In vitro evaluation of a novel bacteriophage cocktail as a preventative for bovine coliform mastitis

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ABSTRACT

The objective of this study was to investigate the potential use of bacteriophage in preventing *Escherichia coli* mastitis on dairies. A cocktail consisting of 4 distinct bacteriophages was generated by screening against 36 *E. coli* isolates from dairy cows in Washington State with clinical mastitis. The bacteriophage significantly inhibited growth of 58% of the Washington State isolates and 54% of *E. coli* mastitis isolates from New York State, suggesting that the cocktail of phages had a relatively broad spectrum of action against relevant strains from 2 distinct geographies. The ability to suppress bacterial growth of these isolates in a liquid growth medium was not affected by the ratio of bacteriophage particles to bacterial cells (multiplicity of infection, MOI). For those *E. coli* that were completely inhibited by the phage cocktail, an MOI as low as 10 had the same effect as 10 µg/mL of ceftiofur on the growth rate of *E. coli* over a 12-h period using optical density measurements. A 3.3- to 5.6-log reduction of growth was achieved when *E. coli* was co-incubated with our phage cocktail in raw milk over a 12-h period at physiologic temperature. A modified gentamicin protection assay using bovine mammary epithelial cells provided a model to test whether bacteriophage could prevent cell attachment and invasion by chronic coliform mastitis strains. Pretreatment of cell cultures with the phage cocktail significantly reduced adhesion and intracellular survival of *E. coli* compared with controls. When combined with a bismuth-based teat sealant, the phage cocktail was able to inhibit bacterial growth when challenged with 1.6×10^3 cfu/mL of a clinical mastitis *E. coli* strain. In vitro results show bactericidal activity by our phage in raw milk and mammary tissue culture systems. Before a bacteriophage-based dry-cow treatment becomes a potential option for dairies, in

vivo studies must be able to demonstrate that a specific dose of bacteriophage can protect cows from experimentally induced *E. coli* mastitis without inducing an inflammatory reaction.

Key words: bacteriophage, *Escherichia coli*, mastitis, dry cow therapy

INTRODUCTION

Bovine mastitis is one of the most common forms of disease in dairy cows worldwide. Mastitis reduces milk yield and increases milk production costs due to discarded milk, preventative and therapeutic expenses, and premature culling (Halasa et al., 2007; Heikkilä et al., 2012). Additionally, mastitis has a significant negative impact on milk quality and animal welfare (Bradley, 2002). *Escherichia coli* is a pathogen frequently associated with bovine mastitis in well-managed dairies, and it often causes severe mammary gland inflammation in the cow (Bradley and Green, 2001; Hogan and Smith, 2003). *Escherichia coli* can also play a role in chronic subclinical IMI, and its persistence is associated with different bacterial strain characteristics that allow intracellular invasion of mammary epithelial cells (Döpfer et al., 1999; Dogan et al., 2012; Lippolis et al., 2014). Broad-spectrum antibiotics have been used systemically or as intramammary treatment for coliform mastitis. Therapeutic trials have shown questionable clinical efficacy of antibiotics against *E. coli* infections during lactation, and their use is often discouraged in such cases (Suojala et al., 2013). Intramammary antibiotics administered during the dry period can help eliminate coliform infections present at the end of lactation and prevent new IMI before calving. Blanket dry-cow therapy with long-acting antibiotics is considered a standard practice on almost all dairies worldwide; however, coliform mastitis remains a significant problem on well-managed farms (Bradley and Green, 2001). The routine prophylactic use of intramammary antibiotics on dairies is now under public scrutiny due to concerns over the transfer of antibiotic resistance genes to human pathogens (Mollenkopf et al., 2010). Organic dairies are not permitted to use dry-cow antibiotics and there is a

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lack of efficacious alternative preventatives for coliform mastitis (Mullen et al., 2013, 2014).

Bacteriophages are viruses that can replicate inside bacteria, thereby suppressing their proliferation. Although the use of bacterial viruses as therapeutic agents was explored as early as 1919 by Felix d'Herelle, the co-discoverer of phages, there has been a recent resurgence of interest in phage therapy due to the emergence of antibiotic-resistant bacteria (Saini et al., 2013; Tsonos et al., 2014). Several animal studies have demonstrated the safe and efficacious use of phage against *E. coli* infections (Brüssow, 2005; Lu and Koeris, 2011). In the 1980s, Smith and Huggins (1983) used coliphages to protect calves, piglets, and lambs from diarrhea after they were given oral doses of enteropathogenic *E. coli* strains. A cocktail of different phages against *E. coli* O157:H7 has been shown to reduce shedding of these bacteria in experimentally infected sheep (Raya et al., 2006, 2011; Callaway et al., 2008). Additionally, bacteriophages have been isolated against *E. coli* associated with postpartum uterine infections with the hope of using phages as an alternative to antibiotics for the treatment of metritis in dairy cows (Bicalho et al., 2010; Santos et al., 2010).

In order for phage therapy to be a viable alternative to antibiotics, several important obstacles need to be overcome (Tsonos et al., 2014). *Escherichia coli* bacteriophages often infect specific strains, which limits their ability to infect a diverse group of disease-causing coliform isolates, thus requiring the use of multiple phages in a cocktail to increase host range specificity (Brüssow, 2005). Phage cocktails are also used in an attempt to combat bacterial resistance that can develop due to evolutionary dynamics between bacteriophages and their hosts (Lu and Koeris, 2011). Although it is easy to demonstrate bacteriophage activity in vitro, relatively few studies have addressed how phages infect bacteria, replicate, and persist under in vivo conditions. For example, in order for phage to be effective in treating or preventing mastitis, it must be able to lyse bacteria in the presence of raw milk and dry-cow secretions. In fact, previous work done with *Staphylococcus aureus* and bacteriophage K showed that phage attachment and lytic activity were suppressed in raw whole milk but not in heat-treated milk (O'Flaherty et al., 2005; Gill et al., 2006).

The objectives of the current study were to determine if a broad host-range bacteriophage cocktail could be made against mastitis-causing *E. coli*, to evaluate coliphage activity in raw milk, to determine whether our cocktail could reduce adhesion and invasion of mammary epithelial cells by chronic coliform mastitis strains, and to test whether a phage-based intramammary ointment could inhibit bacterial growth.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Two collections of clinical *E. coli* strains were used for this study. Washington State *E. coli* strains were initially isolated by the Washington State University College of Veterinary Medicine (Pullman), from cows with clinical coliform mastitis. New York strains were provided by Cornell University College of Veterinary Medicine (Ithaca, NY), and previously classified as either persistent or transient as determined by clinical presentation of mastitis (Dogan et al., 2006, 2012). To distinguish our inoculant bacteria in raw milk from background coliforms, we transformed one of the persistent strains (P5) with a pUC-18 plasmid containing ampicillin resistance (**Amp-R**). Strain P5 was also used for the bismuth-based cream test. Three persistent strains (P4, P5, and P6) were used for tissue culture experiments.

All strains were plated on Levine eosin methylene blue agar (Sigma-Aldrich, St. Louis, MO) for confirmation of coliform morphology, maintained as streaks on tryptic soy agar (**TSA**; Becton Dickinson and Co., Franklin Lakes, NJ) plates, and grown up in tryptic soy broth (**TSB**; Becton Dickinson and Co.) in a 37°C bath with shaking at 200 rpm for the majority of experiments unless otherwise noted.

Bacteriophage Isolation and Characterization

Samples of the primary effluent of wastewater from a local sewage treatment plant were used to isolate bacteriophages using a standard isolation protocol (Carlson, 2005). Samples were centrifuged for 20 min at $1,500 \times g$ at 4°C to remove solids. The supernatant was transferred to sterile Erlenmeyer flasks containing an exponential-phase bacterial culture of one of the Washington State strains and $10 \times$ TSB. The flasks were incubated with shaking for 18 to 24 h. Following incubation, chloroform was added to the flasks to kill any remaining viable bacteria. Lysates were centrifuged for 30 min at $3,800 \times g$. The supernatant was filtered through a 0.45- μ m polyethersulfone filter, and 100 μ L of the phage lysate was plated with its host strain using the agar overlay method (Kropinski et al., 2009). Phage plaques of different morphologies were plucked from the agar plate and resuspended in phage buffer (100 mM NaCl, 100 mM Tri-HCl, 0.01% wt/vol gelatin). Phage plaque preparations were plated a second time on the same host and replucked to ensure phage purity.

A spot lysis test was used to assess the host range of each isolated bacteriophage preparation. Briefly, 10 to 20 μ L of each bacteriophage preparation was spotted

on lawns of each of the 36 Washington State mastitis strains using the agar overlay method (Kropinski et al., 2009). Plates were examined and scored for degree of bacterial clearance. Preparations with the highest degree of clearance on the most strains were selected for the cocktail and subsequently amplified (Carlson, 2005).

Using standard methods, pulsed-field gel electrophoresis was used to determine whether each phage preparation contained multiple or single phages. Restriction fragment length polymorphism analysis was performed on extracted DNA using *EcoRV* (EC 3.1.21.4) to determine whether each phage in the cocktail was distinct (Lingohr et al., 2009). Phage genomes were sequenced using Illumina HiSeq 2000 (Illumina Inc., San Diego, CA) at Oregon State University (Corvallis) with 200× coverage. Reads were assembled using idba version 1.1.1 (<https://lists.debian.org/debian-med/2014/02/msg00172.html>) followed by PriceTI version 1.2 (<http://derisilab.ucsf.edu/software/price/>) and then annotated using Artemis (<http://www.sanger.ac.uk/science/tools/artemis>). Percentage identities were compared with the National Center for Biotechnology Information database using BLAST+ (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Optical Density Growth Curves

To assess the effects of different doses of bacteriophage cocktail on the growth of *E. coli*, optical density growth curves measured at 570 nm (OD_{570}) were generated for each bacterial strain. A culture of each clinical *E. coli* strain was grown to an OD_{570} of 0.3 ($\sim 1 \times 10^8$ cfu/mL) in TSB as previously described. The culture was diluted in TSB to approximately 1×10^6 cfu/mL, and an aliquot of 0.18 mL was added to a 96-well plate previously prepared with 0.02 mL of phage at concentrations that achieved a multiplicity of infection (MOI) of 10, 100, or 1,000. A control of 10 and 1 μ g/mL of the antibiotic ceftiofur was used. The OD_{570} was measured every 30 min for 12 h. All tests were done in triplicate and repeated twice.

Percentage inhibition was calculated for each strain treated with our cocktail based on the measured OD values at 12 h. Percentage inhibition was calculated as follows: $(OD_{\text{negative control}} - OD_{\text{treatment}}) / (OD_{\text{negative control}} - OD_{\text{positive control}}) \times 100$ (De La Fuente et al., 2006). Ceftiofur at 10 μ g/mL was used as a positive control, bacteria without phage or ceftiofur was the negative control, and the phage cocktail at 100 MOI was the treatment. Strains that fell within a complete, partial, or no inhibition category were averaged together to generate a mean growth curve.

Phage Cocktail Activity in Raw Milk

Raw milk was collected from quarters of healthy cows on a dairy in Washington State. Milk was initially examined for inflammatory cells and then plated on TSA plates to test for native bacteria. Only milk samples negative by California Mastitis Test and containing <100 cfu/mL were used. Then, 100- μ L aliquots of different dilutions of an overnight broth culture of strain P5-AmpR were added to 10 mL of raw milk for treatment and control groups. Treatment flasks contained 100 μ L of a 1×10^9 plaque-forming unit (pfu)/mL phage cocktail, whereas control flasks contained the same volume of phage buffer. Flasks were incubated for 12 h with shaking. At the end of 12 h, samples were plated on TSA plates containing 100 μ g/mL of ampicillin. Bacterial survivors were enumerated following overnight incubation.

A 12-h growth curve was performed using the method above, but additional samples were taken and plated every hour. Bacterial colonies and phage plaques were enumerated as previously described. All tests were done in triplicate and repeated twice.

Bacteriophage Prevention of Mammary Epithelial Cell Adhesion and Invasion by *E. coli*

An immortalized bovine mammary alveolar epithelial cell line, MAC-T, was provided by the Virginia Tech Department of Dairy Science (Blacksburg) and was used for adhesion, invasion, and internalization assays (Huynh et al., 1991). Monolayers were incubated at 37°C with 5% CO₂, 95% air (vol/vol) using Dulbecco's modified Eagle medium (DMEM)/Nutrient Mixture F-12 supplemented with 5% fetal bovine serum (vol/vol).

To quantify the total cell-associated bacteria (intracellular bacteria plus surface-adherent bacteria), we performed a standard adhesion assay (Döpfer et al., 2000). Confluent monolayers were grown in 12-well tissue culture dishes to an average concentration of 4×10^5 cells/well. Overnight cultures of clinical *E. coli* strains P4, P5, and P6 (one culture per strain) were pelleted and washed 3 times with PBS and resuspended in DMEM with enough volume to obtain an OD_{570} of 1.0 (Dogan et al., 2006, 2012). Cultured wells were infected at approximately 10 bacteria cells to every MAC-T cell. Phage cocktail was added to treatment wells at an approximate MOI of 100. Controls included infected cells without phage treatment and cells with phage but no bacteria. After 1 h of incubation, DMEM was removed and wells were washed 6 times with PBS to remove nonadherent extracellular bacteria and phage. One milliliter of lysis buffer (0.1% wt/vol Triton X-100 and

0.025% trypsin) was added to each well and incubated for 10 min. Lysates were serially diluted and plated using standard enumeration methods.

An invasion assay was performed as described in the adhesion assay with modifications. After 1 h of incubation, DMEM was removed and replaced with 100 µg/mL of gentamicin-laden DMEM to kill remaining extracellular *E. coli*. Tissue culture dishes were incubated for an additional hour and wells were treated as described in the adhesion assay.

To assess the ability of intracellular phage to inhibit internalized bacteria and replicate following co-interalization, a long-term invasion assay measuring bacteria and phage counts was performed. The standard invasion assay was modified by further incubation of infected monolayers for up to 72 h with gentamicin-laden DMEM. At 2, 24, 48, and 72 h, the numbers of intracellular bacteria and phage were enumerated as previously described. All tissue culture assays were performed in duplicate.

Bacteriophage Inhibition of *E. coli* in a Bismuth-Based Teat Sealant

To test a potential delivery system for bacteriophage within the teat canal, an emulsification of phage and a common teat sealant containing bismuth subnitrate (Orbeseal, Zoetis, New York, NY) was made by combining 2 g of the sealant, 500 µL of Tween 80 (Sigma-Aldrich, St. Louis, MO), and 1 mL of 1×10^9 pfu/mL bacteriophage cocktail. Ten milliliters of TSB containing 1 or 0.6 g of the phage ointment was inoculated with 1.6×10^3 cfu/mL of bacteria in a 50-mL Falcon tube. Ointment with phage buffer was used as a control. All tubes were incubated with shaking for the duration of the experiment. Tubes were sampled at 0, 4, 8, and 12 h, serially diluted, and plated on TSA using standard techniques.

Statistical Analysis

To analyze the effect of the different bacteriophage MOI on the growth curve of 62 *E. coli* isolates, a general linear model (repeated-measures analysis of covariance) was used. The dependent variable was the OD₅₇₀ of the TSB culture; the fixed effects included treatment (different phage concentrations and controls) and time. The interaction of treatment and time was also included in the model. Our data were longitudinally collected and, therefore, had a series of repeated measures (total of 24) of optical density throughout the study period. To account for within-*E. coli* correlation of the optical density, the error term was modeled by imposing a first-order autoregressive (AR-1) covariance structure.

The same statistical method was used to examine the effect of the phage cocktail on the growth curve of a persistent *E. coli* strain in raw milk, except that the dependent variable in these cases was counts (cfu). A Student's *t*-test was used for all other analyses in the study where comparisons were made between 2 groups (treatment and control). All statistical tests were run in JMP Pro (11.0, SAS Institute Inc., Cary, NC) with an $\alpha = 0.05$.

RESULTS

Bacteriophage Isolation, Selection, and Characterization

Seventy-four bacteriophages were initially isolated using 36 clinical coliform mastitis isolates from dairies in Washington State. Each individual phage preparation was then tested for its ability to suppress growth of the 36 clinical isolates using spot lysis tests. Four phages were selected from this group as candidates for a cocktail, based on their distinct broad host ranges against these mastitis strains. Genome sizes of these phages were estimated to be between 140 and 172 kb following analysis by pulsed-field gel electrophoresis. Three of the 4 phage genomes were susceptible to digestion under standard conditions by *EcoRV*. The banding patterns of the genomic fragments were dissimilar. Together, these results demonstrate that each of our phage preparations contained a single bacteriophage genetically distinct from the others.

Genome sequence analysis confirmed this, revealing that the cocktail contained 2 T4-like phage, a rV5-related phage, and a phi92-related phage (Kropinski et al., 2013). Bacteriophage phi92 is known for its broad host range due to its multivalent adsorption apparatus (Schwarzer et al. 2012). All the phages in our cocktail were lytic and did not contain genes that would suggest prophage-like capabilities.

Effect of Bacteriophage Cocktail on Growth Curves of *E. coli* Isolates

Different doses of the bacteriophage cocktail (MOI) were used to measure its ability to suppress growth of the 36 Washington State *E. coli* clinical strains. The phage cocktail demonstrated a broad spectrum of action against these isolates. Twenty-one strains (58%) were completely or partially inhibited by the phage cocktail at 100 MOI (Figure 1a). To assess the applicability of our cocktail to field *E. coli* strains different from those used to isolate our phages, we performed the same growth curve inhibition analysis on a collection of 26 clinical coliform mastitis strains from New York

State. The phage cocktail showed a similar distribution of growth inhibition as the Washington State strains, with 14 (54%) strains showing complete or partial inhibition (Figure 1b). Complete inhibition was defined as >90% reduction of bacterial growth by the phage cocktail at 12 h compared with controls. Partial inhibition was defined as between 10 and 90% reduction, and no inhibition was categorized as <10% growth suppression of bacteria (Figure 2). For all of the *E. coli* strains tested, growth curves were not significantly affected by dose, demonstrating that the range of MOI tested were equally effective in inhibiting growth. All strains were completely susceptible to ceftiofur at 10 µg/mL, but their growth was partially inhibited by ceftiofur at 1 µg/mL (Figure 2). In strains that were completely or partially inhibited, there was no statistically significant difference between our positive control (ceftiofur 10 µg/mL) and our phage cocktail ($P < 0.05$).

Phage Cocktail Activity in Raw Milk

Total bacterial counts in raw milk were measured after 12 h of incubation with the phage cocktail using a range of initial bacterial inoculant doses of a transformed persistent *E. coli* strain. A 3.3- to 5.6-log reduction in bacterial growth compared with control was achieved when our cocktail was challenged with 10^2 , 10^3 , 10^4 , 10^5 , and 10^6 cfu/mL ($P < 0.05$).

In addition, we conducted a 12-h bacterial growth curve with and without the phage cocktail in raw milk.

Bacteriophage titers were also measured to assess phage binding and replication in raw milk. The phage cocktail significantly inhibited bacterial growth compared with negative controls, achieving a 3.75-log reduction in bacterial growth at 12 h ($P < 0.05$). Decreases in counts of colony-forming units corresponded with increases in counts of plaque-forming units, indicating phage lysis of bacteria between 4 and 8 h (Figure 3).

Bacteriophage Prevention of Mammary Epithelial Cell Adhesion and Invasion by *E. coli*

To examine whether our phage could prevent and clear chronic coliform infections, we used tissue culture as an ex vivo model to study the effects of bacteriophage on the ability of *E. coli* to adhere to and invade mammary epithelial cells. Tissue culture plates pretreated with approximately 10^8 pfu of our cocktail reduced mammary epithelial cell adhesion of 3 chronic mastitis *E. coli* strains—by 98.0, 99.6, and 99.7% ($P < 0.05$) for P4, P5, and P6, respectively (Figure 4a). Intracellular invasion of mammary epithelial cells, as measured by the gentamicin protection assay, was reduced for the 3 strains—by 88.3, 98.1, and 74.3% ($P < 0.05$) for P4, P5, and P6, respectively, in the presence of bacteriophage (Figure 4a).

To compare intracellular survival of persistent *E. coli* with and without bacteriophage, a long-term invasion assay was conducted over 3 d. Although we observed no significant difference in bacterial numbers intracel-

Figure 1. Growth inhibition of bacterial isolates from dairies in 2 different states at 12 h, demonstrating that the bacteriophage cocktail has a broad host range against bacteria from diverse locations: (A) 58% of the 36 bacterial isolates from Washington State were completely or partially inhibited; (B) 54% of the 26 isolates from New York State were similarly suppressed.

lularly after 48 and 72 h of incubation with gentamicin, bacteriophage counts were relatively stable throughout the assay and appeared to increase in response to bacte-

rial growth (Figure 4b). When comparing intracellular bacterial counts to negative controls (without phage), P -values for each time point were ≤ 0.05 .

Figure 2. Mean optical density growth curves (OD₅₇₀) for *Escherichia coli* isolates, isolated from mastitic cows from Washington and New York, inoculated with different levels of the bacteriophage cocktail (multiplicity of infection, MOI) and ceftiofur and in the absence of bacteriophage (control). (A) All isolates (n = 62); (B) isolates completely inhibited by the cocktail, as defined by a 91 to 100% decrease in growth at 12 h (n = 28); (C) isolates partially inhibited, as defined by a 10 to 90% decrease at 12 h (n = 7); (D) isolates not inhibited, as defined by a 0 to 9% decrease at 12 h (n = 27). The 95% CI represent the average OD over the entire experimental period. For the control, 95% CI = 0.875–0.833; for ceftiofur 1 µg/mL, 95% CI = 0.833–0.875; and for ceftiofur 10 µg/mL, 95% CI = 0.129–0.137. For all phage doses, 95% CI = 0.104–0.110 for strains completely inhibited (B), 0.300–0.355 for strains partially inhibited (C), and 0.746–0.782 for strains with no inhibition (D).

Figure 3. Growth curve of persistent *Escherichia coli* strain P5-AmpR inoculated with bacteriophage or phage buffer (control) in raw milk. Initial concentrations were $\sim 50,000$ cfu/mL and 2×10^8 pfu/mL for bacteria and phage, respectively. Error bars represent means ± 1 standard error.

Bacteriophage Inhibition of *E. coli* in a Bismuth Subnitrate Teat Sealant

The bacteriophage cocktail was emulsified with a bismuth-based teat sealant (Orbeseal) and its activity tested in liquid growth medium. Using a challenge

dose of 1.6×10^3 cfu/mL of a clinical mastitis strain, our bacteriophage ointment at 10^9 and 6×10^8 pfu was able to reduce bacterial growth by 5.40 and 2.80 logs, respectively, after 12 h of incubation at physiologic temperature (Figure 5). The greatest difference between the treatment and control occurred at the 8-h time point, resulting in a 6.77-log reduction in bacterial counts.

DISCUSSION

Escherichia coli is an opportunistic environmental pathogen, and strains isolated from cows with mastitis are genetically diverse, even from the same farm (Moser et al., 2013). No specific genetic markers or virulence factors have been identified that can distinguish mastitis strains from environmental *E. coli* isolated from dairies (Wenz et al., 2006). Bacteriophages that are isolated using a single clinical strain may not be able to infect different field strains due to high bacterial host specificity. Despite the apparent large heterogeneity among coliform mastitis isolates, our cocktail of 4 different bacteriophages had a greater than expected host range against mastitis-causing *E. coli*. This was demonstrated by its relatively similar activity against clinical *E. coli* mastitis strains from New York State compared with the strains from Washington State that were originally used to isolate the phages. The universal application of a bacteriophage cocktail to help prevent *E. coli* mastitis on a variety of geographi-

Figure 4. (A) Reduction of adhesion and invasion of MAC-T cells by 3 persistent *Escherichia coli* strains (P4, P5, and P6) when pretreated with phage cocktail, expressed as percentage relative to the control. (B) Survivability of internalized bacterial strain P4 and phage with and without bacteriophage (control) expressed in logs of colony- and plaque-forming units. Error bars represent means ± 1 standard error.

Figure 5. Growth curve of *Escherichia coli* strain P5 in tryptic soy broth with 2 concentrations of phage teat sealant emulsions compared with the control.

cally distinct dairies is theoretically possible; however, 27 of the 62 coliform mastitis isolates examined were resistant to our 4 phages. Wider host range activity may be achieved by adding novel phages to the existing cocktail. In addition, phage cocktail combinations that are isolated against coliform strains currently found on a particular farm may provide a more effective and targeted approach.

Bacteriophages are commonly found in raw milk samples and can create problems for cheese and yogurt processors by infecting starter bacterial cultures (Madera et al., 2004; García et al., 2009). Although staphylococcal bacteriophage K is able to inhibit growth of *Staphylococcus aureus* in pasteurized milk, it is unable to affect bacterial growth in raw milk due to bacterial agglutination, which prevents phage binding (O'Flaherty et al., 2005). Heat-treating milk inactivates whey proteins, allowing phage to bind and lyse *Staph. aureus* effectively (Gill et al., 2006). Most recently, it has been shown that *E. coli* can be completely eliminated by phages in raw milk at an incubation temperature of 25°C (McLean et al., 2013). Our results demonstrate that bacteriophage can bind to, replicate, and lyse *E. coli* in the presence of raw milk constituents and significantly reduce bacterial titers after 12 h of growth at physiologic temperature. It appears that in a raw milk medium, bacteriophage interact with *E. coli* differently than they do with *Staph. aureus*, allowing the phage to effectively inhibit the growth of *E. coli* bacteria. Recent successful control programs for the contagious mastitis pathogens *Staph. aureus* and *Streptococcus agalactiae* have increased the relative impact

of environmental mastitis-causing organisms such as *E. coli* and *Streptococcus uberis* (Zadoks and Fitzpatrick, 2009). It is not known whether streptococcal bacteria avoid bacteriophage infection in raw milk in a similar fashion to staphylococcal bacteria.

Certain *E. coli* strains are able to persist in cows via intracellular invasion of mammary epithelial cells while avoiding endosome-lysosome fusion and endosome acidification (Almeida et al., 1996, 2011; Passey et al., 2008). By using cultured bovine mammary epithelial cells, we attempted to analyze the ability of our phage cocktail to prevent chronic coliform mastitis under conditions that approximate the teat canal and mammary gland environment. Preapplication of bacteriophage significantly reduced the ability of *E. coli* to adhere to and invade bovine mammary epithelial cells, suggesting that our cocktail might be used to prevent cases of *E. coli* mastitis. It is unknown whether the phage cocktail is lysing the bacteria before it has a chance to attach and invade the mammary epithelial cells, or whether additional interference mechanisms exist. Previous work has shown that bacteriophage can be co-internalized with bacteria into eukaryotic cells (Hsia et al., 2000; Capparelli et al., 2007; Ray et al., 2009). Our bacteriophage remained viable within mammary epithelial cells even after 3 d, indicating that phage may be able to clear chronic cases that are resistant to host defenses and antibiotic therapy (Hsia et al., 2000).

Preventing rather than treating active *E. coli* IMI has been emphasized because most clinically affected cows mount a rapid immune response that eliminates coliform bacteria from the mammary gland. Dry-cow antibiotic therapy immediately followed by an internal teat sealant is often used to prevent mastitis in dairy herds by clearing up persistent infections at dry-off and preventing new infections before calving (Godden et al., 2003; Bradley et al., 2011). By combining bacteriophage with a nonantibiotic bismuth-based intramammary teat sealant, *E. coli* infections that can cause mastitis can be potentially controlled on organic and conventional dairies with a single dry-cow application. It has been suggested that gram-negative organisms such as *E. coli* cause mastitis in lactation by infecting the cow during her transition period, rather than early in her dry period (Bradley et al., 2011). It is unclear whether bacteriophage activity could be maintained in a cow's teat canal and udder throughout the end of her dry period. Potential adverse consequences of infusing *E. coli* bacteriophage in a dry nonlactating udder has not been thoroughly tested yet; however, previous work with *Staph. aureus* showed a moderate increase in somatic cell counts in lactating quarters infused with phage K compared with controls (Gill et al., 2006). Before field clinical trials can be conducted, it must

be shown that the use of a preventative bacteriophage preparation is safe to use in dry cows and protects them from an experimental *E. coli* infection.

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