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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* isolate*s* 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 × *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× 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 × *g*. The supernatant was filtered through a 0.45-μm polyethersulfone filter, and 100 μL 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 m*M* NaCl, 100 m*M* 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 μL of each bacteriophage preparation was spotted