiotics, it has repeatedly been shown that long-duration therapy is more effective than short-duration therapy (10, 25). The effects of phage infusion into healthy quarters were also examined to

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assess the pharmacokinetics of infused bacteriophages and the impact of phage infusions on the quality of milk.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All bacterial cultures were grown at 37°C on Bacto Trypticase soy broth (TSB) or Trypticase soy agar (TSA) (BD, Franklin Lakes, NJ). *Staphylococcus aureus* strain ATCC 19685 was used for propagating and enumerating bacteriophage K. In order to minimize the possibility that bacterial toxins would contaminate the phage preparations used for treatment, this bacterial strain was assayed for the presence of genes coding for staphylococcal enterotoxins a to e, g, h, and i; exfoliative toxins a and b; toxic shock syndrome toxin; and Panton-Valentine leukocidin and γ-hemolysin by molecular methods (4, 19). For the collection of bovine *S. aureus* isolates and the diagnosis of bovine *S. aureus* mastitis, individual quarter milk samples were collected aseptically and 10 μl was plated onto Columbia base agar containing 5% sheep blood and incubated for 24 to 48 h at 37°C. Presumptive *S. aureus* colonies were identified on the basis of colony morphology and hemolysis; identity was confirmed by the tube coagulase test with rabbit plasma (BD). All *S. aureus* isolates recovered from the study animals were subcultured once and stored frozen at −80°C in TSB containing 15% (vol/vol) glycerol until further use.

Bacteriophage was detected in milk samples by spotting the milk samples on a lawn of *S. aureus* ATCC 19685 (1). The lawns were prepared by flooding a TSA plate with approximately 2 ml of a log-phase *S. aureus* culture (grown to an optical density equivalent to a no. 2 McFarland standard), and the excess culture was removed with a pipette. An aliquot of each sample was serially diluted in SM buffer (100 mM NaCl, 10 mM $MgCl_2$, 0.01% [wt/vol] gelatin, 50 mM Tris-HCl; pH 7.5), and 10 μl of each dilution was spotted onto a lawn. The plates were incubated at 37°C for 18 to 20 h, and the number of plaques was counted. The sensitivity of the *S. aureus* isolates to phage K was determined by spotting 10 μl of phage K at the routine test dilution (RTD) and 100× the RTD onto lawns composed of the bovine *S. aureus* isolates under investigation (6).

Bacteriophage lysate preparation. Bacteriophage K (ATCC 19685-B1) was used for all experiments. Phage stocks were grown and titrated on the phage K propagating strain (ATCC 19685). Phage stocks were titrated by serial dilution in SM buffer and plating by the soft agar overlay method (1). Crude phage lysates were prepared in TSB liquid culture. The crude lysate used for administration was filter sterilized by a vacuum-driven, 0.22-μm-pore-size disposable device (Millipore, Nepean, ON, Canada), titrated, and stored at 4°C until use. Purified phage was prepared by a method modified from the method of Sambrook et al. (29). Briefly, crude TSB lysate was clarified by centrifugation at $13,000 \times g$ for 20 min at 4°C, digested with 1 μg/ml DNase and RNase (Sigma), and precipitated in the presence of 10% polyethylene glycol (PEG) 8000 and 1 M NaCl at 4°C overnight. The PEG was extracted from the precipitate with chloroform, and the phage suspension was purified by separation on a self-generated CsCl gradient (0.75 g/ml CsCl, run at $25,000 \times g$ at 4°C for 24 h) in a Beckman SW28 rotor. In the case of the toxicity and milk alteration trials, the phage particles were purified by a single passage through a CsCl gradient. For the treatment of *S. aureus* mastitis, the phage preparations were purified by a second passage through a CsCl gradient. The purified phage was dialyzed extensively against Hanks' balanced salt solution (HBSS; 137 mM NaCl, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.6 mM $MgSO_4$, 0.5 mM $MgCl_2$, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4), filter sterilized, titrated in triplicate, and stored as a high-titer stock at 4°C until use. Prior to administration, the crude and purified phage stocks were diluted aseptically in sterile HBSS to achieve the desired final concentration of 1×10^{10} PFU/ml.

Toxicity trial. Prior to experimentation with dairy cows, the phage preparations were screened in mice to determine acute toxic effects. All experiments involving animal subjects were carried out in accordance with University of Guelph animal care guidelines. For these experiments, 6- to 8-week-old female BALB/c mice (Charles River, QC, Canada) weighing 18 to 20 g were used. The mice were arbitrarily assigned to one of five treatment groups: 100 μl or 250 μl crude phage, 100 μl or 250 μl purified phage, or 250 μl HBSS as a negative control. There were three mice in all treatment groups except for the HBSS control group, which contained four mice. Mice were cohoused by treatment. The mice were administered a single dose of phage suspension or saline by intraperitoneal injection. The final concentration of the purified phage preparation was 6.8×10^9 PFU/ml; the concentration of the crude phage preparation was 1.7×10^{10} PFU/ml. The mice were observed at regular intervals for up to 96 h postinjection for symptoms of lethargy, ruffling, hunching, and irregular breathing.

Milk analysis. Milk samples were aseptically collected from individual quarters of the study cows. Samples were transported to the laboratory within 2 h after collection and stored refrigerated until analysis. A Somacount 300 automated cell counter (Bentley Instruments, Chaska, MN) was used to determine milk somatic cell counts (SCC). Each quarter sample (10 μl) was plated onto Columbia base agar containing 5% sheep blood and incubated for 24 to 48 h at 37°C. The plates were then examined for the presence of bacteria in five categories: coagulase-negative staphylococci, *S. aureus*, *Streptococcus* spp., coliform bacteria, and *Corynebacterium bovis*. Preliminary determinations of bacterial identity were made by colony morphology and hemolysis. *S. aureus* was confirmed by the tube coagulase test in rabbit plasma; *Streptococcus* spp. were identified by the CAMP test with determination of esculin and inulin production; coliform identity was confirmed by picking a single colony and culturing it in a BBL Enterotube II (BD). The number and type of colonies found per 10-μl aliquot were scored and noted according to a four-interval scale, with a score of 1 indicating 1 to 5 colonies, a score of 2 indicating 6 to 10 colonies, a score of 3 indicating 11 to 50 colonies and a score of 4 indicating over 50 colonies.

Milk alteration trial. A preliminary trial was conducted in May 2005 with healthy, lactating Holstein cows to assess the effects of phage infusion on milk quality. The animals were milked twice daily and housed in a tie-stall arrangement at the Ponsonby Dairy Research Station, maintained by the University of Guelph. Aseptic quarter milk samples were collected from candidate cows 7 days prior to treatment and were examined for SCC and bacterial content, as described above. Cows selected for the study had milk SCCs of less than 1×10^5 cells/ml in all quarters and were culture negative for *S. aureus*, *Streptococcus* spp., and coliform bacteria prior to treatment. Treatments were administered by intramammary infusion at the cow's normal milking time by one of the study authors. At milking time, a milk sample was aseptically collected from each quarter to be treated. The cow was milked out normally and the teat end was disinfected with 70% isopropanol. The treatment was then aseptically infused through the teat canal from a sterile 10-ml disposable syringe fitted with a J-12 canula (Jorgensen Laboratories, Loveland, CO) by insertion of the cannula tip into the teat canal to a depth of 3 to 5 mm (partial insertion method). Following infusion, the mammary gland was massaged upwards briefly to distribute the treatment into the gland.

Individual quarters of six healthy, lactating Holstein cows were randomly assigned to one of four treatments: purified phage, crude phage, HBSS, or no infusion. The purified phage concentration was 8.8×10^9 PFU/ml, and the crude phage concentration was 9.0×10^9 PFU/ml. Cow parity ranged from 1 to 3, with a mean parity of 1.83. Treatments were randomized among quarters. Each quarter received a single 10-ml infusion in the manner described above. Milk samples were collected aseptically immediately before treatment, at the following three consecutive milkings (12, 24, and 36 h post-infusion), and at 7 days postinfusion. Milk samples were analyzed for SCC and bacteriology. All milk samples were also assayed for the presence and the amount of bacteriophage by spotting the samples on a lawn of *S. aureus* ATCC 19685, as described above.

Treatment of subclinical mastitis. The clinical trial used to test the efficacy of phage therapy against subclinical *S. aureus* mastitis was conducted from June to August 2005 with lactating Holstein dairy cows located in seven sites around Guelph, ON, Canada. All sites were enrolled in the CanWest Dairy Herd Improvement Association. Candidate cows were selected on the basis of their history of SCCs and *S. aureus* mastitis. The cows included in the study had been lactating for more than 30 days and were less than 90 days from their expected dry-off dates. Cows which had received antibiotics for any reason within 30 days of the treatment start date were excluded from the study. Quarter milk samples were collected by aseptic technique from candidate cows at 12 to 14 days before treatment and again at 5 to 7 days before treatment and were cultured for *S. aureus*, as described above. Quarters which were positive for *S. aureus* on either of these two pretreatment samplings were considered positive for *S. aureus* mastitis and the animals were recruited into the study. The recruited animals were randomly assigned to the phage-treated or placebo groups and stratified by parity, frequency of pretreatment *S. aureus* isolation, and number of quarters affected by *S. aureus* mastitis; each stratum contained two categories. Stratification by parity assigned cows into primiparous and multiparous groups. Stratification by *S. aureus* isolation frequency assigned cows in which both pretreatment samples cultured positive for *S. aureus* into one group and cows which intermittently shed *S. aureus* into the other. Stratification by number of affected quarters divided cows into single-quarter and multiple-quarter groups. Treatments were assigned by cow.

Treatments were provided as prefilled, labeled, individual-use 10-ml syringes sealed with disposable silicone caps and were stored refrigerated when they were not in use. The sterility of the preparations was checked by inoculation of approximately 1 ml from each batch into 5 ml of sterile TSB, incubation at 37°C
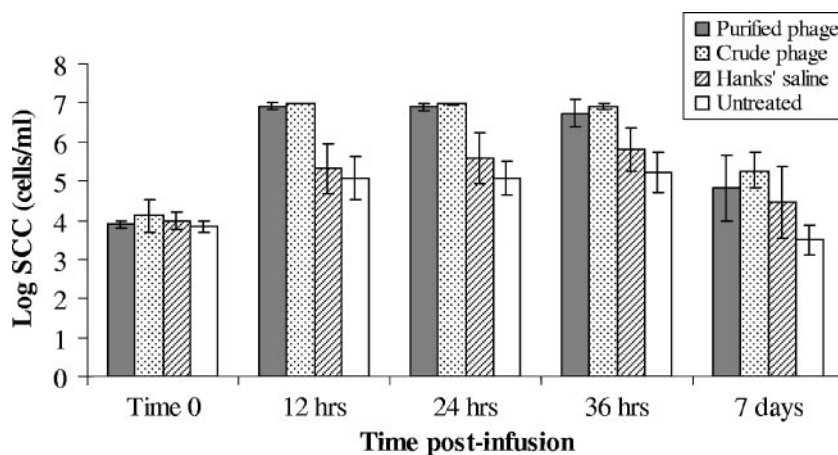
FIG. 1. Effects of a single intramammary infusion of phage K on the healthy lactating quarters of six dairy cows. Data are the mean $\log_{10}$ milk SCC values from quarters which received 10-ml intramammary infusions of a purified phage preparation (8.8×10^9 PFU/ml), crude phage preparation (9.0×10^9 PFU/ml), saline, or no infusion. Time zero indicates the mean SCC of quarters immediately prior to the phage infusion. Error bars represent ± 1 standard deviation.

for 48 h, and observation for the development of visible turbidity. At each site, an extra sentinel syringe filled with the phage treatment was provided; this syringe was returned to the laboratory at the conclusion of the study to confirm the phage concentration and proper storage of the material. The treatment regimen consisted of a 10-ml intramammary infusion of sterile phage suspension in HBSS given at milking time once per day for 5 consecutive days. The concentration of the phage suspensions was 1.25×10^{10} PFU/ml. The negative (placebo) control was a 10-ml infusion of sterile HBSS given once per day for 5 days. The workers administering the treatments were blinded to the treatment assignments. At treatment time, the study animals' teat ends were prepared for milking according to the herd operator's procedure and the cows were milked out normally. Following milking, the teat end was disinfected by scrubbing with a single-use cotton pad saturated with 70% isopropanol. The treatment was then infused from the provided syringe fitted with a J-12 cannula by the partial insertion method. Following infusion, the mammary gland was massaged upwards briefly to distribute the treatment into the gland. The treatments were administered by the herd owners at five sites and by a study technician at two sites. At one site where a study technician was present, aseptic milk samples were collected from the study cows daily at treatment time, immediately prior to milking. Aseptic quarter milk samples were collected from all treated cows at 2 to 7 days, 9 to 14 days, 16 to 21 days, and 23 to 28 days after the end of treatment. The samples were cultured for *S. aureus*, as described above, to confirm bacteriological cure. Quarters which yielded *S. aureus* on any posttreatment sampling were considered not cured.

Data analysis. All data on the SCC and phage counts present in milk samples were log transformed to normalize the data prior to analysis. Pair-wise comparisons of SCC and phage concentration were made between treatment groups within each sampling time by Student's *t* test with independent variance estimates and α equal to 0.05. Statistical comparisons of phage shed into milk were made only for the first postinfusion sample (12 h), due to the large number of undetectable counts in subsequent samples. In the analysis of data from the treatment of subclinical mastitis, differences in cure rates between the phage-treated and the placebo groups were determined by χ^2 analysis with the Yates correction applied. An additive pretreatment disease severity score was calculated for each treated quarter by adding the *S. aureus* colony count scores from each of the three pretreatment quarter milk samples (12). With the sparseness of the data set, multivariable logistic regression could not be performed, as the models would not converge. Statistical correlations were calculated on a per-quarter basis for herd, parity, pretreatment disease severity score, posttreatment *S. aureus* colony count score, treatment type, and cure status by Spearman rank-order analysis. Mann-Whitney U tests were used to compare colony count scores taken pre- and posttreatment for each phage-treated quarter and to compare the colony count scores collected during treatment from phage- and saline-treated quarters within a sampling day. All statistical analyses were conducted by using Statistica, version 4.5 (StatSoft, Tulsa, OK).

RESULTS

S. aureus strain ATCC 19685 was assayed for a number of common *S. aureus*-associated toxin-coding genes; only the gene coding for γ-hemolysin was detected. When the phage preparations were screened for their toxicity by intraperitoneal administration to mice, no adverse reactions to any of the treatments were observed. No significant weight change of the mice in any of the treatment groups was found after 72 h ($P > 0.1$).

The effect of a single phage infusion into healthy lactating quarters was examined. There was no significant difference in SCCs between any of the treatment groups immediately prior to the administration of the treatments in both alteration trials ($P > 0.1$) (Fig. 1). In the alteration trial, a comparison of treatment with purified phage lysate, crude lysate, and saline and no treatment was made. At 12 h following the infusion of the treatments, an approximately 3-log-unit increase in milk SCC was observed in the quarters infused with either the pure or the crude phage lysate (Fig. 1). This increase was significantly different from the SCC of the saline-treated and untreated control quarters ($P < 0.002$). At all sampling times, there was no significant difference between the SCCs of quarters infused with the pure or crude phage lysates, nor was there a difference between the saline-treated and untreated quarters ($P > 0.05$). The SCCs of the phage-infused quarters remained high throughout the 36-h sampling period, and in all cows but one, the SCCs returned to normal levels (ca. 1×10^5 cells/ml) after 7 days. In the one exceptional cow, the SCC had returned to a normal level after 14 days (data not shown).

Viable phage could be detected in milk collected from phage-infused quarters for up to 36 h postinfusion in this experiment (Table 1). The six quarters infused with pure phage shed between 2.5×10^4 PFU/ml and 5.0×10^5 PFU/ml at 12 h postinfusion; the log geometric mean concentration was 4.8 PFU/ml (arithmetic mean, 1.3×10^5 PFU/ml) (Table 1). In the six quarters infused with the crude phage preparation, milk phage concentrations ranged from 4.0×10^3 PFU/ml to 2.7×10^5 PFU/ml at 12 h postinfusion, with a log geometric mean

TABLE 1. Frequency and geometric mean amount of bacteriophage K shed into milk samples after a single 10-ml infusion of purified or crude phage lysate preparation into the quarters of six healthy lactating cows[a]

Sampling time postinfusion	Pure phage		Crude phage		Saline		No treatment	
	No. of quarters shedding	Mean log PFU/ml	No. of quarters shedding	Mean log PFU/ml	No. of quarters shedding	Mean log PFU/ml	No. of quarters shedding	Mean log PFU/ml
Time zero	0	—[b]	0	—	0	—	0	—
12 h	6	4.83	6	4.16	0	—	0	—
24 h	5	3.85	2	3.57	0	—	0	—
36 h	3	3.68	2	3.15	0	—	0	—
7 days	0	—	0	—	0	—	0	—

[a] Saline and untreated quarters are negative controls. The number of quarters shedding represents the number of infused quarters (total $n = 6$) which were shedding a detectable level of phage at a given sampling time; time zero represents a milk sample taken immediately before infusion of the treatments. Means are calculated only from quarters still shedding detectable phage.
[b] —, no phage was detected in any quarter.

concentration of 4.2 PFU/ml (arithmetic mean, 5.3×10^4 PFU/ml). The difference in phage shed between the two phage preparations was not significant at 12 h postinfusion ($P = 0.08$). Both the number of quarters shedding detectable phage and the mean amount of phage shed declined in subsequent samplings (Table 1). No phage was detected in any of the samples taken immediately prior to phage infusion (time zero), nor was phage detected in any sample collected from quarters which did not receive a phage infusion.

The clinical trial performed to assess the ability of phage K to cure preexisting cases of *S. aureus* mastitis enrolled 26 cows. Of these 26 cows, 2 were withdrawn from the trial due to incomplete posttreatment sampling, resulting in the inclusion of 24 cows and 38 infected quarters in the final data analysis. All 38 *S. aureus* isolates collected from the 38 infected quarters were susceptible to phage K at RTD prior to the start of treatment. The phage-treated group consisted of 13 cows and 18 quarters, while the saline-treated group consisted of 11 cows and 20 quarters. The cows were milked twice daily at five sites (housing 16 cows) and three times daily at one site (housing 8 cows). The herd sizes ranged from 45 to 360 animals, with a mean herd size of 133 animals. Statistics on the study animals and their distribution among the two treatment groups is shown in Table 2. Sentinel syringes were returned from four of the six participating sites; all four showed that the phage had not degraded during storage (data not shown). A fifth syringe was returned approximately 1 month after the end of treatment, and the phage concentration in this preparation was approximately 20% lower than the initial concentration of 1.25×10^{10} PFU/ml. Thirty-three posttreatment *S. aureus* isolates, collected from 33 quarters which were not cured, were all sensitive to phage K at RTD.

Phage treatment efficacy was evaluated on the basis of bacteriological cure in all animals enrolled in the study and also by measurement of *S. aureus* shedding by treated quarters in eight enrolled animals located at one trial site. Bacteriological cure was established by repeated posttreatment sampling and culture of milk collected from the treated quarters. Treated cows were sampled weekly for up to 4 weeks after treatment; quarters which cultured positive for *S. aureus* at any time posttreatment were considered uncured and were not sampled further. Quarters which did not produce any *S. aureus* from all four posttreatment samples were considered cured. In the phage-treated group, 3 of the 18 quarters treated (16.7%) were cured. In the saline placebo group, 0 of the 20 treated quarters were cured (Table 2). This difference was not found to be statistically significant by χ^2 analysis ($P = 0.19$). Two phage-treated quarters, located in two different herds, were reported to show signs of clinical mastitis (milk clotting, udder hardness) during treatment; these signs subsided by the conclusion of the treatment regimen. There were no reports of clinical mastitis signs from any of the saline-treated quarters during treatment.

At one trial site, a study technician was able to recover aseptic milk samples from the treated animals on a daily basis. The study site contained eight animals and was the only site at which cows were routinely milked three times daily. Five animals (represented by five quarters) were treated with phage, and three animals (represented by six quarters) were treated with saline. Daily phage infusion into the infected quarters had no appreciable effect on milk SCC over the course of the treatment regimen (data not shown). There was no significant difference between the SCCs of phage-treated and saline-treated quarters at any sampling time ($P > 0.05$). Bacteriological examination of these milk samples showed a daily fluctu-

TABLE 2. Cows, quarters, lactation number, production, lactation duration, and cure rates for 24 cows enrolled in a clinical trial to assess the efficacy of phage therapy in the treatment of established subclinical bovine mastitis caused by *S. aureus*[a]

Treatment group	No. of cows	No. of quarters	No. of cows with the following parity:				Mean parity	Mean days in milk	Mean milk production (kg/day)	No. of quarters cured	% Cure
			1	2	3	4					
Phage	13	18	2	5	5	1	2.38	158.3	33.9	3	16.7
Saline	11	20	1	3	3	4	2.91	207.4	28.2	0	0.0
Total	24	38	3	8	8	5					

[a] Infected quarters were infused once per day for 5 days with either a purified phage preparation or saline placebo.
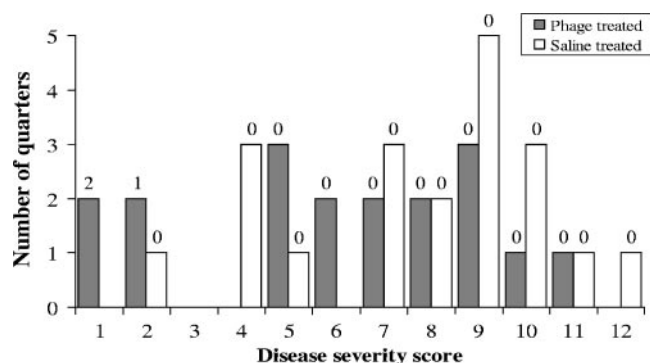
FIG. 2. Distribution of case severity across 38 quarters with *S. aureus* IMIs enrolled in a clinical trial to assess the efficacy of phage therapy for the treatment of bovine *S. aureus* mastitis. Infected quarters were assigned to either phage treatment or saline placebo. Disease severity score represents the summed *S. aureus* colony count scores from three pretreatment samples. The numbers over the bars indicate the number of cured quarters in that treatment and severity score category.

ation in the amount of *S. aureus* shed by quarters during the treatment trial (data not shown). The colony count scores from the phage-treated and saline-treated quarters were compared within each day; no significant within-day differences were found between the phage and the saline groups ($P > 0.05$).

The distribution of cures arranged by the severity of the preexisting mastitis case is shown in Fig. 2. The three cured quarters had among the lowest additive disease severity scores. All three of the cured quarters were also not shedding detectable levels of *S. aureus* on the first day of treatment. Analysis of the data by Spearman rank-order correlation found significant negative correlations between cure and the degree to which a given quarter was shedding *S. aureus* on the start day of treatment ($P = 0.0038$), as well as cure and the pretreatment disease severity score of the quarter ($P = 0.0035$). There was no significant correlation between treatment regimen and the degree to which a given quarter was shedding *S. aureus* on the first posttreatment sampling ($P = 0.64$). Within the 18 phage-treated quarters, no significant difference was found between the colony count scores for *S. aureus* shed on the treatment start date and at 1 week posttreatment ($P = 0.63$).

DISCUSSION

The administration of both the purified and crude phage lysates to mice by intraperitoneal injection had no observable effect on mouse health. This observation confirms those noted by Matsuzaki et al. (22). However, the administration of the same phage preparations to lactating dairy cattle by intramammary infusion elicited a dramatic immune response, as measured by increased milk SCCs (Fig. 1). At no time was a mastitis pathogen detected in any of the quarter milk samples collected, confirming that the elevated SCCs were not due to the introduction of pathogens into the mammary gland. Infusions of crude phage lysate or phages purified by precipitation and ultracentrifugation elicited similar SCC responses, suggesting that the phage particles themselves were able to elicit a sudden rise in SCCs in the infused quarters. Quarter SCCs at or below 2×10^5 to 2.5×10^5 cells/ml are considered healthy

for most practical purposes (31). By this criterion, the SCCs of all phage-treated quarters returned to normal levels by 2 weeks postinfusion. In contrast to healthy quarters which had been infused with the phage preparations, quarters which were experiencing *S. aureus* mastitis did not exhibit any significant rise in SCCs following phage infusion, suggesting that quarters with elevated SCCs are not as sensitive to the introduction of phage as healthy quarters.

While a detailed examination of the mammary gland immune response to phage infusion was beyond the scope of this study, it is clear that the bovine mammary gland is able to react strongly to the presence of bacteriophage K. The majority of somatic cells present in normal milk are leukocytes, particularly macrophages. When the mammary gland becomes inflamed, the milk SCC increases rapidly; most of the newly recruited somatic cells are phagocytic neutrophils, accompanied by a smaller proportion of T and B lymphocytes (15). A specific or innate immune response against phage K could trigger somatic cell recruitment. However, the presence of preformed antiphage immunoglobulin G1 (IgG1) or IgM in the gland would imply that all animals had prior exposure to phage K or an antigenically related phage. All cows used in this portion of the study had no known history of *S. aureus* mastitis, and three of the six animals used were in their first lactation. Furthermore, no *S. aureus* phages were detected in any milk sample collected from animals which did not receive phage infusions. Clearly, a more thorough understanding of the mammary gland's immune response to phage infusion would benefit the application of phage therapy in the treatment of mastitis.

Bacteriophage which was infused into the quarters of healthy animals was detectable in the milk for up to 36 h after infusion (Table 1). This observation was confirmed in a second pilot study which sampled four animals at 12, 24, 36, 48, 60, and 84 h postinfusion (data not shown). The amount of phage detected in milk sampled from phage-infused quarters was far less than that which would be expected from simple dilution in the milk produced by the gland. Assuming that the average quarter has a total volume of 6 liters, a phage concentration of approximately 1×10^7 PFU/ml would be expected in the first postinfusion sample. In the preliminary trial, however, the amount of active phage shed into the milk from infused quarters was 2 to 3 log units lower than this expected value. As noted above, the presence of phage-inactivating antibodies in the mammary gland was not determined in this study. Repeated intramammary infusions of bacteriophage T4 have been shown to elicit local IgG, IgM, and IgA production 8 to 12 days after the first infusion (8). It is not clear if phage-inactivating antibodies could be produced in the mammary gland within 12 h of phage infusion.

Due to the elevated SCCs observed in the preliminary trial, a phage preparation which was purified by two passages through a CsCl gradient was selected for use in the clinical trial to treat *S. aureus* mastitis. It has been suggested by some workers that the treatment of *S. aureus* IMIs with chemical antibiotics may be enhanced if they are used in combination with a *S. aureus* bacterin (20). The crude phage lysate administered in the preliminary trial is essentially a combination of a bactericidal agent (bacteriophage) and a bacterin produced by the lysis of the host *S. aureus* cells during phage propagation. It

was the intention of the authors to avoid the complicating factor of the coadministration of a bacterin with the phage preparation, which could potentially make it difficult to determine to what extent cure was effected by the phage, the bacterin, or a combination of both. This concern was also raised in the early phage therapy literature (16).

For the treatment of subclinical *S. aureus* mastitis with bacteriophage, a 5-day course of daily intramammary infusion was used in a placebo-controlled, stratified design. The quarters treated by phage therapy did not exhibit a significant cure rate or reductions in either disease severity or *S. aureus* shedding. It is not likely that the cure rate obtained by phage therapy in this study is of clinical significance, as the three quarters which were cured were experiencing extremely mild forms of subclinical *S. aureus* mastitis (Fig. 2). This inverse correlation between case severity and cure rate has been noted by other workers in the evaluation of chemical antibiotics (10, 12) and probably represents the higher likelihood that such cases will cure spontaneously.

It is clear that mutation of the bacteria resulting in resistance to phage K was not responsible for the lack of a significant cure rate, as such resistance was not observed in any of the *S. aureus* isolates collected from infected quarters before or after treatment. The possibility that phage-treated quarters were cured and subsequently reinfected by *S. aureus* is also highly unlikely, as previous studies examining the antibiotic treatment of *S. aureus* IMIs found that changes in bacterial strains pre- and posttreatment are rare events (28). Therefore, the lack of treatment efficacy is due to some other factor.

As shown in this study, the pharmacokinetics of bacteriophage K following infusion into the mammary gland are complicated by the apparent inactivation of phage in the mammary gland following infusion and the ability of phage K to elicit an increase in the quarter SCC under some conditions. Additionally, a reduction in the binding rate of phage K to *S. aureus* which is independent of phage inactivation has been observed in milk or milk whey (14, 24). There is some evidence that this rate reduction is mediated by whey proteins which attach to the *S. aureus* cell surface and inhibit phage binding (14). *S. aureus* has also been shown to aggregate when it is grown in milk or milk whey (3, 24), which may confer some protection to the cells against phage attack.

In conclusion, the efficacy of bacteriophage in the treatment of bovine mastitis caused by *S. aureus* appears to be limited under the treatment conditions studied. The 5-day course of high-titer phage infusions was not sufficient to overcome the barriers to phage-mediated bacterial lysis which are present in the bovine mammary gland, which include the inhibitory effects of raw milk. The pharmacokinetic study showed that phage may persist within an infused quarter for 36 h after treatment but that the effective concentration of phage present in the quarter is significantly less than that predicted by simple dilution of the phage preparation by the milk contained in the gland. Phage is also able to elicit an increased SCC in healthy quarters, and the degree of inflammation may affect the amount of free phage available. Clearly, further work is required if phage therapy is to be successfully practiced in the treatment of bovine mastitis caused by *S. aureus*.

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