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9

Successes and Failures of Bacteriophage Treatment of Enterobacteriaceae Infections in the Gastrointestinal Tract of Domestic Animals

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1. Introduction

Bacteriophages are numerous in the ecosystem and play a central role in bacterial ecology (Ashelford et al., 2000; Brabban et al., 2005; Breitbart et al., 2003; Danovaro et al., 2001; Fuhrman, 1999). Bacteriophages have frequently been isolated from various environmental sources and the gastrointestinal tract of animals (Adams et al., 1966; Bielke et al., 2007a; Breitbart et al., 2003; Callaway et al., 2003, 2006; Dhillon et al., 1976; Filho et al., 2007; Higgins et al., 2008; Klieve & Bauchop, 1988; Klieve & Swain, 1993; Kudva et al., 1999; Orpin & Munn, 1973; Raya et al., 2006; Smith & Huggins, 1982). Breitbart and co-workers (2003) found bacteriophages to be the second most abundant uncultured biological group in their analysis of human feces and Furhman (1999) suggests that bacteriophages could be responsible for as much as 50% of bacterial death in surface waters. It has been suggested that bacteriophages in cattle help maintain microbial diversity and balance, allowing the ecology of the gut, particularly the rumen, to adapt to changes in feed and water intake (Klieve and Swain 1993; Swain et al., 1996). Bacteriophages lytic for *E. coli* and *Salmonella* were isolated from cattle feedlots with no correlation between presence of *E. coli* O157:H7 or *Salmonella* and bacteriophages against the specific pathogen (Callaway et al., 2006). *Salmonella* targeted bacteriophages were isolated from *Salmonella*-positive poultry farms, with bacteriophages found at only one *Salmonella*-negative farm. A total of seven bacteriophages were isolated from farms that were *Salmonella*-positive, and two bacteriophages from the single *Salmonella*-negative chicken house (Higgins et al., 2008). This might suggest that as environmental *Salmonella* increased, a near-simultaneous increase in bacteriophages may have also occurred. The hypothesis corresponds with other reports where it was found that bacteriophages within treated animals remained in the animal for the duration of the infection, but once the bacterial host was no longer present, the presence of bacteriophages also rapidly dropped (Barrow et al., 1998; Calloway et al., 2003; Hurley et al., 2008; Smith and Huggins, 1987).

Because bacteriophages are a natural component of gastrointestinal microbial populations, they are presumably a potentially effective control strategy against bacterial pathogens. However, *in vivo* attempts have yielded mixed results (Bach et al., 2003; Bielke et al., 2007b,

Hurley et al., 2008; Kudva et al., 1999; O'Flynn et al., 2004; Higgins et al., 2007; Smith and Huggins, 1983, 1987; Toro et al., 2005). This chapter will review both successes and failures in research aimed to reduce enterobacterial infections of the gastrointestinal tract. During the last approximately 60 years, there have been sporadic published reports of efficacy in treating Enterobacteriaceae infections systemically and within the gastrointestinal tract. While a number of reports have rather consistently indicated that systemic or tissue-associated infections were treatable by parenteral administration of appropriate bacteriophage cocktails, reports of successful treatment of enteric Enterobacteriaceae are much more sporadic, and are interspersed with a number of reports of failed attempts for enteric treatment. The present chapter will discuss selected successes and failures and describe the possible differences in these studies and the potential for development of more effective strategies.

Bacteriophages can be regarded as natural enemies of bacteria, and therefore are logical candidates to evaluate as agents for the control of bacterial pathogens. Bacteriophages can be selected to kill bacterial pathogen target cells, and not affect desired bacteria such as starter cultures, commensals in the gastrointestinal tract or on skin, or accompanying bacterial flora in the environment. Bacteriophages harbor the potential for precise targeting of bacterial contamination, without compromising the viability of beneficial microorganisms in the habitat. Additionally, since bacteriophages are generally composed entirely of proteins and nucleic acids, the eventual breakdown products consist exclusively of amino acids and nucleotides, and, unlike antibiotics and antiseptic agents, their introduction into and distribution within a given environment may be seen as a natural process. With respect to their potential application for the biocontrol of pathogens, it should be considered that bacteriophages are the most abundant self-replicating units in our environment, and are present in significant numbers in water and foods of various origins, and most surfaces in our environment (Sulakvelidze and Barrow, 2005). A test of the safety of bacteriophages when administered orally to human volunteers revealed no adverse side effects (Bruttin and Brüssow, 2005). Very low levels of bacteriophage were found in the serum, suggesting low passage from the intestinal lumen to the blood flow, liver enzymes were not affected by bacteriophage ingestion, and no antibodies to the bacteriophages were detected. Mai and co-workers (2010) noted that treatment of mice with anti-*Listeria* bacteriophages did not significantly affect gastrointestinal microflora diversity. Additionally, Carlton et al. (2005) reported no adverse effects in rats after five continuous days of oral bacteriophage administration, suggesting that bacteriophages can in fact be regarded as safe.

Prior to the discovery of antibiotics, bacteriophages were researched as bacterial control agents (For a review, see Alisky et al., 1998). However, a lack of understanding of mechanisms resulted in therapeutic difficulties and resulted in poor experimental results. When treating bacterial infections, the goal is to take advantage of the lytic cycle of bacteriophages, rather than the lysogenic cycle in which bacteria are not killed (Figure 1). With an increase in bacterial pathogens that are resistant to traditional antibiotics, the scientific community has developed a renewed interest in using bacteriophages and they are currently being investigated in numerous laboratories and companies as alternative treatments for a variety of problems. Indeed, for some specific applications, bacteriophage therapy holds significant promise, and there is growing evidence that bacteriophage may be effective for some applications, with the caveat that these viruses are incredibly specific by definition, and selection of product for specific applications may be of critical importance.

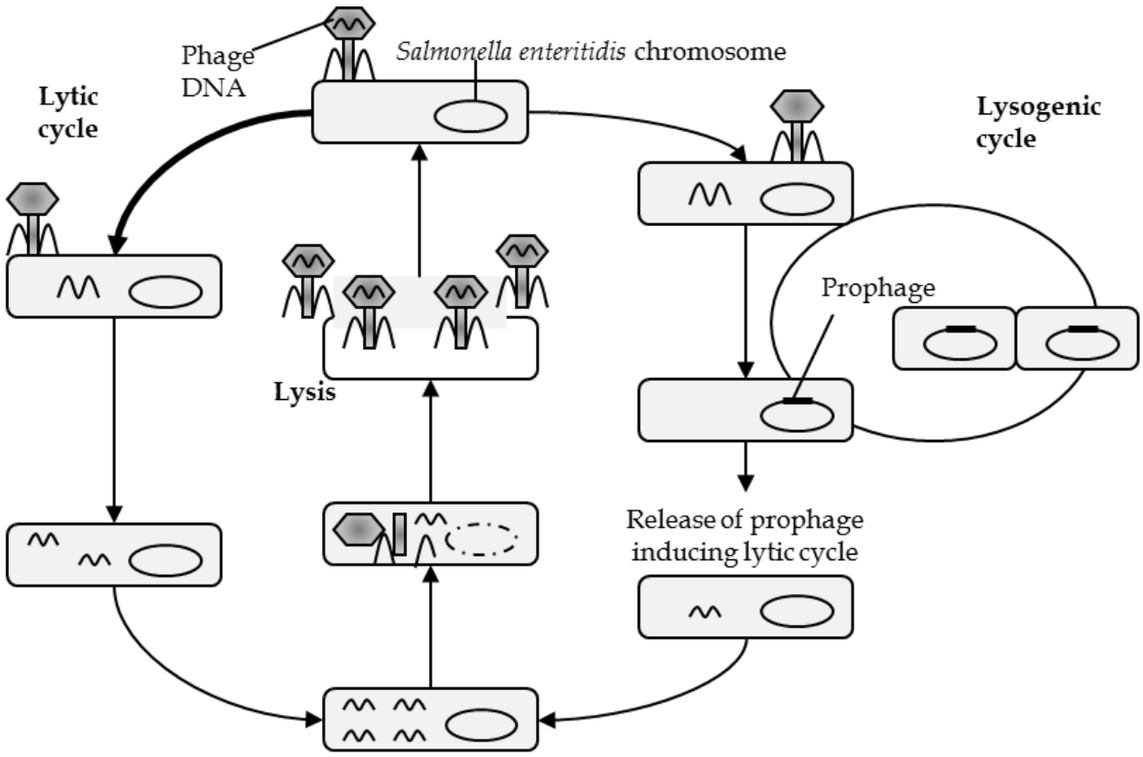

Figure provided by Dr. Jack Higgins.

Fig. 1. **Life Cycle of Bacteriophages.** Bacteriophages are capable of entering either a lytic cycle, in which the bacteriophage inserts its DNA into the host cell and replicates to make multiple copies of the bacteriophage virus before lysing the cell wall, and releasing daughter viruses to repeat the cycle. Bacteriophages can also enter a lysogenic cycle. Instead of using the cell to replicate large numbers of virus particles, the bacteriophage genome can remain in the host's genome and replicate through binary fission of the cell. Some lysogenic bacteriophages are capable of entering into the lytic cycle, while others will remain lysogenic.

2. Successes

The bacteriocidal effects of bacteriophages have long been studied for their usefulness in treating gastrointestinal infections. Early studies originating from the former Soviet Union, Eastern Europe, and Western Asia suggested bacteriophages could prevent and treat *Vibrio cholera* infections (Dubos et al., 1943; Dutta, 1963; Marčuk et al., 1971; Sayamov, 1963). In the 1980s, Slopek and co-workers (1983a-b, 1984, 1985a-c, 1987) published numerous papers showing the promising results of treating septic patients with bacteriophages. While the validity of these studies has been questioned, in part due to relaxed scientific rigor in these regions during the time when these studies were completed (Alisky et al, 1998; Merril et al., 2003) and are not often cited by bacteriophage researchers in recent years, they have served as an inspiration for continued research into the possibility that bacteriophages can cure gastrointestinal diseases in humans and animals.

Some bacteriophage research has also focused on the treatment of animals to cure a variety of diseases. In relatively recent years, Smith and Huggins (1982) compared the efficacy of

bacteriophages with that of antibiotics in treating both generalized and cerebral infections in mice. They isolated anti-K1 bacteriophages that were able to lyse K1+ *E. coli*. When administered by intramuscular injection at the same time as, or eight hours after, infection with *E. coli*. These bacteriophages were able to cure infection, even when used at a low titer. The same effects were seen with intracerebrally infected mice treated with bacteriophages 16 hours after infection. The bacteriophages were more effective than numerous types of antibiotics at curing mice. Smith and Huggins (1983) also successfully used bacteriophage therapy to treat calves, pigs, and lambs that had been infected with *E. coli*. They selected a bacteriophage that would lyse *E. coli* and also selected a second bacteriophage that would lyse *E. coli* cultures that had become resistant to the first bacteriophage. Key to the success of this selection method was the idea that, by selecting a bacteriophage that affected the K antigens of *E. coli*, resistance would require a modification to an important component of virulence for the cell. Resistant *E. coli* strains had different colony morphology on agar plates and were K-negative. Treatment consisted of two bacteriophages, one that resulted in a K-negative strain as resistance was developed, and a second to lyse the K-negative cells. The combination of bacteriophages to combat resistance was better able to prevent death in calves with diarrhea than a single bacteriophage or no bacteriophage treatment. Sheng et al. (2006) followed a similar method to select bacteriophages against *E. coli* O157:H7. They selected bacteriophage that attached to LPS on the cell surface so that resistant cells had to change LPS expression with the idea that it would decrease the pathogenicity of the bacteria. Resistant cultures had rough colony morphology instead of the usual smooth mucoid texture of many typical pathogenic Enterobacteriaceae. When bacteriophage KH1, selected to attach to LPS, was administered alone it did not reduce *E. coli* O157:H7 recovery in sheep. However, when combined with another bacteriophage, recovery of *E. coli* O157:H7 was reduced. In 1987, Smith and Huggins used bacteriophages to treat calves with *E. coli*-caused diarrhea. They selected their bacteriophages by administering *E. coli* to a calf followed by a bacteriophage cocktail. Bacteriophages able to survive the gastrointestinal tract were collected in the feces 24 hours post-administration. These bacteriophages were used to treat subsequent calves. Calves given bacteriophages within 24 hours of the onset of diarrhea recovered within 20 hours. Also, sick calves placed on bedding that had been sprayed with bacteriophages recovered from diarrhea. Smith and Huggins noted that during the period of disease, bacteriophages continued to persist in the feces, but after recovery, bacteriophage numbers dropped dramatically.

Biswas et al. (2002) successfully cured vancomycin-resistant *Enterococcus faecium*-infected mice with bacteriophage therapy. Mice were treated with bacteriophages just 45 minutes after infection with bacteria. Treatment at a multiplicity of infection (MOI) level of 0.3 to 3.0 was able to cure all of the infected mice. However, lower multiplicity of infection ratios (MOI) of 0.03 to 0.003 resulted in just 60% and 40% survival of mice, respectively. They also noted that bacteriophage treatment could be delayed for up to five hours after infection. However, if treatment was delayed for 18 or 24 hours, only 50% recovery was seen. In other studies, preparations of the appropriate bacteriophage have been able to protect mice and guinea pigs against systemic infections with strains of *Pseudomonas aeruginosa* and *Acinetobacter baumanii* and have been able to inhibit the rejection of skin grafts caused by *P. aeruginosa* infections (Soothill 1992, 1994). Interestingly, a very low MOI of 10^{-6}, one bacteriophage to one million bacteria, was able to protect mice against infection with *P. aeruginosa* (Soothill, 1992).

While each of these studies was successful at curing the infected animals, treatment was given simultaneously with the bacteria, or just a few hours after infection. These would not be practical conditions for treating disease under real world conditions because it is often not known that an animal is sick until clinical signs are noticed several days after the infection begins. Furthermore, key to the success of these experiments, is knowledge that the selected host is susceptible to the bacteriophage (or combination of bacteriophages) which was administered. As discussed below, multiple researchers have focused on improving food safety by treating animals pre-harvest with bacteriophages targeted against foodborne pathogens.

One such pathogen has been *Escherichia coli* O157:H7 in ruminants. Much research has investigated the ability of bacteriophages to treat *E. coli* O157:H7 infections in sheep and cattle. Enterohemorrhagic *E. coli*, such as *E. coli* O157:H7, cause severe enterohemorrhagic enteritis, renal uremic syndrome, and are capable of causing death, especially among young children, immune-suppressed individuals, and the elderly (Gyles, 2007; Rangel et al., 2005). Cattle and sheep are the primary sources of waterborne and foodborne cases, and with an infectious dose of approximately 100 cells, control of this pathogen is of utmost importance to meat processors (Besser et al., 1999; Chapman et al., 2001; Grauke et al., 2002; Wells et al., 1991; Zhao et al., 1995). Like other *E. coli*, the primary site of infection for cattle and sheep is the hindgut. Grauke et al. (2002) noted a correlation between positive fecal samples and isolation from the rumen and duodenum while Naylor et al. (2003) found the primary site of infection in the recto-anal junction. Other reports have confirmed the rectum and cecum as primary sites of infection in cattle (Buchko et al., 2000; Dean-Nystrom et al., 1999). These findings suggest that oral bacteriophage treatment, with bacteriophages selected for anaerobic activity, may affect *E. coli* O157:H7 colonization in sheep and cattle. In fact, multiple studies have focused on the application of anaerobically active bacteriophages to ruminants for the control of *E. coli* O157:H7, as described below.

Bacteriophage CEV1, isolated from sheep with short and transient *E. coli* O157:H7 infections, was found to be lytic against 20 pathogenic strains of *E. coli* and had both aerobic and anaerobic *in vitro* activity. Oral administration of CEV1 to infected sheep, three days post-infection, showed reduced levels of *E. coli* O157:H7 in the ruminal, cecal, and rectal contents two days after bacteriophage treatment (Raya et al., 2006). Similarly, rectal administration of bacteriophage KH1 and SH1 to cattle infected with *E. coli* O157:H7 was able to reduce the levels of recovered pathogen. The same bacteriophages eliminated detectable levels of *E. coli* O157:H7 in mice (Sheng et al., 2006). In 2008 Calloway et al. isolated bacteriophages from cattle feces and noted an effectiveness of these bacteriophages at reducing *E. coli* O157:H7 levels throughout the intestine of sheep. Bacteriophages were selected *in vitro* for their ability to lyse *E. coli* O157:H7, and eight different bacteriophages were included in the culture to combat resistance by the bacteria. While each of these studies effectively reduced the levels of *E. coli* O157:H7 in sheep and cattle, the researchers noted that bacteriophages may not prove a long-term treatment and application should be considered immediately prior to processing for maximum effectiveness. The principle reason for this is that as with antimicrobial chemicals, serial applications have often led to selection of bacteriophage-resistant bacteria.

Like *E. coli* O157:H7, researchers have attempted to reduce multiple serovars of *Salmonella* in poultry, a frequent source of cases of *Salmonella* in humans. *Salmonella enterica* serovars

continue to be among the most important foodborne pathogens worldwide due to the considerable human rates of illness reported and the wide range of hosts that are colonized by members of this genus, which serve as vectors and reservoirs for spreading these agents to animal and human populations (CDC 2005, 2006a,b, 2007, 2008a,b). Furthermore, public concern for the appearance of resistant strains to many antibiotics, particularly among zoonotic pathogens such as common *Salmonella* isolates, is also challenging several sectors of agriculture to find alternative means of control (Boyle et al., 2007). In the United States, it is estimated that 1.4 million humans contract salmonellosis and that the annual cost of this illness, including lost productivity, is $3 billion annually (WHO, 2006). In the year 2004, surveillance data indicated that the greatest number of foodborne illnesses was caused by *Salmonella*, comprising 42% of all laboratory diagnoses (FoodNet, 2005). Because many of these illnesses are associated with poultry and poultry products (Bean & Griffin, 1990; Persson & Jendteg, 1992), the reduction of microbial contamination during the production of poultry is important. Further considerations for bacteriophage treatment to control *Salmonella* in poultry are issues associated with antiobiotic treatment. Poultry harboring *Salmonella* infection can be treated with antibiotics with some success (Goodnough & Johnson, 1991; Muirhead, 1994). However, Manning and co-workers (1994, 1992) reported increased *Salmonella* colonization when chickens were treated with selected antibiotics, possibly due to reduction of normal bacterial flora in the gastrointestinal tract that serve as a natural barrier to *Salmonella* infection. Additionally, Kobland et al. (1987) and Gast et al. (1988) have recovered antibiotic resistant *Salmonella* from experimentally challenged birds treated with antibiotics. Recently, the United States Food and Drug Administration (FDA) has banned the use of enrofloxacin in poultry production because of concerns regarding an increase in resistant *Campylobacter* infections in humans (FDA, 2005). Recently, a ban on antibiotics and coccidiostats was put in place by European Parliament Council Directive 1831/2003. The regulation stated that antibiotics, other than coccidiostats and histomonostats, had to be removed from feed by the end of 2005, and that anticoccidial substances would be prohibited by 2013. After these dates, medical substances in animal feeds will supposedly be limited to therapeutic use by veterinary prescription (European Parliamant and of the Council, 2003). Thus, it is increasingly important that effective and inexpensive methods or products to treat bacterial infections in food production animals be developed.

Recently, Toro et al. (2005) reported using a combination of bacteriophages and competitive exclusion to treat *Salmonella*-infected chickens. They were able to reduce recovery of *Salmonella* Typhimurium (ST) from the ceca of chickens. In the successful experiments, chickens were challenged with ST during the course of treatment with bacteriophages. However, treatment with bacteriophages was not better than treatment with a competitive exclusion product. And, combination of competitive exclusion and bacteriophages did not further reduce ST recovery. Similarly, Filho and co-workers (2007) reported that administration of bacteriophage cocktails could temporarily reduce the incidence of *Salmonella* recovery in broiler chickens, but by 48 hours post-treatment there was no difference between treated and non-treated controls. Additionally, combining the bacteriophage cocktail with a probiotic culture had no effect on *Salmonella* recovery when compared to bacteriophages alone. The pattern was also noted by Higgins et al. (2007) when *Salmonella* was reduced to zero recovery, but 48 hours after administration recovery from bacteriophage-treated birds increased to higher than non-treated control groups.

In addition to control of paratyphoid *Salmonella*, much research has been completed on the study of bacteriophages in poultry for other diseases. The first reports of bacteriophage therapeutics in poultry were made by d'Herelle in 1922 when he reported successful treatment of at least 19 barnyards affected by fowl typhoid. Bacteriophages administered were selected specifically to target the pathogens involved. The pathogens causing the typhoid were either one or a combination of five different bacterial species. The data reported in these studies is anecdotal, with the statement "The sick recovered and the epizootic stopped at once" comprising all the results for those studies. However, treatment of a highly pathogenic and systemic host-adapted *Salmonella*, in this case, might be more effective as even a temporary reduction in pathogen levels could buy a critical amount of time for acquired immunity and eventual recovery of infected birds from the disease. This could be distinctly different than the case with the essentially non-pathogenic (for poultry) common paratyphoid isolates that are commonly implicated in food borne disease of humans. Supporting this hypothesis, other attempts to alleviate bacterial disease in poultry have focused on non-paratyphoid *Salmonella*. In 1926, Pyle isolated bacteriophages specific for *Salmonella pullorum* from the feces of birds affected with the disease. He demonstrated that even after 120 passages, strains of bacteriophage that were not initially lytic did not become lytic. Additionally he observed that two bacteriophage strains which were initially lytic became more lytic over 60 serial passages. Two experiments employing the lytic bacteriophages ensued. *Salmonella pullorum* and bacteriophages were injected simultaneously into the pectoral muscle of chickens in one treatment group, with the treated group receiving bacteriophage eight hours post-challenge. When compared to controls that received no bacteriophage treatment, mortality was delayed in both treatment groups. In the second experiment, the bacteriophages and *S. pullorum* were administered or the bacteriophages were administered eight hours post-challenge in the drinking water. Again, when compared to controls which did not receive bacteriophage treatment, the onset of mortality was delayed.

Berchieri et al. (1991) treated birds infected with ST with bacteriophages and found that the levels of ST could be reduced by several $\log_{10}$, and mortality associated with this unusually pathogenic ST was reduced significantly. However, ST was not eliminated, and returned to original levels within six hours of treatment. Also, the bacteriophages did not persist in the gastrointestinal tract for as long as the *Salmonella* was present. In fact, bacteriophages persisted only as long as they were added to the feed. In order to be effective, bacteriophages had to be administered in large numbers, and soon after infection with ST. Similar to reports below (Hurley et al., 2008), the bacteriophages may have been killing the bacteria via lysis from without and, instead of infecting and replicating within the cell, the bacteriophages may have been killing the ST by an excess of penetration from the bacteriophages. This may explain the decline in bacteriophage numbers despite the presence of a host for replication.

Multiple researchers have investigated the possibility of curing *E. coli* infections in poultry. In 1998 Barrow et al. prevented morbidity and mortality in chickens using bacteriophages lytic for *E. coli*. When chickens were challenged intramuscularly with *E. coli* and simultaneously treated with 10^6 – 10^8 pfu of bacteriophages, mortality was reduced by 100%. This study also demonstrated that bacteriophages can cross the blood brain barrier, and furthermore can amplify in both the brain and the blood.

Huff et al. (2003) used bacteriophages to treat airsacculitis caused by *E. coli* in chickens. Marked efficacy was achieved when administering bacteriophage with the bacterial challenge inoculum, by injection in the thoracic air sac. However, drinking water administration of the same bacteriophages was ineffective at preventing the manifestation of the disease syndrome. This indicated that it is important to deliver bacteriophages directly to the site of infection. It was also shown that an aerosol treatment of bacteriophages, followed by an *E. coli* challenge on the same day, the next day, or three days later reduced morbidity and mortality associated with respiratory infection (Huff et al., 2002a). Thus, the study demonstrated a prophylactic ability of bacteriophages in the respiratory tract. However, given the evidence that bacteriophages do not typically remain in an environment without an appropriate host (Ashelford et al., 2000; Fiorentin et al., 2005; Hurley et al., 2008; Oot et al., 2005), prophylaxis could be difficult without continued administration or by knowing an animal had been exposed.

In summary, there are few current reports of efficacy of bacteriophage treatment in chickens other than when treatment was administered at the same time as bacterial challenge or via injection. Outside of experimentally controlled situations, it is not usually possible to treat a disease at the same time as the challenge. Also, it is not practical to treat commercial poultry flocks by individual injection, though highly valuable breeder flocks might warrant the time and money involved. However, such of these limited successes do not necessarily translate into effective enteric treatments. Host-associated pressure against pathogen infections may predispose systemic bacteriophage therapy toward success. In these cases, where bacteriophages are used to treat systemic or tissue-associated infections, an acute efficacy of merely reducing the infection load by 90% or more, could greatly reduce mortality and reduce the duration and magnitude of disease by allowing time for acquired immunity in the animal host. In the intestinal lumen, host pressures against the infection may not be as severe and many Enterobacteriaceae are capable of free living status within the gut without eliciting robust acquired immune responses from the infected animal. In these cases, a temporary reduction in enteric colonization may not be as likely to be curative, as discussed below.

3. Failures

As the history of published successful bacteriophage treatments of enteric disease is reviewed, it is readily evident that such reports, while often dramatic in effect, are relatively sporadic during the last approximately 60 years. Given that experimental failures frequently are not published, as the cause of failure can often not be ascertained, the authors suspect that history is replete with unpublished examples of failures to treat enteric Enterobacteriaceae infections. Still, some reports of failures, or incomplete successes, have been documented and are described below.

Bacteriophage KH1, shown to lyse 12 of 16 *E. coli* O157 strains tested, originally showed promise as results of *in vitro* tests demonstrated an ability to lyse bacterial cultures, by plaque formation, at both 37 °C and 4 °C (Kudva et al., 1999). However, when administered to *E. coli* O157:H7 infected sheep, bacteriophage KH1 did not effectively reduce levels of recovered pathogen, despite continued recovery of bacteriophages from the feces for eight days post-treatment (Sheng et al., 2006). Aerobic, instead of anaerobic, selection may have played a key role in the ability of this bacteriophage to effectively eliminate intestinal

carriage of *E. coli* O157:H7. Multiple researchers have suggested anaerobic environments can affect bacteriophage activity (Bach et al., 2003; Kudva et al., 1999; Raya et al., 2006; Tanji et al., 2005). When Bach and co-workers (2003) tested the effects of bacteriophage DC22 on *E. coli* O157:H7 in an *in vitro* fermentation system prior to treating infected sheep, bacteriophage DC22 only decreased microbial levels in the artificial ruminant set up at high multiplicities of infection (MOI), and failure of the bacteriophage to replicate and increase PFU over the course of 120 hours suggests that the bacteriophages may have reduced *E. coli* by lysis from without rather than by infecting, replicating within, and lysing the cells. Subsequent *in vivo* studies in sheep did not result in decreased shedding of *E. coli* O157:H7, and bacteriophages were found in the feces for only two days post-treatment. Similarly, Tanji et al., (2005) administered a bacteriophage with promising *in vitro* test results to *E. coli* O157:H7 mice with little success. These studies reinforce the need to understand how *in vitro* conditions relate to the *in vivo* infection parameters and show the need for an appreciation for the ecosystem where the bacteriophage will be used.

In silico modeling was used by Hurley et al. (2008) to predict parameters for treating *Salmonella*-infected chickens with bacteriophage SP6 in an attempt to better comprehend the biological factors of the luminal ecosystem, *Salmonella*, and bacteriophages, and how they interact within the gastrointestinal tract. Among the factors considered were varying growth rates, feed and water intake, and *Salmonella* resistance to the bacteriophages. The results of these *in silico* test results were considered when an *in vivo* challenge was designed. However, after bacteriophage treatment *Salmonella* was detected at levels that did not differ from control groups not treated with bacteriophages. In fact, bacteriophage infection may have selected for resistant bacteria because half of the *Salmonella* isolates from a treated group were resistant to bacteriophage SP6 on day 29, one day after the second dose of bacteriophage treatment. Moreover, many of the *Salmonella* cultured from other samples of bacteriophage-treated birds showed at least a partial resistance to bacteriophages, with only partially clear plaques forming on soft agar overlays when, prior to bacteriophage treatment, the *Salmonella* isolate was susceptible and clear plaques routinely formed on soft agar overlays. The authors also noted a steady decrease in bacteriophage excretion, despite continued high levels of *Salmonella* recovery within the cecum. This data is similar to the results of Fiorentin et al. (2005), where *Salmonella* continued to be detected 21 days after inoculation, but bacteriophage levels had declined to undetectable levels. In another related study, bacteriophage treatment resulted in higher levels of *Salmonella* recovery in turkeys 48 hours post-treatment after an initial decrease in *Salmonella* at 6, 12, and 24 hour post-treatment time points (Higgins et al., 2007). These bacteriophages were selected for ability to survive low pH, to simulate passage through the ventriculus of poultry, and were administered with $Mg(OH)_2$ to aid adhesion of bacteriophages to the cell walls of bacteria (Eisenstark, 1967). The authors also noted that bacteriophage resistance was common in all cultures.

Our laboratory and others have demonstrated that resistance to bacteriophages selected against *Salmonella* isolates quickly occurs, often in a single passage (Bastias et al., 2010; Hurley et al., 2008; Fiorentin et al., 2005). When bacteriophage cocktails of 71 different bacteriophages selected for treatment of experimental *Salmonella* Enteritidis infections in chickens, a brief reduction in enteric colonization was noted during the first 24 hours, but rebound levels were similar to controls within 48 hours, even with repeated or continuous dosage of the bacteriophage cocktail (Higgins et al., 2007). Because of the demonstrated

temporary reduction in enteric colonization in these studies, effective bacteriophages were demonstrably able to pass to the lower gastrointestinal tract. As continued treatments failed to maintain this reduction, development of resistance by the enteric *Salmonella* Enteritidis is the most likely explanation.

In order to potentially deliver higher levels of bacteriophage, several attempts to protect the bacteriophage cocktail through the upper gastrointestinal tract were made in our laboratory. Pre-treatment of infected poultry with antacid preparations designed to reduce the acidity of the proventriculus (the true stomach of birds) were successful in increasing the number of administered bacteriophage that successfully passed into the intestinal tract, but this treatment did not improve the outcome of bacteriophage treatment of *Salmonella* Enteritidis infection (Higgins et al., 2007).

An alternative approach is to select for alternative non-pathogenic bacteriophage hosts which could potentially "carry" bacteriophage through the gastrointestinal tract and, with continuous dietary administration of the non-infected alternative host bacterium, provide a means of amplification within the gut of the host (Bielke et al., 2007a). Bielke and co-workers (2007b) demonstrated that non-pathogenic alternative hosts can be selected for some bacteriophages that were originally isolated using a *Salmonella* Enteritidis target. This approach, which has potential utility for amplification of large numbers of phage without the necessity to thoroughly separate bacteriophage from a pathogenic target host, was also used to create a potential "Trojan Horse" model for protecting the bacteriophages through the upper gastrointestinal tract, thus potentially providing a vehicle for enteric amplification of those surviving bacteriophages. In these studies, neither the Trojan Horse approach, nor the continuous feeding of the alternative host bacteria as a source of enteric amplification, were effective in producing even more than a transient reduction in enteric *Salmonella* infections.

Through these failures, many investigators have concluded that the escape of even a minority of target bacteria within the enteric ecosystem allows for almost immediate selection of resistant target bacteria and rebound to pre-treatment levels of infection may even exceed the levels of non-treated controls in some cases.

4. Potential strategies to overcome failures

Bacteriophage resistance is an important component of therapy to overcome before bacteriophages can really be a viable antimicrobial for infection. The generation time for bacteria is typically short enough that mutants with bacteriophage resistance can emerge within hours (Higgins et al., 2007; Lowbury and Hood, 1953). One possible strategy to overcome this problem is administration of multiple bacteriophage isolates for treatment. Smith and Huggins (1983) selected a bacteriophage against *E. coli* K^+, and then subsequently selected a bacteriophage against a resistant strain of *E. coli* K^+. The combination of these two bacteriophages reportedly cured calves, pigs, and lambs of intestinal colibacillosis. Despite this success, resistance is difficult, if not impossible, to predict and combining the correct cocktail of bacteriophages to overcome resistance would be a blind guess in most cases. This, combined with the highly selective nature of individual bacteriophage isolates and even cocktails, as described above, is discouraging from the perspective of enteric therapeutic development, especially for very low level or opportunistic pathogens.

The most success is likely to come from treating points in the system that are continually bombarded with bacteria that have not been previously subjected to the bacteriophages being used for treatment. Also important for this system is keeping exposure of the bacteria to bacteriophages to a minimal amount of time. If the bacteriophages interact with the bacteria for long periods of time, the bacteria will become resistant as repeatedly demonstrated in the above discussion. Food and meat processing facilities are an excellent example. As live animals enter a slaughter/processing facility, the bacteria have not likely been exposed to the bacteriophages used to treat the infection, thus greatly increases the chances of success. Similar potential exists for hatchery applications, and applications to food products, as discussed below.

Higgins and co-workers (2005) successfully treated turkey carcasses at a processing facility with bacteriophages specific to the *Salmonella* to which they were infected. This process was effective when either an autogenous bacteriophage treatment targeted to the specific *Salmonella* strain infecting the turkeys was used, or a cocktail of nine wide host-range *Salmonella*-targeting bacteriophage were used. Similarly, a bacteriophage treatment for cattle carcass contamination has been effective at reducing the *E. coli* 0157:H7 load at processing has been developed and commercially licensed in the United States. These successes avoid development of bacteriophage resistance by applying treatment at a single point during production, in an environment where proliferation of the target organism is extremely limited. In this way, since the target organism is never intentionally exposed twice to the same treatment, resistance is unlikely to ever increase beyond the naturally-occurring resistance to the bacteriophage (or cocktail) used.

One of the most well documented successes of published treatment of enteric Enterobacteriaceae infections with bacteriophages was the study of Smith and Huggins (1983) as described above. It is notable that in this successful study, the bacteriophage cocktail used was a combination of two bacteriophages, but the second was isolated using the target organism which was resistant to the first bacteriophage. This approach of selecting for bacteriophage isolates using target bacteria that are resistant to sequential bacteriophage treatments was not used in the work of Higgins et al. (2007), or in several other published studies. Higgins and co-workers (2007) used a collection of bacteriophages, independently isolated from different sources and with several different plaque morphologies, suggesting that a number of different bacteriophages were employed – and failed to persistently reduce enteric colonization. Similarly, application of a bacteriophage combination failed to reduce *S.* Enteritidis PT4 infections in broilers (Fiorentin et al., 2005). However, some cocktails have been successful. A combination of three bacteriophages isolated from feces of patients infected with *E. coli* O157:H7 were applied to contaminated processed beef for reduction of the pathogen (O'Flynn et al., 2004). In 2006, Sheng et al. reported that a combination of two bacteriophages worked better at reducing *E. coli* O157:H7 in ruminants than each bacteriophage administered solely. Perhaps, with a defined method to select for bacteriophages that have become resistant to bacteriophages, a combination can overcome the resistance issue. However, the resistance acquired by the pathogen would have to be predictable and consistent. Smith and Huggins' (1983) method to first apply a bacteriophage specific for an antigen on the cell surface was successful, and may prove to be a procedure that could consistently overcome the issue of resistance in bacteriophage therapy. However, the ability to simultaneously target a broad range of wild-type isolates under field conditions has not been explored.

It is possible that one of the most notable exceptions to the many failures to treat enteric Enterobacteraceae infections during recent years, that of Smith and Huggins (1983), provides a singular clue as to the potential for enhancing the likelihood of enteric Enterobacteriaceae efficacy. It is possible that selection of multiple bacteriophages for the same target cell phenotype results in selection of bacteriophages that are effective through identical mechanisms of adhesion, penetration, replication, and release. When new bacteriophages are isolated for efficacy against sequentially resistant isolates of the target bacteria, and these are combined for administration as a cocktail, the ability of the target cell to shift phenotype may be severely limited, resulting in a much larger proportion of target cell reduction, thereby increasing the probability of elimination or cure. Multiple researchers have noticed a change in colony morphologies of *E. coli* that had become resistant to bacteriophages selected to adhere to key-components of pathogenicity of the organism, such as lipopolysaccharides (O'Flynn et al., 2004; Sheng et al., 2006). This change in morphology may relate to a decreased ability to cause infection because the bacteria may be inhibited as a result of the bacteriophage resistance.

Another consideration for bacteriophage treatment could be the application of bacteriophages to foods post-processing. Multiple researchers have noticed a successful reduction of foodborne pathogens on meats, and fruits (Bielke et al., 2007c; Higgins et al., 2005; Leverentz et al., 2001, 2003; O'Flynn et al., 2004). Treating processed poultry carcasses with different bacteriophages was able to eliminate *S.* Enteritidis (Bielke et al., 2007c) or field isolates of *Salmonella* to below detection limits (Higgins et al., 2005). A mixture of three different bacteriophages reduced the levels of *E. coli* O157:H7 detected on processed beef, though the bacteriophage treatment was not as effective at temperatures below 30 °C, making them ineffective at refrigeration temperatures (O'Flynn et al., 2004). Leverentz et al. (2001, 2003) successfully reduced the levels of *Salmonella* and *Listeria monocytogenes* on selected fruits with bacteriophage application. A loss of effectiveness was seen on cut fruits, possibly due to low pH of the fruit flesh. While this research appears promising, the studies do not report the long-term susceptibility of the contaminating bacteria to the bacteriophages. Perhaps, selection of a cocktail of bacteriophages to combat resistance, and for the ability to lyse bacteria at refrigeration temperatures could result in successful reduction of these foodborne pathogens.

5. Conclusions

While bacteriophage treatment of enteric infections has had some success, failures do occur and the system has not yet been perfected. Chemical antibiotics are often effective against multiple species of bacteria and do not require specific selection to treat infections. Unlike bacteriophages, which tend to be at least somewhat host specific and may not even kill bacterial isolates within the same species. Still, with the rise of antibiotic resistance, bacteriophages may be able to offer a line of defense in situations for which antibiotics are not available, or are not effective. For example, with the restriction or elimination of antibiotics usage in food animals, researchers have been investigating the possibility of bacteriophages to control foodborne pathogens. With the realization that resistance of pathogenic isolates of bacteria to bacteriophages can, and do, emerge, perhaps the best application would be to apply the bacteriophages immediately prior to slaughter. Thus, the

pathogen could be effectively reduced in the gastrointestinal tract, and subsequently reduce the risk of contamination during processing. With the frequency of reported failures, and the assumption that many are not reported, bacteriophage therapeutics has not been perfected well enough for widespread application. In addition to resistance, safety, specificity, and long-term effectiveness must be demonstrated, and although several products have been licensed in the United States and elsewhere, procedures for demonstration of these characteristics are not well established, providing an additional regulatory burden for commercialization.

Clearly, widespread bacteriophage treatments with Enterobacteriaceae within the gastrointestinal tract have not been adopted for any animal species during the last 60 years and successful research in this area has been modest and sporadic. Nevertheless, the occasional reports by reputable scientists in solid journals must indicate that there is potential for improved therapeutic efficacy of bacteriophages for this purpose. With the diminution of new antimicrobial pharmaceuticals and the widespread resistance among many pathogenic enteric Enterobacteriaceaes, a breakthrough in this area is sorely needed.

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Bacteriophages have received attention as biological control agents since their discovery and recently their value as tools has been further emphasized in many different fields of microbiology. Particularly, in drug design and development programs, phage and prophage genomics provide the field with new insights. Bacteriophages reveals information on the organisms ranging from their biology to their applications in agriculture and medicine. Contributors address a variety of topics capturing information on advancing technologies in the field. The book starts with the biology and classification of bacteriophages with subsequent chapters addressing phage infections in industrial processes and their use as therapeutic or biocontrol agents. Microbiologists, biotechnologists, agricultural, biomedical and sanitary engineers will find Bacteriophages invaluable as a solid resource and reference book.

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