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Bacteriophages for prophylaxis and therapy in cattle, poultry and pigs

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Abstract

The successful use of virulent (lytic) bacteriophages (phages) in preventing and treating neonatal enterotoxigenic *Escherichia coli* infections in calves, lambs and pigs has prompted investigation of other applications of phage therapy in food animals. While results have been very variable, some indicate that phage therapy is potentially useful in virulent *Salmonella* and *E. coli* infections in chickens, calves and pigs, and in control of the food-borne pathogens *Salmonella* and *Campylobacter jejuni* in chickens and *E. coli* O157:H7 in cattle. However, more rigorous and comprehensive research is required to determine the true potential of phage therapy. Particular challenges include the selection and characterization of phages, practical modes of administration, and development of formulations that maintain the viability of phages for administration. Also, meaningful evaluation of phage therapy will require animal studies that closely represent the intended use, and will include thorough investigation of the emergence and characteristics of phage resistant bacteria. As well, effective use will require understanding the ecology and dynamics of the endemic and therapeutic phages and their interactions with target bacteria in the farm environment. In the event that the potential of phage therapy is realized, adoption will depend on its efficacy and complementarity relative to other interventions. Another potential challenge will be regulatory approval.

Keywords: *Escherichia coli*, *Salmonella*, *Campylobacter*, *Staphylococcus*, O157:H7, bacteriophage, bacteriophage therapy

Introduction

Increasing concerns about antimicrobial resistance in animal and human pathogens and expanding knowledge of the mechanisms and epidemiology of transmission of antibiotic resistance have driven the recent search for novel alternatives to antimicrobial drugs in humans and animals. The need for alternatives for animal use has been further accentuated by regulatory actions such as the recent ban on the use of sub-therapeutic levels of antibiotics in animal production in the European Union.

Although bacteriophage (phage) therapy is one such alternative, it is not novel. Soon after their discovery by Twort (1915) and d'Herelle (1917) phages were used to control avian typhoid caused by *Salmonella* Gallinarum. Human applications soon followed, and by 1930–1940 phages were commonly used therapeutics, particularly in Georgia, Russia and Poland, and also in the USA (Summers, 2005). Interest in phage therapy declined in the west following the introduction of antibiotics, but has increased dramatically, particularly with recent research indicating that phage therapy can be effective in treating

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serious infections caused by antibiotic resistant pathogens such as vancomycin-resistant *Enterococci* (Biswas *et al.*, 2002) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Matsuzaki *et al.*, 2003). Details of these recent, as well as early applications of phage therapy in humans, animals and animal models can be found in numerous reviews (see for example, Weber-Drabowska *et al.*, 2000; Sulakvelidze *et al.*, 2001; Barrow, 2001; Summers, 2005; Sulakvelidze and Barrow, 2005; Kropinski, 2006; Parisien *et al.*, 2008).

The following review is focused on recent research in applications of phages for treatment and prevention of bacterial infections of cattle, swine and poultry. These have evolved largely from the excellent studies by Williams Smith and colleagues on *Escherichia coli* infections in mice, calves, pigs and lambs. Although these researchers explored phage therapy for pathogens of food animals, many recent veterinary applications have addressed zoonotic pathogens such as *Salmonella* spp., *Campylobacter jejuni* and *E. coli* O157:H7 that have food animals as natural reservoirs. Information on other phage-related therapies such as use of phage-encoded enzymes and bacterial vaccines generated by phage infection or by phage-encoded enzymes can be found in recent comprehensive reviews (Fischetti, 2005; Sulakvelidze and Barrow, 2005; Kropinski, 2006; Parisien *et al.*, 2008).

Pertinent aspects of phage biology

Phages are highly plentiful in nature and share a common ecology with their bacterial hosts (Brüssow and Kutter, 2005). Consequently they are found in the alimentary tracts of animals and humans, and in foods, soil, water, sewage and associated environmental niches. Because of their ubiquitous presence, they are generally considered safe, an assumption that is strongly supported by the early widespread use of phages in humans, as well as by recent experimental studies in animals and humans.

Phages can be grouped as having either virulent (lytic) or temperate life cycles. Those for therapeutic use are lytic, exhibiting a self-replicating virulent infectious cycle that results in rapid degradation of host cell DNA, phage replication, and lysis with release of tens to hundreds of progeny phages (Guttman *et al.*, 2005). In contrast, the life cycle of temperate phages involves integration of the phage genome into the host cell DNA as a prophage, where it replicates along with the host cell DNA without lysing the bacterial host. The prophage can, however, revert to the lytic cycle with release of progeny phages. Clearly, temperate phages do not offer the therapeutic potential as lytic phages. In fact, activated temperate phages may be undesirable because they can transfer virulence and other undesirable genes from one bacterium to another by transduction.

The therapeutically desirable lytic, self-replicating infectious cycle of virulent phages is derived largely from *in vitro* studies with favorable ratios of phages to the target host bacteria [multiplicity of infection (MOI)]. At an ideal MOI, phages amplify themselves and kill the target hosts by repeated cycles of replication until the host is eliminated. Under other circumstances, the phage–host interaction may have different outcomes and be more self-limiting. At very high MOIs, the host may be killed by 'lysis from without', in which attachment of many phages to a single bacterial cell results in lysis without phage replication. In addition, extensive lysis may reduce the numbers of susceptible hosts to below the 'phage proliferation threshold' (Payne and Jansen, 2003), where the numbers of target bacteria are insufficient to sustain phage replication. In other situations, phages and susceptible bacteria may co-exist in an oscillating equilibrium of a predator–prey relationship. Co-existence with relatively stable numbers of hosts and phages may reflect the emergence of phage resistant sub-populations (Mizoguchi *et al.*, 2003) or that phages and bacteria occupy different niches such as the lumen, mucous layer or epithelial surface of the gut (Connerton *et al.*, 2004; Chibani-Chennoufi *et al.*, 2004).

Another important characteristic that influences the therapeutic potential of phages is their specificity, which determines their host ranges among targeted and non-targeted bacteria. Most phages offer much greater specificity than antimicrobial drugs, targeting only specific subtypes within a species, serovar or serogroup. Others may have a broad host range, such as the *Salmonella* phage, Felix O1, which infects most *Salmonella* serovars (Kallings, 1967). Phage specificity is determined largely by the interaction between binding sites on their tail fibers and one or more receptors on the cell surface of the host bacterium, which may include lipopolysaccharides, proteins, capsular polysaccharides, flagella and pili. While a narrow spectrum of activity is desirable to avoid unwanted effects on commensal flora, the ideal therapeutic phage would infect multiple pathogens carrying common surface receptors. A recent report of two phages that lyse multiple serotypes of two different pathogenic groups of *E. coli*, but few non-pathogenic *E. coli* (Viscardi *et al.*, 2008) indicates that such phages occur in nature.

Phage therapy in cattle

Neonatal E. coli *infections*

In their initial studies in mice, Smith and Huggins (1982) showed that a single dose of phage R was at least as effective as one or more doses of antibiotics in preventing death due to *E. coli* septicemia. Soon after, they explored the use of phages for control of enterotoxigenic *E. coli* (ETEC) infections in neonatal calves, pigs and lambs (Smith and Huggins, 1983; Smith *et al.*, 1987a). These studies have been reviewed in detail (Barrow, 2001;

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Sulakvelidze and Barrow, 2005) and key outcomes of their work with ruminants are summarized in Table 1. Briefly, they showed that the severity and mortality of neonatal ETEC diarrheal disease could be prevented by selected phages given orally, sprayed on pen litter or transmitted from pens occupied previously by phage-treated animals. They also found that naturally emerging phage-resistant strains lacking the virulence-associated K antigen were less virulent than the parent strains, but resistant K-positive strains retained their virulence. Factors such as pH, colostral phage antibodies and body temperature on phage viability and efficacy were also investigated (Smith *et al.*, 1987b). The sheer quantity and quality of the work in these reports demands that interested readers consult the original papers.

Furthering the work of Smith and colleagues, Barrow *et al.* (1998) examined the effect of phage R in experimental *E. coli* septicemia in colostrum-deprived calves. Whereas two calves inoculated orally with virulent $K1^+$ *E. coli* H247 (O18:K1:H7) rapidly became moribund and required euthanasia 36 h after infection, three of four calves given 10^{10} PFU of phage R intramuscularly 8 h after the same challenge remained healthy. Phage treatment did not alter numbers of strain H247 in the feces or blood, but delayed its entry into the blood for 1 day as titers of phage R in feces and blood increased to high titers 16–24 h after treatment. One day after challenge, phage resistant $K1^+$ isolates of H247 were identified in the feces of the one treated calf that became ill, and a day later predominated in the blood of this calf. However, the illness in this calf was considered to be due to absorption of toxins from the gut rather than septicemic disease caused by the resistant mutants. Phage treatment 8 h after infection thus appeared to control disease severity but not prevent infection, as also seen in other studies (Smith and Huggins, 1983; Smith *et al.*, 1987a).

E. coli *O157:H7 infection*

As the predominant serotype of Shiga toxin-producing *E. coli* associated with severe disease in humans, *E. coli* O157:H7 is a major public health concern (Rangel *et al.*, 2005). Healthy cattle and other ruminants are important natural reservoirs of this organism, shedding it in their feces intermittently or for short periods (Besser *et al.*, 1997). Transmission to humans occurs most frequently through meats, milk, water, fresh produce, animals and environmental sources contaminated directly with manure of infected cattle. Hence, there is considerable interest in on-farm interventions to control this organism in cattle, including phage therapy. Several studies of *E. coli* O157:H7 phage therapy intended for cattle have been conducted in sheep, which serve as a convenient model.

Evaluation of three lytic *E. coli* O157:H7-specific phages, KH1, KH4 and KH5, *in vitro* revealed that complete lysis occurred within 8 h at 37°C and 4°C, but only with a mixture of the three phages at high MOI ($\sim 10^3$) and incubation with aeration (Kudva *et al.*, 1999). In a subsequent 21-day trial, phage KH1 was ineffective in sheep when given orally at a dose of 1.3×10^{11} PFU orally 1, 9, 10 and 11 days after infection with *E. coli* O157:H7, despite the presence of the phage in feces at 10^5–10^6 PFU g^{-1} during this time (Sheng *et al.*, 2006). Similarly, a high dose (10^{13} PFU) of phage DC22, which eliminated *E. coli* O157:H7 from a model rumen fermenter in 4 h, had no significant effect on shedding of *E. coli* O157:H7 by experimentally infected lambs over 27 days (Bach *et al.*, 2008). Fecal phage counts declined from 10^6 PFU g^{-1} on day 3 to undetectable levels on day 13.

Sheng *et al.* (2006) investigated rectal treatment of cattle infected by rectal inoculation because of reports that the recto-anal junction is the primary site of colonization of cattle by *E. coli* O157:H7 (Naylor *et al.*, 2003). On day 0, 7 days after recto-anal inoculation of 10 steers with a four-strain mixture of *E. coli* O157:H7, five steers were given phages SH1 and KH1 (25 ml of 10^{10} PFU ml^{-1}) by rectal infusion and swabbing, and in drinking water at 1.8 to 5.4×10^6 PFU ml^{-1} for 4 days. Counts of phages and *E. coli* O157:H7 were monitored for 16 days in recto-anal mucosal swab samples (RAMS). On day 0, counts of *E. coli* O157:H7 were 10^4–10^5 CFU $RAMS^{-1}$, giving an initial MOI of $\sim10^5$ to 10^6. Subsequent average *E. coli* O157:H7 counts were significantly lower in treated than control calves on days 1–10, by ~1.5 log units $RAMS^{-1}$. Phage counts peaked at $\sim10^6$ PFU $RAMS^{-1}$ on day 3, and then declined steadily to $\sim10^2$ PFU $RAMS^{-1}$ on day 16. Despite persistence of the phages and the initial reductions in *E. coli* O157:H7 counts, four of the five treated calves remained infected. This result differed considerably from that of a mouse experiment included in the same report, in which three oral treatments with SH1 alone or mixed with KH1 eliminated *E. coli* O157:H7 from feces within 2–6 days. The authors concluded that the mixture of phages KH1 and SH1 can reduce but not eliminate shedding of *E. coli* O157:H7 by cattle. Although the recto-anal junction is a preferred site of *E. coli* O157:H7 colonization, rectal phage administration is impractical in cattle, and the phages are likely to be excreted promptly. Also, this route would not be effective initially on the target organism at other sites, although both *E. coli* O157:H7 and the phages would likely be transmitted at lower numbers via the fecal–oral route.

Two brief trials lasting 2–4 days indicated that numbers of *E. coli* O157:H7 in feces and/or intestinal contents of sheep or cattle were reduced significantly soon after similar high doses of different phages. In four sheep treated once orally with 10^{11} PFU of phage CEV1 3 days after challenge, *E. coli* O157:H7 populations 2 days later were reduced significantly by 2–3 log units in cecal and rectal but not rumen contents (Raya *et al.*, 2006). In the second short study (Callaway *et al.*, 2008), a mixture of

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Table 1. Summary of studies by H. W. Smith and colleagues on phage therapy of neonatal enterotoxigenic *E. coli* (ETEC) infections in calves and lambs

Animals	Objective	Challenge	Applications of phages	Observations	Reference
Calves, <24 h old	Reduce morbidity, disease severity and mortality.	ETEC O9:K30.99; 3×10^9 CFU, orally.	1. Mix of phages B44/1, B44/2, 10^{11} PFU, once orally 1 or 8 h after infection, before onset of diarrhea. 2. Mix of phages B44/1, B44/2, 10^{11} PFU, once orally after onset of diarrhea. 3. Mix of phages B44/1, B44/3, 10^{11} PFU, once orally after onset of diarrhea.	1. No diarrhea in treated calves; greatly reduced mortality and *E. coli* numbers. Appearance of $K30^-$ mutants. 2. Diarrhea in all calves, 67% mortality. High numbers of B44/1-resistant mutants in calves that died. 3. Diarrhea in all calves, mortality reduced to 15% mortality due to action of B44/3 against B44/1-resistant mutants.	Smith and Huggins (1983)
Lambs, <24 h old	Reduce morbidity, disease severity and mortality.	ETEC O8:K85.99; S13, 3×10^8 CFU orally.	Phage S13, 10^9 or 10^{10} PFU, once orally 8 h after infection.	Incidence of diarrhea reduced by 50%, mortality from 25% to 0%. Greatly reduced *E. coli* numbers in feces and the intestinal tract.	Smith and Huggins (1983)
Calves, <24 h old	Reduce or prevent morbidity, mortality severity of diarrhea and septicemia.	Several trials with individual and multiple ETEC strains, various doses, orally.	1. Individual and multiple strain-specific phages, each at 10^5 PFU, once orally, before or at time of challenge or at onset of diarrhea. 2. Different oral doses of phages at different times in relation to challenge. 3. Phages given in milk (10^5 PFU l^{-1}) at different times in relation to challenge. 4. Phages remaining in an uncleaned room after housing phage treated infected calves. 5. Phages sprayed on litter in room at $\sim10^5$–10^9 PFU m^{-2}. 6. Investigation of phage resistant mutants.	1. Individual or multiple phages highly effective in reducing diarrhea and mortality due to individual strains. A mix of six phages effective against infection with multiple strains, but less so than against individual strains. 2. Disease greatly reduced with doses of 10^2 or 10^5 phages given 6 h or 10 min before infection and up to 10–12 h after infection. 3. Phages in milk very effective when given 4 h before, or up to 10–12 h after infection. 4. Phage contamination of room prevented diarrhea in calves infected 3 h after placement in rooms. 5. Spraying phages on litter prevented disease in calves infected 6 h or 0 h before, and 3 h after placement on the litter. 6. K^- phage resistant mutant *E. coli* less virulent than parent K^+ strains. Emergent K^+ phage resistant mutants as virulent as the parent strain and treatable with readily generated mutant phages.	Smith *et al.* (1987a)
Calves, 5–7 days old	Investigate factors affecting oral phage therapy of calves.	Various ETEC strains.	Several phages tested *in vitro* or *in vivo* with respect to effects of: 1. Stomach pH on phage viability. 2. Antibodies to phages in colostrum on phage activity. 3. Optimal temperature for virulence.	1. Phage inactivated at normal abomasal pH of 3 or less. Feeding milk or milk containing calcium carbonate elevated abomasal pH and prevented phage inactivation for 2.5 or 5 h, respectively. 2. Phage antibodies in colostrum were inactivated at normal abomasal pH but active against phages in the intestines when the abomasal pH was increased by feeding milk or colostrum containing calcium carbonate. 3. The body temperature of newborn calves, being slightly higher than the optimal *in vitro* temperature for virulence of most tested phages, may influence phage virulence *in vivo*.	Smith *et al.* (1987b)

eight phages (total >10^{12} PFU) was given by oral gavage to 10 sheep on days 2 and 3 after challenge with *E. coli* O157:H7. Compared to those in controls, fecal *E. coli* O157:H7 counts were reduced significantly by 1.5–2 log units g^{-1} 24 h after the first dose, but not at 12 and 24 h after the second dose, suggesting the initial effect was transient. Most sheep continued shedding an average of ~10^4 CFU g^{-1} of feces 24 h after two treatments. At autopsy on day 4, reductions in numbers of *E. coli* O157:H7 were significant in cecal but not rectal or ruminal contents. However, counts in feces collected immediately before euthanasia were ~100 times higher than in rectal contents, suggesting that phage treatment may not have been effective at colonization sites at the recto-anal junction. Also of note was the finding from a second experiment that a MOI of 1 was more effective than MOIs of 0.1, 10 and 100 in reducing counts of the challenge strain in ruminal, cecal and rectal contents.

Both of these brief trials suggest that phage therapy shortly before slaughter may reduce the risk of carcass contamination by lowering the fecal load of *E. coli* O157:H7. However, such an approach does not address environmental dissemination of the organism during the rearing and growing phases of production, or perhaps contamination of hides, which contributes substantially to carcass contamination.

While none of the above studies resulted in elimination of *E. coli* O157:H7 from cattle or sheep, some of us (Waddell *et al.*, 2000) have had more encouraging results in experimentally infected calves. Weaned 7–8-week-old calves infected orally on day 0 with 3×10^9 CFU of *E. coli* O157:H7 were treated or not with a total of 10^{11} PFU of a mixture of six phages on days −7, −6, −1, 0 and 1. The phages were given in calf milk replacer containing calcium carbonate to buffer stomach acid, and the dose on day 0 was given 4 h before the bacterial inoculum. While most untreated calves shed *E. coli* O157:H7 in their feces for at least 12–16 days, the treated calves stopped shedding this organism abruptly after day 8, when the concentrations of phages in their feces had increased sharply to 10^9–10^{11} PFU g^{-1}. Such dramatic increases in phage numbers did not occur in uninfected control calves given only the phages, indicating extensive replication of the phages in the treated animals. No adverse effects of phage therapy were observed clinically or on monitoring of total fecal coliform and *E. coli* counts, and no phage-resistant *E. coli* O157:H7 were isolated over the 14–16 days of the trial.

The very encouraging results with calves suggest that phage therapy can effectively eliminate *E. coli* O157:H7 under some circumstances. Treatment may have application early in the cattle production cycle, since initial infection of beef cattle often occurs in calves less than 12 weeks of age (Gannon *et al.*, 2002). Additional treatments at periods of peak transmission later in the production cycle may help reduce the overall prevalence and environmental dissemination. However, treatment of young adult beef cattle with a subset of four of these phages reduced but did not eliminate shedding of *E. coli* O157:H7 from experimentally infected cattle (Niu *et al.*, 2008). Possibly, age-related differences in development of the gastro-intestinal tract may in part explain the differences in efficacy in calves and adult cattle. Although the phage dose for the adult cattle was increased at least 10-fold, this would hardly account for dilution in the substantially larger volume of the contents of the adult rumen and intestinal tract. Also, since adult cattle are likely to have experienced greater exposure to *E. coli* O157:H7 infection (Laegreid *et al.*, 1999; Gannon *et al.*, 2002) they are more likely than calves to have levels of immunity that may limit colonization and replication of *E. coli* O157:H7 to levels below those required to support phage amplification in the gut.

Bovine mastitis caused by S. aureus

Clinical and sub-clinical mastitis remains a major milk production-associated disease among dairy cattle worldwide. Treatment most often involves repeated intramammary infusions of antibiotics into affected quarters via the teat canal. *S. aureus* is a common cause of mastitis in cattle, and poses particular challenges because of relatively low cure rates, antimicrobial resistance and sub-clinical persistence in herds (Makovec and Ruegg, 2003; Luby and Middleton, 2005). Concern also exists because of transmission of antibiotic resistant *S. aureus*, including MRSA from animals to humans (Lee, 2003). Although phage therapy was effective against lethal MRSA infection in mice (Matsuzaki *et al.*, 2003), a recent study of phage therapy of *S. aureus* mastitis in cattle was less encouraging (Gill *et al.*, 2006a). Five daily intramammary infusions with 10^{11} PFU of *S. aureus* phage K in udder quarters with sub-clinical *S. aureus* mastitis resulted in cure of only 3 of 18 quarters, a non-significant effect compared with saline-treated controls (cure rate 0/20 quarters). *S. aureus* counts in milk fluctuated after treatment, as did the counts in saline treated animals, and recovered *S. aureus* isolates were susceptible to lysis by phage K. Possible reasons for the lack of efficacy included enzymatic inactivation of phages in the mammary gland, inhibition of the binding of active phages to the target bacteria by milk proteins, and phage aggregation by milk proteins (Gill *et al.*, 2006a, b). Given these limitations, it would appear that phage therapy of bovine mastitis has limited potential.

Phage therapy in chickens

E. coli *infections*

Colibacillosis is a serious problem in poultry production causing mortality and carcass condemnations (Barnes

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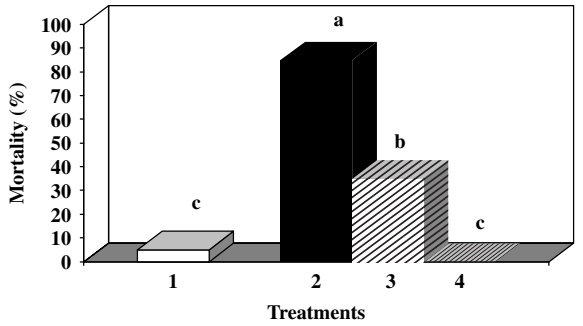

Fig. 1. Effect of mixing *E. coli* with bacteriophages prior to challenge Treatments: 1, Control □; 2, *E. coli*, 10^4 CFU ■; 3, *E. coli*, 10^4 CFU, mixed with 10^4 PFU of bacteriophages ▨; 4, *E. coli*, 10^4 CFU, mixed with 10^8 PFU of bacteriophages ▨. Values represent the means of two replicate pens of 10 birds per pen. Values with different letters differ significantly ($P \leq 0.05$).

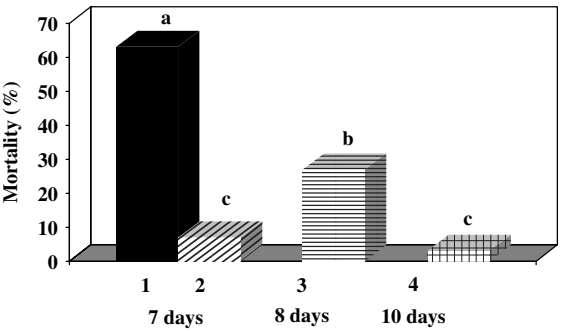

Fig. 2. Efficacy of a bacteriophage aerosol spray to prevent colibacillosis. Treatment 1 ■, birds challenged with *E. coli* only. Treatments 2 ▨, 3 ▤, and 4 ⊞, birds sprayed with bacteriophages at 7 days of age prior to challenging with *E. coli* at 7, 8, or 10 days of age, respectively. Values represent the mean of four replicate pens of 10 birds per pen. Values with different letters differ significantly ($P \leq 0.05$).

et al., 2003). Initial infection is thought to occur in the respiratory tract as airsaculitis that quickly becomes septicemic, resulting in considerable mortality. In exploring applications of phage therapy, Barrow *et al.* (1998) found that phage R, active against $K1^+$ *E. coli* (Smith and Huggins, 1982) was highly effective in both prevention and treatment of experimental *E. coli* septicemia and meningitis in chickens. Intramuscular (i.m.) inoculation of 10^6 PFU of phage R fully protected newly hatched and 3-week-old chickens against death due to septicemia following simultaneous i.m. inoculation of 10^6 CFU of *E. coli* O18:K1:H7 strain H247. Also, a higher dose (10^8 PFU) prevented death due to meningitis following intracranial inoculation of 10^3 CFU of the same bacterium. Titers of *E. coli* H247 in the blood, spleen and brain were much lower in treated than in untreated birds, and marked increases in titers of phages in samples from treated birds indicated dissemination and replication of the phages after administration. Similar phage treatments given 1–2 days before i.m. challenge were highly effective prophylactically, and if given therapeutically at the onset of clinical signs, reduced mortality by 70%.

E. coli of serogroup O2 are a frequent cause of airsaculitis and septicemia, prompting some of us, William Huff and colleagues, to investigate phage therapy to prevent and treat infections caused by this serogroup. Phages virulent for a pathogenic *E. coli* O2:NM strain were evaluated when given by various routes at different times in relation to challenge. In all experiments, the challenge was inoculation of $\sim 10^4$ CFU of an *E. coli* O2:NM strain into one thoracic airsac (Piercy and West, 1976). In initial experiments, different numbers of the selected phage were mixed with *E. coli* prior to inoculation. The mortality in birds challenged only with *E. coli* was 85% (Fig. 1), whereas a mixture containing 10^4 PFU significantly reduced mortality to 35%, and a mixture containing 10^8 PFU completely protected the birds (Huff *et al.*, 2002a). Although this experimental design was artificial, it suggested that phages might be used to prevent colibacillosis and it demonstrated the importance of phage dose for therapeutic efficacy. In addition, this model provides a relatively simple method to screen phages for efficacy *in vivo*.

Because colibacillosis frequently begins as a respiratory infection, 7-day-old chicks were treated with an aerosol spray of phages as a preventative measure immediately, or 1 or 3 days before challenge. Mortality was significantly lower in all groups of birds treated prior to challenge than in challenged but untreated birds (Fig. 2), suggesting that an aerosol administration of phages could provide protection for at least 3 days (Huff *et al.*, 2002b).

To evaluate phages for treatment rather than prevention, they were given by aerosol spray or i.m. inoculation at intervals following challenge of 7-day-old birds (Huff *et al.*, 2003a). The aerosol spray was not effective, but i.m. administration of phages given immediately, 24 h or 48 h after challenge significantly reduced mortality compared with that in untreated, challenged birds (Fig. 3). Additional research showed that multiple i.m. doses of phages enhanced phage treatment efficacy (Huff *et al.*, 2003b). Blood levels of phages were determined after aerosol or i.m. administration to unchallenged birds, in order to determine if sufficient numbers would reach the blood to combat the septicemic phase of colibacillosis. Few birds treated by aerosol administration had detectable levels of phages in the blood at intervals up to 24 h after treatment. However, after i.m. inoculation, titers of phages in blood remained above 10^4 PFU ml^{-1} in all five birds for up to 6 h after administration, and at 24 h, were $\sim 10^2$ PFU ml^{-1} in four of the five birds (Huff *et al.*, 2003a). Intramuscular inoculation of phages is therefore likely to be far more effective than aerosol administration in treatment of the septicemic phase of colibacillosis. Another study (Huff *et al.*, 2004) demonstrated that a combination of i.m. inoculation of phages and a low

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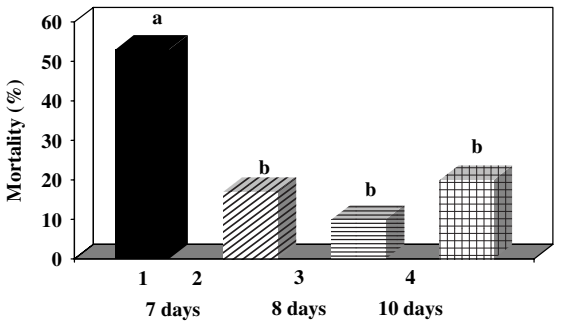

Fig. 3. Efficacy of intramuscular administration of bacteriophage to treat colibacillosis. Treatment 1 ■, birds challenged with *E. coli* and not treated with bacteriophage. Treatments 2 ▨, 3 ▤, and 4 ⊞, birds challenged with *E. coli* and treated with bacteriophage immediately (7 days of age), 24 h (8 days of age), or 48 h (9 days of age) after *E. coli* challenge, respectively. Values represent the mean of four replicate pens of 10 birds per pen. Values with different letters differ significantly ($P \leq 0.05$).

dose of enrofloxacin given in drinking water potentially enhanced the efficacy of treatment of colibacillosis.

Together, these studies of phage therapy of respiratory and septicemic O2 *E. coli* infections of chickens indicate that prevention and treatment by phage therapy may be feasible, although with potential limitations similar to those noted by Barrow *et al.* (1998). To be effective preventatively, high numbers of phages are needed at the sites of infection at the time of exposure or systemic infection. High numbers are also likely to be required in the blood within a day or so of infection if phages are to be effective as treatments. In poultry farms, aerosol administration might provide protective levels in the respiratory tract but not the levels required for treatment. Since intramuscular administration is not practical or economically feasible in large modern poultry farms, a focus on preventative use would have the greatest potential.

Salmonella *infections*

Poultry products are a major source of human salmonellosis, due to widespread sub-clinical carriage of *Salmonella* by poultry, resulting in high rates of carcass contamination. In addition, certain serovars such as *S.* Typhimurium and *S.* Enteritidis can be highly virulent in young chickens and older birds. The emergence of multi-drug resistant subtypes of several *Salmonella* serovars affecting poultry, other food animals and humans has intensified the search for alternative non-antibiotic control strategies for these important enteric pathogens.

In a brief early report (Taylor and Silliker, 1958), treatments of hatching eggs infected with *S.* Chittagong, *S.* Pullorum or *S.* Typhimurium with a broad-range *Salmonella* phage and/or serovar-specific phages improved the percent hatch of fertile eggs from <45% to >70%. However, despite this apparent success, this technology does not appear to have been explored further, and more recent studies have focused on post-hatch applications of phage therapy.

Berchieri *et al.* (1991) found that 10^4 PFU of phage AB2 given orally to newly hatched chicks immediately after challenge with *S.* Typhimurium F98 and in feed containing 10^3 PFU g^{-1} for 7 days was ineffective in reducing cecal content levels of the challenge strain. The phage did however replicate to high titers at 4 days post-infection (p.i.), but was undetectable after the levels of F98 fell below 10^6 CFU ml^{-1} of cecal contents. Resistance to phage AB2 was frequent, occurring in 34–82% of 50 isolates of F98 on each of p.i. days 2, 4, 7 and 10. The rough O antigen phenotype of all resistant isolates may have contributed to the persistence of high numbers of *S.* Typhimurium, although this was not considered to be the case. No phage AB2 neutralizing antibodies were detected in sera collected from these chickens at 32 days p.i. In contrast, a different phage, 2.2, significantly reduced the overall mortality rate over 21 days from 56% to 20%, and was also effective against two other strains. However, this result was only achieved with a dose of 10^{11} PFU. Reductions of up to >2 log units in counts of the challenge strain in the crop and small intestine occurred during the first 3–6 h, but were transient, and counts in the liver were almost 1 log unit lower than in the controls after 24 and 48 h.

The above study illustrates several of the issues in phage therapy. Both phages were virulent *in vitro*, but AB2 was not effective *in vivo*, despite evidence of replication in the gut. Phage 2.2 was effective *in vivo*, but only at a high dose. The need for high doses and the early and transient nature of the decreases in target organism perhaps suggest lysis from without or only one cycle of replication. Treatment also appeared to limit invasion and colonization of the liver. However, *S.* Typhimurium was not eliminated and its persistence at increasing titers up to 24 h after treatment may reflect that the *Salmonella* had escaped from phage predation by establishing intracellular infection.

A recent study evaluated three broad host range phages against *S.* Enteritidis, *S.* Typhimurium and *S.* Hadar as a pre-harvest treatment in 36-day-old broiler chickens (Atterbury *et al.*, 2007). Two days after oral challenge of groups with *S.* Enteritidis, *S.* Typhimurium or *S.* Hadar, they were treated orally with 10^9 PFU (trial 1) or 10^{11} PFU (trial 2) of corresponding phages 151, 10 or 25, respectively, in an antacid buffer. Counts of *S.* Enteritidis and *S.* Typhimurium in cecal contents over 6 days were reduced significantly by >4 and >2 log units, respectively, but only at the higher dose (10^{11} PFU). These substantial reductions could potentially reduce the risk of product contamination at slaughter. Phage 25 had no effect on counts of *S.* Hadar, despite its strong lytic activity against this strain *in vitro*. The frequencies of phage resistant colonies isolated from treated birds in trial 1 (*S.* Enteritidis, 21%; *S.* Typhimurium, 10%; *S.* Hadar, 23%) were about

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twice those of isolates from untreated infected birds, and except for *S.* Hadar, were even greater after treatment with the higher phage dose. Tested resistant isolates did not retain their resistant phenotype when sub-cultured five times, and colonized the ceca of chickens as effectively as the original strains. Overall, these findings indicated that a high phage dose was potentially effective as a pre-harvest intervention to reduce the levels of *S.* Enteritidis and *S.* Typhimurium in cecal contents of broiler chickens shortly before the age of slaughter. Although the high phage dose generated higher proportions of phage resistant strains, resistance did not persist for long in the absence of phage *in vitro* or *in vivo*, and perhaps could be circumvented by treatments with a mixture of phages.

The substantial reductions in *Salmonella* reported above were rarely or inconsistently achieved in several other studies, although evidence for an effect of phage therapy was noted. Interpretation of results is somewhat challenging however, due to different methods, doses and modes of administration, age of chickens and outcomes measured. For example, after chickens challenged with *S.* Enteritidis were treated orally with two phage cocktails, either alone or in combination, rates of isolation of *S.* Enteritidis from cecal tonsils after 24 h were reduced significantly from 100% in untreated controls to 45–70%. However, the effect was transient since isolation rates were not significantly lower in treated (85–100%) compared with control chickens (100%) 48 h after treatment (Andreatti Filho *et al.*, 2007).

In a longer term study, counts of *S.* Enteritidis in cecal homogenates 14 days after challenge of day-old chickens were reduced by ~0.3–1.3 log units following five trials of various oral treatments with four individual phages 3 h after challenge (Sklar and Joerger, 2001). However, these reductions were significant in only two trials, in which counts of *S.* Enteridis in cecal contents exceeded 10^4 CFU g^{-1}. Similarly, an oral dose of 10^{11} PFU of a mixture of three phages reduced *S.* Enteritidis counts in cecal contents up to 3.5-fold over 25 days, and rates of colonization of the liver and spleen, but the reductions were not significant (Fiorentin *et al.*, 2005). In another study, oral treatment of chickens with a low dose of a mixture of three broad host range phages 3 days before and 3 days after challenge with *S.* Typhimurium resulted in marginally improved weight gain and moderate but inconsistent reductions in numbers of the challenge strain in ileal and cecal samples (Toro *et al.*, 2005). Also, the same authors found that a commercial competitive exclusion product (Protexin) was at least as effective as phage treatment alone, and had no synergistic effect when combined with phage treatment. Similar results were obtained when a commercial probiotic, Floramax-B11, was compared to a mixture of 45 phages (WT45) either alone or in combination with the phages in treatment of day-old chicks challenged with *S.* Enteritidis (Andreatti Filho *et al.*, 2007).

Campylobacter *infections*

Campylobacteriosis, particularly caused by *C. jejuni*, is the most common food-borne bacterial enteritis in developed countries. *Campylobacters* colonize the intestinal tracts of healthy animals of many species, including foods animals such as poultry, pigs and cattle. Poultry meat poses a major public health risk since broiler chickens are heavily colonized with *C. jejuni* at slaughter, resulting in high rates of carcass contamination during processing, frequently at levels greater than 10^5 CFU per carcass (Jorgensen *et al.*, 2002). Quantitative risk assessment (Rosenquist *et al.*, 2003) indicates that the incidence of human *Campylobacter* infections could be reduced substantially if the numbers of *Campylobacter* on poultry meat are reduced by 2 log units. Recent evidence suggests that this objective might be achieved with pre-harvest phage therapy. Moreover, the presence of naturally occurring *Campylobacter* phages in commercial broiler flocks correlates with a significant >1 log unit reduction in the numbers of *C. jejuni* in the cecal contents of broiler chickens (Atterbury *et al.*, 2005). These and many other aspects of *Campylobacter* phages have been reviewed in detail by Connerton *et al.* (2008).

The first report of phage therapy for *C. jejuni* in broiler chickens employed both preventative and therapeutic phage administration (Wagenaar *et al.*, 2005). In the preventative trial, broiler chickens 10 days old were challenged with *C. jejuni* strain C356 on day 4 of a 10-day course of oral gavage with 0.4 to 2×10^{10} PFU of phage 71. In the therapeutic trial, the same dose of phage 71 was given daily for 6 days commencing 5 days after challenge at 15 days of age. Counts of phages and the challenge strain were monitored in cecal contents until the chickens were 39 days old. Preventative treatment delayed but did not prevent colonization. Levels of *C. jejuni* were initially 2 log units lower than in controls, and then stabilized at ~1 log unit lower than in the controls. Phage and *C. jejuni* counts showed alternating fluctuations, consistent with alternating cycles of host replication and clearance with phage amplification, as occurs in naturally infected flocks (Atterbury *et al.*, 2005). After therapeutic phage treatment, counts of *C. jejuni* were immediately reduced by >3 log units for several days then stabilized at ~1 log unit lower than in the controls. Based on this greater efficacy following therapeutic dosage, a similar approach was applied to chickens challenged 10 days before the usual age of slaughter (~42 days). Birds were given a mixture of two phages, 71 and 69, orally for 4 days commencing 7 days after challenge. Following an initial drop of 1.5 log units, counts stabilized at 1 log unit lower than in control birds. These results were interpreted as encouraging for the use of phage therapy immediately pre-slaughter as a means of reducing the risks of human campylobacteriosis. To overcome the tendency towards the phage–host equilibrium and development of a

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resistant host subpopulation under farm conditions, it was suggested that pre-harvest treatment would be more effective with fresh lytic phages rotated in different production cycles.

Loc Carillo *et al.* (2005) reported a comprehensive study of phage therapy to reduce the *C. jejuni* burden in chickens. Groups of chickens infected with *C. jejuni* strains HPC5 or GIIC8 that became heavily colonized within 5–7 days were treated at 25 days of age with two candidate phages, CP8 or CP34, given as a single oral dose of 10^5, 10^7, or 10^9 PFU in an antacid buffer containing calcium carbonate. In the first 24 h, doses of 10^5 and 10^7 PFU of CP 8 were more effective than 10^9 PFU in reducing cecal colonization. With 10^7 PFU, levels of the challenge strains in contents of the upper intestine, ceca and lower intestine fell 0.5 to >5 log units, then rose after 2–3 days to levels ~1–2 log units lower than in controls. Phage CP34 was clearly more effective than CP8 against strain HPC5, despite *in vitro* evidence that CP8 was virulent for this strain. CP8 replicated in the intestinal tract without significantly affecting the overall population of strain HPC5. However, phage CP8 proved very effective against strain GIIC8, with reductions of 5.6 log units 24 h after treatment. After 5 days, levels of the challenge strain in cecal and lower intestinal contents were still significantly lower than in controls by ~2 log units. Resistance to phage CP34 was detected less frequently in isolates of HPC5 from chickens (4%) compared with HPC5 isolates from *in vitro* experiments (11%). Two of the CP34-resistant HPC5 isolates from chickens were less able to colonize chickens, and 97% of 90 isolates recovered from birds infected with these resistant strains had reverted to their phage sensitive state. As in the study by Wagenaar *et al.* (2005), these findings suggest that phage therapy holds promise for control of *C. jejuni* in poultry as a food-borne pathogen. The results emphasize the need for well-designed *in vivo* as well as *in vitro* evaluation of candidate phages, phage dose, and the merit in investigating the emergence and characteristics of phage-resistant hosts. Most importantly, it demonstrates the substantial influence of individual phage–host combinations on the effectiveness of phage therapy.

Phage therapy in pigs

ETEC infections

The first of the few studies of phage therapy in pigs were the excellent experiments conducted by Williams Smith in the United Kingdom on treatment of experimental ETEC diarrhea in neonatal pigs (Smith and Huggins, 1983). They assessed the efficacy of a mixture of two phages against diarrhea induced in neonatal pigs by infection with ETEC strain P433 (O20:K101:987P+). One phage, P433/1, used the K101 antigen as a receptor and lysed strain P433 and the other, P433/2, was lytic for K101-negative mutants of strain P433 that arose spontaneously at high frequency *in vitro* and were resistant to phage P433/1. Both phages were highly lytic *in vitro*, with as few as nine particles of P433/1 and four particles of P433/2 required to completely lyse broth cultures of their respective hosts.

In the first experiments, 14 newborn pigs fed bovine colostrum were inoculated orally at 6 h of age with *E. coli* P433 along with a non-pathogenic *E. coli* and a *Lactobacillus*. Seven of the pigs were treated with 10^{10} PFU of each of the two phages at the onset of diarrhea, 13–16 h after infection. The untreated pigs developed severe diarrhea; four died after 26–65 h and the remaining three pigs had severe diarrhea lasting 44 to 84 h. Fecal counts of ETEC P433 increased to ~10^9 CFU g^{-1} within 3–7 h, remained at this level for 24 h then declined to ~10^8 CFU g^{-1} at the end of the study (96 h). In contrast, none of the treated pigs died, diarrhea was mild and lasted 7–13 h, and fecal counts of ETEC P433 declined rapidly from ~10^8 CFU g^{-1} at the time of treatment to 10^5 CFU g^{-1} 5 h after treatment. Fecal titers of the phages, predominantly P433/1, remained at 10^6 PFU g^{-1} or higher throughout the study. Phage 433/1-resistant K-negative mutants were detected in several treated pigs, but always at low numbers compared with ETEC P433. Interestingly, in subsequent experiments, phage P433/1 was essentially as effective alone as it was when given with P433/2, whereas phage P433/2 alone was ineffective. Also, the K-negative phage-resistant mutants did not induce diarrhea in pigs. Smith and Huggins (1983) noted that because several K antigens are associated with ETEC in pigs, phages that target colonizing pili (F4, F5, F6 or F18) would cover a wider range of porcine ETEC and might be more practical.

Interest in phage therapy for neonatal ETEC infections declined after the development of effective control with pilus-based vaccines administered to pregnant sows. However, ETEC infections in weaned pigs continue to be a major economic problem for pig producers. Furthermore, widespread resistance of ETEC to antimicrobial agents has lead to renewed interest in phage therapy. Recently, Jamalludeen *et al.* (2007) isolated phages active against O149 ETEC, now the most common and widespread porcine ETEC, worldwide. Following characterization of the phages, nine were selected for further evaluation. Six phages (GJ1–GJ6) lysed 99–100% of 85 strains of O149:H10 ETEC but only 0–12% of 42 O149:H43 ETEC strains. Phage GJ1 was completely sequenced and shown to lack genes for toxins and for lysogeny (Jamalludeen *et al.*, 2008). Three other phages (GJ7–GJ9) that were selected on an O149:H43 ETEC host strain lysed 86–98% of 42 O149:H43 ETEC and 2–53% of O149:H10 ETEC. The nine phages were not specific for O149 *E. coli* as they lysed 6–68% of the 72 strains of the ECOR collection and 3–41% of 37 non-O149 ETEC.

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Subsequently, phages GJ1–GJ7 were evaluated individually and in some combinations for prophylaxis and treatment of experimental infection of pigs with an O149:H10 ETEC strain (Jamalludeen *et al.*, submitted for publication). Parameters that were measured were severity and duration of diarrhea, weight change, and excretion of the challenge ETEC. Oral administration of 10^9 PFU of phages GJ1–GJ6 individually in prophylactic mode, 15 min before challenge, significantly decreased the composite diarrhea score (a combination of duration and severity), excretion of the challenge ETEC and weight loss in treated compared with untreated, challenged pigs. Prophylactic treatment with a mixture of phages GJ1, GJ2 and GJ7 (10^9 PFU of each phage) also resulted in a significant reduction in the composite diarrhea score and a reduction in shedding of the challenge ETEC, but the decrease in the challenge ETEC was not significant. In therapeutic mode, three doses of a mixture of phages GJ1 and GJ6 (10^8 PFU of each phage) were given orally at 6-h intervals beginning 24 h after challenge with ETEC O149:H10. Treatment was associated with significant decreases in diarrheal scores and excretion of the challenge ETEC as well as with weight gain compared with weight loss in the control pigs. Levels of the phages in feces increased to as high as 10^{11} PFU g^{-1} within 1–2 days after treatment and gradually declined to $\sim 10^3$ PFU g^{-1} by the end of the experiment on day 6. In a separate trial in unchallenged pigs, the levels of phages in feces increased markedly when sodium bicarbonate was given orally just prior to the phages, suggesting that antacids protected the phages from the low pH of the stomach.

Additional work is needed to optimize doses, prepare the phages in a suitable formulation for field applications, and evaluate their effectiveness under field conditions. Post-weaning ETEC diarrhea is a good target for alternatives to antibiotics because of the high frequency of multiple drug resistance of ETEC (Maynard *et al.*, 2003) and the association of virulence and drug resistance genes on plasmids (Boerlin *et al.*, 2005). Smith's notion of seeking phages that use the colonizing pili as receptor has particular merit for ETEC in post-weaning diarrhea, since F4 and F18 pili are the only colonizing pili that are frequently associated with ETEC that cause this disease in pigs.

Salmonella *infections*

The only reported evaluation of phage therapy for *Salmonella* in pigs was a pre-harvest food safety intervention to reduce contamination of pork products that might occur following the dissemination of *S.* Typhimurium during shipping, lairage and slaughter (Lee and Harris, 2001). Pigs challenged intranasally with 5×10^8 CFU of *S.* Typhimurium were treated 3 h later by both oral and i.m. routes with either 2×10^9 PFU of phage Felix-O1 or a placebo. Six hours after treatment, enumeration of the challenge strain in tonsils, ileocecal lymph nodes, lung, liver, spleen, cecum and rectal contents revealed *S.* Typhimurium was still present at high numbers in the intestinal contents of pigs in both groups, but levels in the tonsils and ceca were reduced significantly. Further details in a patent (Harris and Lee, 2003) suggest that multiple dose variations on this treatment regime in the 24 h before shipping and slaughter can reduce the levels of *S.* Typhimurium in these and other tissues. As in other species, i.m. inoculation of phages may not be feasible in modern swine production.

Related applications of phages

Phage control of zoonotic pathogenic bacteria in foods

While there has been little or no adoption of phage therapy at the farm level, considerably greater interest has evolved in the food processing industry, where control of zoonotic pathogens immediately pre-harvest and during processing is a major focus. For example, pre-slaughter treatments of animal hides with phages against *E. coli* O157:H7 and *Salmonella* to reduce their entry into the food chain are acceptable to the US Food and Drug Administration (see http://omnilytics.com/news). Phages also reduce the load of zoonotic pathogens in some food products during or after processing, although efficacy has varied (Greer, 2005; Higgins *et al.*, 2005; Hudson *et al.*, 2005; Sulakvelidze and Barrow, 2005; Hagens and Loessner, 2007). Significant reductions of *C. jejuni, Salmonella* and other bacteria that do not replicate at refrigeration temperatures required high MOIs, suggesting that bacterial killing was by lysis from without. High doses of phages against *Listeria monocytogenes*, which replicates at refrigeration temperatures, have also proved effective in some foods (Carlton *et al.*, 2005) and the US Food and Drug Administration recently approved use of a commercial phage preparation to control *Listeria* spp. in ready-to-eat meat and poultry products (http://www.cfsan.fda.gov/~lrd/fr060818.html).

Current status of phage therapy in cattle, poultry and pigs

Research into phage therapy in cattle, poultry and pigs has addressed two major applications; reducing the impact of infections caused by animal pathogens on animal health and production, and control of zoonotic human pathogens by reduction of the bacterial load spread from animal reservoirs to foods and other vehicles of transmission to humans. While the objectives of these applications differ in some respects, both include potential preventative and therapeutic uses of phages, and they

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share a number of common requirements for successful development. Perhaps the most important requirement is a clear understanding of the epidemiology and pathogenesis of infection, the diversity of the target organisms, and the ability of candidate therapeutic phages to match this diversity in their common ecology.

Currently, the most promising animal health applications of phage therapy appear to be for acute intestinal, respiratory and systemic infections of young cattle, poultry and pigs caused by *E. coli* and *Salmonella*. Despite the rigor and early success in these studies, phage therapy for these and other animal diseases has not been developed further. Although this is largely due to the adoption of alternatives such as improved management practices, new vaccines and prophylactic use of antimicrobial drugs, it may also reflect some of the limitations of phage therapy, as pointed out by Barrow (Barrow *et al.*, 1998; Barrow, 2001). In addition to knowledge of the epidemiology of the agent, these included: the need for broad host range phages, the efficacy of phages in fluid as opposed to other matrices, the importance of timing phage use to critical points in infection and pathogenesis, and the need for practical and economic modes of administration. Some of these challenges are discussed below.

Interest in phage therapy for zoonotic pathogens in animals intensified more recently, when heightened awareness of the impact of food-borne infections with *S.* Enteritidis, *E. coli* O157:H7 and *C. jejuni* coincided with increasing concerns about antimicrobial resistance (Van den Bogaard and Stobberingh, 2000). As a result, the studies of this application of phage therapy are relatively recent and vary widely in scope, designs and outcome. Also, most used newly isolated, partially characterized phages against one or only a few different strains of these very diverse target organisms. While these differences make interpretation difficult, there are several consistent observations. High doses of phages given orally can reduce intestinal levels of the target organisms by >1–2 log units for 1–3 days. Although transient, this effect can substantially reduce the pathogen load entering the food chain if treatment is administered immediately prior to slaughter. Currently, such applications appear to be most successful for *C. jejuni* in poultry. Treatment immediately pre-slaughter does not, however, impact farm prevalence, environmental dissemination of the organism, or hide or feather contamination. To effect control at the farm level, the same or greater reductions, and preferably elimination of the target organism would be required. However, elimination appears to be an unlikely outcome of phage therapy, as for most other interventions.

Challenges and opportunities

Further development of phage therapy for animal applications faces a number of practical and biological challenges that will require considerable research. The dose and route of administration are important economic and practical considerations in agriculture. The most practical route for all species is via feed or drinking water. Given the sensitivity of many phages to the low pH of the stomach, encapsulation of phages with pH protectants (Waddell and Johnson, 2004; Ma *et al.*, 2008) would reduce inactivation of phages after ingestion and hence the total required phage dose. If required, i.m. inoculation may be feasible for calves, pigs and dairy cattle, but not for large-scale treatment of beef cattle. Large-scale i.m. inoculation of poultry, for example, during outbreaks of colibacillosis, is also not feasible, except perhaps in small, valuable breeder flocks. However, preventative injection of hatching eggs and spray treatment of the hatchery could possibly prevent the early onset of colibacillosis and colonization by *Salmonella*. Also, spray treatment of litter with phages (Smith *et al.*, 1987b) may reduce horizontal transmission of these and other organisms.

On a cautionary note, on-farm phage treatments will contribute to the diverse populations of host-specific phages that exist in these environments (Atterbury *et al.*, 2005; Hansen *et al.*, 2007; Ott *et al.*, 2007; Callaway *et al.*, 2008) and promote the emergence of phage resistant mutants or new genotypes of the target organism. This may reduce the effectiveness of the therapeutic phages, requiring alternating use of different phage cocktails in successive flocks or herds. Also, natural exposure and/or repeated use of phages, especially parenterally, may lead to development of phage neutralizing antibodies, reducing phage efficacy (Smith *et al.*, 1987b; Huff *et al.*, unpublished data).

The selection of therapeutic phages is crucial for their efficacy in the field. The high specificity of phages is a major advantage over antibiotics with respect to preservation of the normal flora, but it also has limitations. Most lytic phages are specific for a narrow range of subtypes within target pathogens. Consequently, phage cocktails may be required, particularly in preventative applications against potentially diverse animal or zoonotic pathogens, and to combat the emergence of phage resistant strains. Alternatively, phages with a broad host range can be selected by extensive screening to identify those that lyse a range of pathogens with common surface receptors, such as those that target multiple isolates of two different pathogenic groups of *E. coli* (Viscardi *et al.*, 2008). Such phages could also be engineered to target multiple receptors (Kropinski, 2006). Fortunately, in most studies where phage resistant mutants have been sought and characterized, they have been less virulent or do not become predominant. Furthermore, phage resistant mutants can be used to select the most effective phages (Yoichi *et al.*, 2004).

Since *in vivo* and *in vitro* virulences of phages frequently do not correlate, informative evaluations of candidate phages require well-planned and rigorous *in vivo* studies in appropriate models. Poultry, pigs and

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sheep are reasonably manageable for controlled experimental trials, except with pathogens such as *E. coli* O157:H7 that require higher levels of biocontainment. The use of cattle however has obvious practical and economic challenges. Mice have been used in several studies of phage therapy for *E. coli* O157:H7 (see, for example, Sheng *et al.*, 2006), but their suitability as a model for cattle is questionable because they differ so greatly from ruminants in gastro-intestinal anatomy, physiology and microbiology. Also, because of age-related development, the age of experimental subjects within a species, would best match the intended age of application, as has been done in some of the above studies of phage therapy for *C. jejuni* in chickens. Additional challenges in animal experimentation in some cases include the selection and availability of the most appropriate infection models. For example, in control of *E. coli* O157:H7 in cattle, naturally occurring 'super shedders' are likely the best candidates for phage therapy (Matthews *et al.*, 2006) but are difficult to obtain in sufficient numbers.

The selected phages and the phage formulations for therapeutic use must be safe. Phages are generally considered innocuous, although their ability to carry and transduce virulence related and other unwanted genes are a concern. This possibility can be largely eliminated by testing phages for undesirable genes or by multiplex PCR or currently economical whole genome sequencing. Most of the animal studies in cattle, poultry, and pigs have used crude phage lysates produced in the target pathogenic hosts without any untoward effects, despite the presence of endotoxins, bacterial debris, in some cases, exotoxins. For large-scale commercial production, it would be safer to propagate phages in non-pathogenic or at least toxin-negative hosts. Also, for parenteral use, removal of endotoxin would reduce any associated risk, as would the use of lysis-deficient phages (Matsuda *et al.*, 2005).

Conclusions

Phage therapy has potential for control of both animal and zoonotic pathogens in cattle, pigs and poultry. The information gained from recent studies provides a basis for much needed comprehensive and rigorous investigation of this potential. Hopefully, this will include more complete characterization of candidate phages, efficacy studies with administration by feasible routes of administration, and measured outcomes appropriate for the intended application. Additionally, thorough investigation of phage resistance and other dynamics of phage–host interactions in animal environments is essential. If the potential of phage therapy holds, its acceptance will depend on its efficacy, safety and cost relative to other interventions, and its complementarity with them. Complementarity is an important consideration, as it is likely that effective control of animal and zoonotic pathogens will require multiple interventions. Should these criteria be met, phage therapy will also require regulatory approval. As mentioned previously, the US Food and Drug Administration and the US Department of Agriculture have shown some flexibility in approving the use of phages for control of *L. monocytogenes* in ready-to-eat meat and poultry products, and not objecting to pre-harvest phage treatment of hides to reduce *Salmonella* and *E. coli* O157:H7 contamination. Whether this flexibility will extend to use in animals in the US and elsewhere remains to be seen. Most opinions acknowledge that the regulatory requirements in most Western countries are not geared to deal with therapeutic agents that multiply and the prompt introduction of new phages when resistance develops, as has been practiced in Eastern Europe for decades.

References

Andreatti Filho RL, Higgins JP, Higgins SE, Gaona G, Wolfenden AD, Tellez G and Hargis BM (2007). Ability of bacteriophages isolated from different sources to reduce *Salmonella enterica* serovar *enteritidis in vitro* and *in vivo*. *Poultry Science* **86**: 1904–1909.

Atterbury RJ, Dillon E, Swift C, Connerton PL, Frost JA, Dodd CE, Rees CE and Connerton IF (2005). Correlation of *Campylobacter* bacteriophage with reduced presence of hosts in broiler chicken ceca. *Applied and Environmental Microbiology* **71**: 4885–4887.

Atterbury RJ, Van Bergen MA, Ortiz F, Lovell MA, Harris JA, De Boer A, Wagenaar JA, Allen VM and Barrow PA (2007). Bacteriophage therapy to reduce *Salmonella* colonization of broiler chickens. *Applied and Environmental Microbiology* **73**: 4543–4549.

Bach SJ, McAllister TA, Veira DM, Gannon VPJ and Holly RA (2008). Effect of bacteriophage DC22 on *Escherichia coli* O157:H7 in an artificial rumen system (Rusitec) and inoculated sheep. *Annual Review of Microbiology* **52**: 89–101.

Barnes JH, Vaillancourt JP and Gross WB (2003). Colibacillosis. In: Saif YM (ed.) *Diseases of Poultry*. Ames, IA: Iowa State University Press, pp. 631–656.

Barrow P, Lovell M and Berchieri A (1998). Use of lytic bacteriophage for control of experimental *Escherichia coli* septicemia and meningitis in chickens and calves. *Clinical and Diagnostic Laboratory Immunology* **5**: 294–298.

Barrow PA (2001). The use of bacteriophages for treatment and prevention of bacterial disease in animals and animal models of human infection. *Journal of Chemical Technology and Biotechnology* **76**: 677–682.

Berchieri Jr A, Lovell MA and Barrow PA (1991). The activity in the chicken alimentary tract of bacteriophages lytic for *Salmonella typhimurium*. *Research in Microbiology* **142**: 541–549.

Besser TE, Hancock DD, Pritchett LC, McRae EM, Rice DH and Tarr PI (1997). Duration of detection of fecal excretion of *Escherichia coli* O157:H7 in cattle. *Journal of Infectious Diseases* **175**: 726–729.

Biswas B, Adhya S, Washart P, Paul B, Trostel AN, Powell B, Carlton R and Merril CR (2002). Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant *Enterococcus faecium* [erratum appears in *Infection and Immunity* 2002;**70**(3):1664]. *Infection and Immunity* **70**: 204–210.

https://doi.org/10.1017/S1466252308001576 Published online by Cambridge University Press

Boerlin P, Travis R, Gyles CL, Reid-Smith R, Janecko N, Lim H, Nicholson V, McEwen SA, Friendship R and Archambault M (2005). Antimicrobial resistance and virulence genes of *Escherichia coli* isolates from swine in Ontario. *Applied and Environmental Microbiology* **71**: 6753–6761.

Brüssow H and Kutter E (2005) Phage Ecology. In: Kutter E and Sulakvelidze A (eds) *Bacteriophages Biology and Applications*. Boca Raton, FL: CRC Press, pp. 129–163.

Callaway TR, Edrington TS, Brabban AD, Anderson RC, Rossman ML, Engler MJ, Carr MA, Genovese KJ, Keen JE, Looper ML, Kutter EM and Nisbet DJ (2008). Bacteriophage isolated from feedlot cattle can reduce *Escherichia coli* O157:H7 populations in ruminant gastrointestinal tracts. *Foodborne Pathogens and Disease* **5**: 183–191.

Carlton RM, Noordman WH, Biswas B, de Meester ED and Loessner MJ (2005). Bacteriophage P100 for control of *Listeria monocytogenes* in foods: genome sequence, bioinformatic analyses, oral toxicity study, and application. *Regulatory Toxicology and Pharmacology* **43**: 301–312.

Chibani-Chennoufi S, Bruttin A, Dillmann ML and Brussow H (2004). Phage–host interaction: an ecological perspective. *Journal of Bacteriology* **186**: 3677–3686.

Connerton IF, Connerton PL, Barrow P, Seal BS and Atterbury RJ (2008). Bacteriophage therapy and *Campylobacter*. In: Nachamkin I, Szymanski CM and Blaser MJ (eds) *Campylobacter*. Washington, DC: ASM Press, pp. 679–693.

Connerton PL, Loc Carrillo CM, Swift C, Dillon E, Scott A, Rees CE, Dodd CE, Frost J and Connerton IF (2004). Longitudinal study of *Campylobacter jejuni* bacteriophages and their hosts from broiler chickens. *Applied and Environmental Microbiology* **70**: 3877–3883.

d'Herelle F (1917). Sur un microbe invisible antagoniste des bacilles dysentériques. *Comptes rendus Académie Sciences* **165**: 373–375.

Fiorentin L, Vieira ND and Barioni Jr W (2005). Oral treatment with bacteriophages reduces the concentration of *Salmonella enteritidis* PT4 in caecal contents of broilers. *Avian Pathology* **34**: 258–263.

Fischetti VA (2005). Bacteriophage lytic enzymes: novel anti-infectives. *Trends in Microbiology* **13**: 491–496.

Gannon VP, Graham TA, King R, Michel P, Read S, Ziebell K and Johnson RP (2002). *Escherichia coli* O157:H7 infection in cows and calves in a beef cattle herd in Alberta, Canada. *Epidemiology and Infection* **129**: 163–172.

Gill JJ, Pacan JC, Carson ME, Leslie KE, Griffiths MW and Sabour PM (2006a). Efficacy and pharmacokinetics of bacteriophage therapy in treatment of subclinical *Staphylococcus aureus* mastitis in lactating dairy cattle. *Antimicrobial Agents and Chemotherapeutics* **50**: 2912–2918.

Gill JJ, Sabour PM, Leslie KE and Griffiths MW (2006b). Bovine whey proteins inhibit the interaction of *Staphylococcus aureus* and bacteriophage K. *Journal of Applied Microbiology* **101**: 377–386.

Greer GG (2005). Bacteriophage control of foodborne bacteria. *Journal of Food Protection* **68**: 1102–1111.

Guttman B, Raya R and Kutter E (2005). Basic phage biology. In: Kutter E and Sulakvelidze A (eds) *Bacteriophages Biology and Applications*. Boca Raton, FL: CRC Press, pp. 29–66.

Hagens S and Loessner MJ (2007). Application of bacteriophages for detection and control of foodborne pathogens. *Applied Microbiology and Biotechnology* **76**: 513–519.

Hansen VM, Rosenquist H, Baggesen DL, Brown S and Christensen BB (2007). Characterization of *Campylobacter* phages including analysis of host range by selected *Campylobacter* Penner serotypes. *BMC Microbiology* **7**: 90.

Harris DL and Lee N (2003). Compositions and methods for reducing the amount of *Salmonella* in livestock. US Patent No. 6,656,463.

Higgins JP, Higgins SE, Guenther KL, Huff W, Donoghue AM, Donoghue DJ and Hargis BM (2005). Use of a specific bacteriophage treatment to reduce *Salmonella* in poultry products. *Poultry Science* **84**: 1141–1145.

Hudson JA, Billington C, Carey-Smith G and Greening G (2005). Bacteriophages as biocontrol agents in food. *Journal of Food Protection* **68**: 426–437.

Huff WE, Huff GR, Rath NC, Balog JM, Xie H, Moore Jr PA and Donoghue AM (2002a). Prevention of *Escherichia coli* respiratory infection in broiler chickens with bacteriophage (SPR02). *Poultry Science* **81**: 437–441.

Huff WE, Huff GR, Rath NC, Balog JM and Donoghue AM (2002b). Prevention of *Escherichia coli* infection in broiler chickens with a bacteriophage aerosol spray. *Poultry Science* **81**: 1486–1491.

Huff WE, Huff GR, Rath NC, Balog JM and Donoghue AM (2003a). Evaluation of aerosol spray and intramuscular injection of bacteriophage to treat an *Escherichia coli* respiratory infection. *Poultry Science* **82**: 1108–1112.

Huff WE, Huff GR, Rath NC, Balog JM and Donoghue AM (2003b). Bacteriophage treatment of a severe *Escherichia coli* respiratory infection in broiler chickens. *Avian Diseases* **47**: 1399–1405.

Huff WE, Huff GR, Rath NC, Balog JM and Donoghue AM (2004). Therapeutic efficacy of bacteriophage and Baytril (enrofloxacin) individually and in combination to treat colibacillosis in broilers. *Poultry Science* **83**: 1944–1947.

Jamalludeen N, Johnson RP, Friendship R, Kropinski AM, Lingohr EJ and Gyles CL (2007). Isolation and characterization of nine bacteriophages that lyse O149 enterotoxigenic *Escherichia coli*. *Veterinary Microbiology* **124**: 47–57.

Jamalludeen N, Kropinski AM, Johnson RP, Lingohr E, Harel J and Gyles CL (2008). Complete genomic sequence of bacteriophage phiEcoM-GJ1, a novel phage that has myovirus morphology and a podovirus-like RNA polymerase. *Applied and Environmental Microbiology* **74**: 516–525.

Jorgensen F, Bailey R, Williams S, Henderson P, Wareing DR, Bolton FJ, Frost JA, Ward L and Humphrey TJ (2002). Prevalence and numbers of *Salmonella* and *Campylobacter* spp. on raw, whole chickens in relation to sampling methods. *International Journal of Food Microbiology* **76**: 151–164.

Kallings LO (1967). Sensitivity of various *Salmonella* strains to felix 0–1 phage. *Acta Pathologica et Microbiologica Scandinavica* **70**: 446–454.

Kropinski AM (2006). Phage therapy – everything old is new again. *Canadian Journal of Infectious Diseases and Medical Microbiology* **17**: 297–306.

Kudva IT, Jelacic S, Tarr PI, Youderian P and Hovde CJ (1999). Biocontrol of *Escherichia coli* O157 with O157-specific bacteriophages. *Applied and Environmental Microbiology* **65**: 3767–3773.

Laegreid WW, Elder RO and Keen JE (1999). Prevalence of *Escherichia coli* O157:H7 in range beef calves at weaning. *Epidemiology and Infection* **123**: 291–298.

Lee JH (2003). Methicillin (oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. *Applied and Environmental Microbiology* **69**: 6489–6494.

Lee N and Harris DL (2001). The Effect of Bacteriophage Treatment as a Preharvest Intervention Strategy to Reduce the Rapid Dissemination of Salmonella typhimurium in Pigs. *American Association of Swine Veterinarians (AASV)*, Perry, IA: AASV, p. 555.

Loc Carillo C, Atterbury RJ, el Shibiny A, Connerton PL, Dillon E, Scott A and Connerton IF (2005). Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler

chickens. *Applied and Environmental Microbiology* **71**: 6554–6563.

Luby CD and Middleton JR (2005). Efficacy of vaccination and antibiotic therapy against *Staphylococcus aureus* mastitis in dairy cattle. *Veterinary Record* **157**: 89–90.

Ma Y, Pacan JC, Wang Q, Xu Y, Huang X, Korenevsky A and Sabour PM (2008). Microencapsulation of bacteriophage felix O1 into chitosan-alginate microspheres for oral delivery. *Applied and Environmental Microbiology* **74**: 4799–4805.

Makovec JA and Ruegg PL (2003). Antimicrobial resistance of bacteria isolated from dairy cow milk samples submitted for bacterial culture: 8,905 samples (1994–2001). *Journal of the American Veterinary Medical Association* **222**: 1582–1589.

Matsuda T, Freeman TA, Hilbert DW, Duff M, Fuortes M, Stapleton PP and Daly JM (2005). Lysis-deficient bacteriophage therapy decreases endotoxin and inflammatory mediator release and improves survival in a murine peritonitis model. *Surgery* **137**: 639–646.

Matsuzaki S, Yasuda M, Nishikawa H, Kuroda M, Ujihara T, Shuin T, Shen Y, Jin Z, Fujimoto S, Nasimuzzaman MD, Wakiguchi H, Sugihara S, Sugiura T, Koda S, Muraoka A and Imai S (2003). Experimental protection of mice against lethal *Staphylococcus aureus* infection by novel bacteriophage phi MR11. *Journal of Infectious Diseases* **187**: 613–624.

Matthews L, McKendrick IJ, Ternent H, Gunn GJ, Synge B and Woolhouse ME (2006). Super-shedding cattle and the transmission dynamics of *Escherichia coli* O157. *Epidemiology and Infection* **134**: 131–142.

Maynard C, Fairbrother JM, Bekal S, Sanschagrin F, Levesque RC, Brousseau R, Masson L, Lariviere S and Harel J (2003). Antimicrobial resistance genes in enterotoxigenic *Escherichia coli* O149:K91 isolates obtained over a 23-year period from pigs. *Antimicrobial Agents and Chemotherapeutics* **47**: 3214–3221.

Mizoguchi K, Morita M, Fischer CR, Yoichi M, Tanji Y and Unno H (2003). Coevolution of bacteriophage PP01 and *Escherichia coli* O157:H7 in continuous culture. *Applied and Environmental Microbiology* **69**: 170–176.

Naylor SW, Low JC, Besser TE, Mahajan A, Gunn GJ, Pearce MC, McKendrick IJ, Smith DG and Gally DL (2003). Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. *Infection and Immunity* **71**: 1505–1512.

Niu YD, Xu Y, McAllister TA, Rozema EA, Stephens TP, Bach SJ, Johnson RP and Stanford K (2008). Comparison of fecal versus rectoanal mucosal swab sampling for detecting *Escherichia coli* O157:H7 in experimentally inoculated cattle used in assessing bacteriophage as a mitigation strategy. *Journal of Food Protection* **71**: 691–698.

Oot RA, Raya RR, Callaway TR, Edrington TS, Kutter EM and Brabban AD (2007). Prevalence of *Escherichia coli* O157 and O157:H7-infecting bacteriophages in feedlot cattle feces. *Letters in Applied Microbiology* **45**: 445–453.

Parisien A, Allain B, Zhang J, Mandeville R and Lan CQ (2008). Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides. *Journal of Applied Microbiology* **104**: 1–13.

Payne RJ and Jansen VA (2003). Pharmacokinetic principles of bacteriophage therapy. *Clinical Pharmacokinetics* **42**: 315–325.

Piercy DW and West B (1976). Experimental *Escherichia coli* infection in broiler chickens: course of the disease induced by inoculation via the air sac route. *Journal of Comparative Pathology* **86**: 203–210.

Rangel JM, Sparling PH, Crowe C, Griffin PM and Swerdlow DL (2005). Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982–2002. *Emerging Infectious Diseases* **11**: 603–609.

Raya RR, Varey P, Oot RA, Dyen MR, Callaway TR, Edrington TS, Kutter EM and Brabban AD (2006). Isolation and characterization of a new T-even bacteriophage, CEV1, and determination of its potential to reduce *Escherichia coli* O157:H7 levels in sheep. *Applied and Environmental Microbiology* **72**: 6405–6410.

Rosenquist H, Nielsen NL, Sommer HM, Norrung B and Christensen BB (2003). Quantitative risk assessment of human *Campylobacter*iosis associated with thermophilic *Campylobacter* species in chickens. *International Journal of Food Microbiology* **83**: 87–103.

Sheng H, Knecht HJ, Kudva IT and Hovde CJ (2006). Application of bacteriophages to control intestinal *Escherichia coli* O157:H7 levels in ruminants. *Applied and Environmental Microbiology* **72**: 5359–5366.

Sklar IB and Joerger RD (2001). Attempts to utilize bacteriophage to combat *Salmonella enterica* serovar Enteritidis infection in chickens. *Journal of Food Safety* **21**: 15–29.

Smith HW and Huggins MB (1982). Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. *Journal of General Microbiology* **128**: 2–18.

Smith HW and Huggins MB (1983). Effectiveness of phages in treating experimental *Escherichia coli* diarrhoea in calves, piglets and lambs. *Journal of General Microbiology* **129**: 2659–2675.

Smith HW, Huggins MB and Shaw KM (1987a). The control of experimental *Escherichia coli* diarrhoea in calves by means of bacteriophages. *Journal of General Microbiology* **133**: 1111–1126.

Smith HW, Huggins MB and Shaw KM (1987b). Factors influencing the survival and multiplication of bacteriophages in calves and in their environment. *Journal of General Microbiology* **133**: 1127–1135.

Sulakvelidze A and Barrow P (2005). Phage therapy in animals and agribusiness. In: Kutter E and Sulakvelidze A (eds) *Bacteriophages Biology and Application*. Boca Raton, FL: CRC Press, pp. 335–380.

Sulakvelidze A, Alavidze Z and Morris Jr JG (2001). Bacteriophage therapy. *Antimicrobial Agents and Chemotherapy* **45**: 649–659.

Summers W (2005). Bacteriophage research: early history. In: Kutter E and Sulakvelidze A (eds) *Bacteriophages Biology and Applications*. Boca Raton, FL: CRC Press, pp. 5–27.

Taylor WI and Silliker JH (1958). Hatching of eggs. U.S. Patent No. 2,851,006.

Toro H, Price SB, McKee AS, Hoerr FJ, Krehling J, Perdue M and Bauermeister L (2005). Use of bacteriophages in combination with competitive exclusion to reduce *Salmonella* from infected chickens. *Avian Diseases* **49**: 118–124.

Twort FW (1915). An investigation on the nature of the ultramicroscopic viruses. *Lancet* **189**: 1241–1243.

Van den Bogaard AE and Stobberingh EE (2000). Epidemiology of resistance to antibiotics links between animals and humans. *International Journal of Antimicrobial Agents* **14**: 327–335.

Viscardi M, Perugini AG, Auriemma C, Capuano F, Morabito S, Kim KP, Loessner MJ and Iovane G (2008). Isolation and characterisation of two novel coliphages with high potential to control antibiotic-resistant pathogenic *Escherichia coli* (EHEC and EPEC). *International Journal of Antimicrobial Agents* **31**: 152–157.

Waddell T, Mazzocco A, Johnson RP, Pacan J, Campbell S, Perets A, MacKinnon J, Holtslander B and Poppe C (2000). Control of *Escherichia coli* O157:H7 infection of calves by

bacteriophages. In: *Proceedings of the 4th International Symposium and Workshop on Shiga toxin* (*verocytotoxin*)*-producing* Escherichia coli (*VTEC 2000*) *Kyoto, Japan*. 29 October–2 November 2000 [abstract].

Waddell TE, Johnson RP and Mazzocco A (2004). Methods and compositions for controlled release of bioactive compounds. U.S. Patent Application No. 60/463,319.

Wagenaar JA, Van Bergen MA, Mueller MA, Wassenaar TM, Carlton RM (2005). Phage therapy reduces *Campylobacter jejuni* colonization in broilers. *Veterinary Microbiology* **109**: 275–283.

Weber-Dabrowska B, Mulczyk M and Gorski A (2000). Bacteriophage therapy of bacterial infections: an update of our institute's experience. *Archivum Immunologiae et Therapiae Experimentalis* **48**: 547–551.

Yoichi M, Morita M, Mizoguchi K, Fischer CR and Tanji Y (2004). The criterion for selecting effective phage for *Escherichia coli* control. *Biochemical Engineering Journal* **19**: 221–227.

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