Evaluation of bacteriophages for prevention and treatment of diarrhea due to experimental enterotoxigenic *Escherichia coli* O149 infection of pigs

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1. Introduction

There are concerns that increasing antimicrobial resistance of normal flora and enterotoxigenic *Escherichia coli* (ETEC) in pigs (Dunlop et al., 1998; Noamani et al., 2003; Fairbrother et al., 2005) may compromise the therapeutic use of antimicrobials in pigs and threaten human health through transfer of drug resistance genes to zoonotic pathogens. There is therefore a need for safe and practical alternatives to antimicrobials for prophylaxis and therapy in pigs.

Bacteriophages (phages) are non-hazardous self-replicating agents that increase their numbers as they destroy target bacteria (Sulakvelidze et al., 2001; Huff et al., 2005). In the 1980s, excellent studies on phage therapy were carried out by Smith and colleagues, using *E. coli* infection in mice and farm animals (Smith and Huggins, 1982, 1983;...
Smith et al., 1987a, 1987b). Treatment of infected mice with phages was more effective than treatment with antibiotics, and phages were effective when they were administered before or after infection (Smith and Huggins, 1982). The protective effect of phages was also shown against E. coli diarrhea in neonatal pigs and calves (Smith and Huggins, 1983; Smith et al., 1987b). Recently, the effectiveness of phages against Salmonella Typhimurium (Bercieri et al., 1991; Fiorentin et al., 2005), and Escherichia coli in poultry has also been reported (Huff et al., 2004, 2005). Development of bacterial resistance to therapeutic phages and inactivation of orally administered phages are concerns, but there are strategies to circumvent these problems (Smith et al., 1987b; Sulakvelidze et al., 2001).

In vitro screening of phages for efficacy and rapid multiplication is valuable in selection of therapeutic phages (Smith and Huggins, 1982). We therefore used in vitro tests to characterize seven phages that lyse porcine ETEC of O group 149, the most common cause of post-weaning ETEC diarrhea in pigs, worldwide (Jamalludeen et al., 2007). The objective of the current study was to determine the efficacy of these phages individually and in combination in prevention and treatment of experimental O149 ETEC diarrhea in weaned pigs.

2. Materials and methods

2.1. Media

Brain Heart Infusion Broth (BHIB), and MacConkey (MAC) agar were purchased from Difco Laboratories (Detroit, MI) and prepared according to the manufacturer’s instructions. Blood agar was purchased from Fisher Scientific, Nepean, ON, Canada. LB broth, LB agarose, and LB top agarose were prepared as described previously (Jamalludeen et al., 2007). SM buffer was as described by Sambrook et al. (1989).

2.2. Bacterial strains

The challenge strain for experimental infection of pigs was O149:H10:F4 ETEC strain JG280, a hemolytic E. coli with genes for Sta, Stb, LT, and EAST-1 (Noamani et al., 2003). This strain was isolated in 1999 from an Ontario pig with post-weaning diarrhea (PWD) and was obtained from Gallant Custom Laboratory, Cambridge, Ontario. It was also used for detection of phages in pig feces, together with O149:H43:F4 ETEC strain 3220, a 1983 Ontario isolate which is hemolytic and carries genes for LT, Stb, and EAST-1. The organisms were streaked on blood agar, checked for purity, tested for agglutination in O149 antiserum, and then aliquots were frozen at −70 °C in a milk-based freezing solution (Harris, 1954). As required, frozen stock cultures were plated overnight on MAC agar at 37 °C and single colonies were cultured in BHIB for 18–20 h at 37 °C with shaking at 150 rpm. For experimental infections, BHIB cultures of ETEC strain JG280 were sedimented by centrifugation at 5000 × g, then resuspended in 0.01 M phosphate-buffered saline, pH 7.2 (PBS) and adjusted spectrophotometrically to an OD₆₀₀ of 1.4, equivalent to approximately 10¹⁰ CFU/ml. The concentrations of the bacterial suspensions were confirmed by standard plate counts.

2.3. Phages

Seven lytic phages, designated GJ1–GJ7 (Jamalludeen et al., 2007), were evaluated in this study. Phages GJ1–GJ6 were previously found to be highly effective in vitro against ETEC O149:H10:F4 strain JG280, whereas phage GJ7 was most effective against ETEC O149:H43:F4 strain 3220 (Jamalludeen et al., 2007). The phages were propagated in E. coli strain JG280 or strain 3220 in LB top agarose, and prepared as previously described (Jamalludeen et al., 2007), yielding approximately 10⁹ PFU/ml in SM buffer.

2.4. Animals

Pigs (n = 101) were obtained from the University of Guelph swineherd, weaned at 3 weeks of age, transferred to the Animal Isolation Facility at the Ontario Veterinary College, and allowed to acclimatize for 2 days before commencement of experiments. They were housed in groups of up to five pigs, fed a standard non-medicated ration for post-weaning pigs and had water ad libitum. They were weighed at the commencement and end of the experiments. Fecal samples were collected from the rectum prior to and at intervals after infection and treatment. At the end of the experiments, or earlier if required for humane purposes, the pigs were euthanized and necropsied. Tissues were collected at necropsy for the enterocyte adhesion test (see below), which determines the presence or absence of the F4 ETEC receptor, and hence susceptibility to ETEC infection. Results for pigs whose enterocytes failed to bind the challenge strain, GJ280, were removed from the trial data and analyses. All animal care and procedures complied with the requirements of the Canadian Council on Animal Care.

2.5. Experimental trials

Of the five trials described below, Trials 1 and 3 evaluated the efficacy of the selected phages in preventing development of diarrhea when given shortly after exposure to ETEC (i.e. prophylactic use). In Trial 1, six phages were evaluated individually, and in Trial 3, a mixture of three phages was evaluated for prophylactic use. Trial 2 did not include treatment of ETEC-infected pigs with phages but was conducted to determine if treatment of pigs with an antibiotic and an antacid prior to oral inoculation with ETEC or the phages would increase the diarrheal response to ETEC infection, and improve the survival of phages in the gastrointestinal tract. All subsequent trials included these modifications. Trials 4 and 5 evaluated a mixture of two phages for alleviating diarrhea that developed after experimental ETEC infection (i.e. therapeutic use).

2.5.1. Trial 1: prophylactic efficacy of individual phages

Twenty-eight pigs were allocated into six groups of four or five for challenge and treatment with individual phages GJ1–GJ6, and another 15 pigs were challenged but not
treated. The pigs were weighed prior to challenge and just before euthanasia. Each pig was inoculated orally by syringe with approximately $10^{10}$ CFU of O149:H10:F4 ETEC strain JG280, suspended in 5 ml of PBS. Fifteen minutes later, pigs in the six treatment groups were inoculated orally by syringe with $10^{10}$ PFU of phages GJ1–GJ6 in 2 ml of SM buffer, one phage per group. The pigs were monitored for 6 days, during which the occurrence, duration and severity of diarrhea were recorded daily, and fecal samples were collected daily for detection and estimation of the levels of shedding of the challenge strain and phages, as described below. The pigs were then euthanized and necropsied for collection of samples for the enterocyte adhesion test. The severity of diarrhea was scored daily by a method similar to that described by Jensen et al. (2006). A score of 0 was assigned when the feces were firm and normally shaped, a score of 1 was given when the feces were soft but able to retain some shape, a score of 2 was given when the feces were brown and liquid, and a score of 3 was assigned when there was frequent passage of watery feces. Pigs with severe illness resulting in death or euthanasia on humane grounds were given the maximum score of 18 (score of 3 for 6 days). These daily scores were used to calculate individual mean diarrhea scores (sum of daily scores divided by the number of days with diarrhea), and composite diarrhea scores (mean diarrhea score multiplied by the duration of diarrhea in days), and group means for these parameters, where appropriate. Assessment of the effect of treatment also included weight change during the study, and the levels of shedding of the challenge strain ETEC and phages. Challenge ETEC recovered from the pigs were tested for susceptibility to the phage that had been administered.

2.5.2. Trial 2: modifications to the challenge and treatment protocols

This trial was designed to determine if antibiotic (Van der Stede et al., 2003) and antacid treatments (Smith et al., 1987b) prior to inoculation with ETEC strain JG280 would result in a more severe diarrheal response in ETEC-challenged pigs and improved phage survival in the gastro-intestinal tract of pigs given only the phages. All 11 pigs were given 0.5 ml of florfenicol (300 mg/ml, Schering Canada Inc, Pointe-Claire, Quebec) intramuscularly on two consecutive days, followed by 1 day without antibiotic treatment. The next day, all pigs were given 60 ml of 1.4% sodium bicarbonate orally, followed 15 min later by oral inoculation with either ETEC strain JG280 only (five pigs) or phages GJ1–GJ6 only (one pig per phage). Inoculations with bacteria or phages, monitoring, scoring and other aspects of the trial were as described for Trial 1.

2.5.3. Trial 3: prophylactic efficacy of a mixture of three phages

Twenty pigs were challenged orally with $10^{10}$ CFU of ETEC strain JG280, as described in Trial 1, after the florfenicol and antacid pre-treatments described in Trial 2. Fifteen minutes after challenge, 10 pigs were each inoculated orally as described in Trial 1 with a mixture of phages GJ1, GJ2 and GJ7, each at $10^{9}$ PFU. The criteria used for assessing diarrhea, weight change, excretion of phage and challenge ETEC and the enterocyte adherence test were as described for Trial 1.

2.5.4. Trials 4 and 5: therapeutic efficacy of a mixture of two phages

To evaluate the therapeutic, as opposed to prophylactic use of phage therapy, the phages were not administered until 24 h after challenge, after the onset of diarrhea. Ten pigs were challenged orally with $10^{10}$ CFU of ETEC strain JG280 as described in Trial 1 and following the florfenicol and antacid pre-treatments described in Trial 2. At 24 h after challenge, the severity of diarrhea was scored as described in Trial 1, and fecal samples were taken to detect and enumerate the ETEC strain JG280 and total E. coli (see below). The pigs were divided randomly into two groups of five pigs, one in which pigs received no treatment and the other in which pigs were treated with phages. Phage treatment comprised of an oral inoculation as described in Trial 1 with a mixture of phages GJ1 and GJ6, each at a dose of $10^{8}$ PFU, three times at 6 h intervals. Sodium bicarbonate was administered once, prior to the first inoculation with phage. The pigs were monitored daily for 5 days after treatment, and then euthanized. As in other trials, daily clinical observations, diarrhea scores, shedding of ETEC strain JG280 and phages, weight change during the experiment, and the results of the enterocyte adherence tests were used to assess the effectiveness of phage treatment. Trial 5 was a repeat of Trial 4, with nine challenged and untreated control pigs and eight challenged pigs treated with a mixture of phages GJ1 and GJ6, as in Trial 4.

2.6. Detection and quantitation of bacteria in feces

Fecal samples were collected by swab from the rectum, stored on ice and processed within 2 h of collection. Samples collected during the adaptation period were tested for the presence of hemolytic O149 ETEC by mixing 1 g of feces in 9 ml of PBS, plating the mixture on MAC agar and blood agar and incubating the plates overnight at 37 °C. If hemolytic colonies were present, five were tested for agglutination with O149 antiserum. After challenge, the levels of the challenge strain JG280 in feces were estimated as percentage recovery of the hemolytic, O149-positive challenge strain relative to other E. coli. Fecal samples were diluted 10-fold in PBS and selected dilutions were plated on blood agar and MAC agar plates. The plates were incubated overnight at 37 °C, and examined for hemolytic and non-hemolytic E. coli-like colonies. Hemolytic E. coli-like colonies on blood agar were tested for agglutination with O149 antiserum. The percentage recovery of the challenge strain was calculated by using the total numbers (per plate) of hemolytic, O149-positive E. coli-like colonies on blood agar, and of E. coli-like colonies on MAC agar. Daily estimates for individual animals were used to calculate the individual and group mean percentage recoveries of the challenge strain over the 6 days of the experiments. Total fecal E. coli counts, determined in Trials 4 and 5, were estimated by counts of E. coli-like colonies from serial 10-fold dilutions of feces on MAC agar. Selected O149-positive E. coli isolated from the pigs after challenge...
were tested by a spot test (Kutter and Sulakvelidze, 2005) for their susceptibility to phages GJ1 and GJ6.

2.7. Detection and quantitation of phages in feces

A 1-g quantity of feces was added to 9 ml of sterile PBS and 0.2 ml of chloroform was added. The mixture was held for 15 min at room temperature, centrifuged at 4000 × g for 10 min and then filtered through a 0.45 μm membrane filter (Fisher Scientific). For pre-treatment samples, the filtrate was tested for phages lytic for ETEC strains GJ280 and 3220 by using a spot test (Kutter and Sulakvelidze, 2005), with these two bacteria as hosts. For post-treatment samples, the filtrate was diluted tenfold (10^-1 to 10^-8) in PBS. A lawn of host bacteria was spread on an LB agarose plate and allowed to dry for about 5 min and then 10 μl of the filtrate dilutions were dropped on quadrants of the plate and allowed to dry. The theoretical detection limit of this assay was 10^3 PFU/g of feces. The plates were incubated at 37 °C for 6–8 h then were examined for lysis in the areas on which the drops of filtrate had been placed. The phage titre was determined by counting the number of plaques in the highest dilution in which plaques could be observed. A negative value was recorded when there were no plaques in the 10^-1 dilution, representing a titre of less than 10^3 PFU/g.

2.8. Enterocyte adhesion test

Immediately after euthanasia, 6 days post-challenge, a sample of small intestine was collected to determine whether the F4+ challenge E. coli could adhere to enterocytes from each pig (Python, 2003). A 5-cm portion of small intestine approximately 20 cm from the ileo-cecal junction was removed, opened longitudinally, and immersed in ice-cold PBS, pH 6.8, containing EDTA (3.72 g/l). Epithelial cells were removed by scraping the mucosal surface with a microscope slide and added to 40 ml of PBS containing 2% formaldehyde. Large fragments were allowed to sediment, and cells in the supernatant were washed twice by centrifuging for 10 min at 200 × g and resuspended in 10 ml PBS. The final suspension was diluted to approximately 10^5–10^6 cells/ml in PBS containing 2% α-mannose. A 1-ml volume of enterocyte suspension was mixed with 1 ml of a broth culture of ETEC strain JG280, that had been diluted 1:30 from an overnight culture then incubated for 4 h at 37 °C to reach an OD_600 of 0.9. The mixture of enterocytes and bacteria was incubated with gentle shaking in a water bath at 37 °C for 45 min. Following incubation, 20 μl of the suspension were placed on a slide, allowed to dry, fixed with 95% methanol, stained with Giemsa, and examined microscopically. A pig was considered positive for the binding of the challenge ETEC, and therefore susceptible to infection, if more than 15% of the cells bound at least five bacteria (Python, 2003). Pigs with negative results in this test were considered not susceptible to the challenge ETEC and their data were not included with the results.

2.9. Statistical analysis

The general linear mixed (GLM) procedure and ANOVA using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) were used to compare the parameters for response in the treatment groups with those in the control group and the association between parameters of the phage-treated groups as compared with the control group in each trial. ANOVA was used for analysis of shedding of E. coli and phage, whereas GLM was used for all other analysis. A difference was considered significant when the P value was less than or equal to 0.05.

3. Results

For all the trials, hemolytic colonies were sometimes found on pre-challenge culture of feces from the pigs. In no case did the hemolytic colonies agglutinate in O149 antiserum. Phages active against the O149 ETEC were not detected in any of the pigs prior to the trials. The outcome of the enterocyte adherence assays resulted in exclusion of the data from two pigs in trial 1 (one control and one treated with phage JG2) and one treated pig in trial 5.

3.1. Prophylactic efficacy of individual phages (Trial 1)

ETEC JG280-infected pigs treated prophylactically with individual phages GJ1–GJ6 had significantly lower group mean weight changes (GLM, P ≤ 0.0001–0.0101), and group mean composite diarrhea scores (GLM, P ≤ 0.0001–0.02) compared to infected but untreated pigs (Fig. 1). Analysis by the GLM revealed that treatment with individual phages GJ1, GJ2 and GJ5 also resulted in significant reductions in group duration of diarrhea (P = 0.0114–0.0329) and group mean diarrhea scores (P = 0.0146–0.0264), and that pigs treated with phage GJ6 had lower group mean diarrhea
score ($P = 0.0191$) but not a shorter mean duration of diarrhea (Fig. 1). The mean percentage excretion of the challenge ETEC within all phage-treated groups (range of group means, 2.75–37.26%) was significantly lower (ANOVA, $P < 0.0001$ for GJ1–GJ5 and 0.0002 for phage GJ6) than that of the infected but untreated controls (group mean, 83.31%). Phages that were lytic for the challenge ETEC were not detected in fecal samples from any of the pigs that had received phages. The challenge bacteria that were recovered from the feces of the pigs that received phages remained susceptible to the phages that had been administered.

3.2. Modifications to the challenge and treatment protocols (Trial 2)

Administration of florfenicol to the pigs for 2 days prior to ETEC challenge and sodium bicarbonate orally 15 min before challenge did not alter the clinical outcome of challenge with ETEC GJ280 (Table 1). Group means of weight change, duration of diarrhea, diarrhea score and composite diarrhea score of challenged pigs (Table 1) were not significantly different ($P = 0.48$, 0.72, 0.4, and 0.47, respectively) from those of the control group in trial 1 (Fig. 1). Also, shedding of the challenge ETEC strain (group mean, 77.32%, Table 1) was similar to that of the control group in trial 1 (group mean, 83.31%, above). However, the modified protocol appeared to influence phage survival. In contrast to the results of trial 1, in which the phages were not detectable in the feces of treated pigs, all six phages were detectable for 1–3 days in the feces of all six treated pigs that received the phages but no ETEC, at levels ranging from $1.5 \times 10^6$ on day 1 to $7 \times 10^3$ on day 3.

3.3. Prophylactic efficacy of a mixture of three phages (Trial 3)

Following the modified challenge and treatment protocol described in trial 2, one group of 10 pigs received ETEC GJ280 alone and the other group of 10 received ETEC GJ280 and a mixture of three phages (GJ1, GJ2, and GJ7). The mixture of phages was effective in significantly reducing the mean duration of diarrhea, mean diarrhea score and the mean composite diarrhea score (GLM, $P \leq 0.0001$, 0.0020 and 0.0020, respectively) (Fig. 2), but the difference in shedding of the challenge ETEC was not significant ($63.45\%$ control group and $37.8\%$ treated group, $P$ value $= 0.14$). Phages were detected in the feces of all the pigs that had received the phage mixture. The phages were detected for 1–6 days in most pigs at titres of $10^4$–$10^{11}$ PFU/g, with the highest mean titres on days 2 and 3.

3.4. Therapeutic efficacy of a mixture of two phages (Trials 4 and 5)

In trial 4, all 10 pigs developed diarrhea by 24 h after challenge and were divided into two groups of five pigs each. In trial 5, all nine pigs in the control group and seven of the eight pigs in the phage treatment group developed diarrhea at 24 h post-ETEC challenge. The one pig which did not develop diarrhea by 24 h, or at any time, gave negative results in the enterocyte adhesion test, and was excluded from the analysis. The severity of diarrhea in pigs assigned to the treated and untreated groups, based on their diarrhea scores on day 1, 24 h after challenge but before treatment, was not significantly different in either trial (ANOVA, $P = 0.74$). Analysis of the combined data for trials 4 and 5 (Fig. 3) showed that pigs given the three treatments with the mixture of phages at 6 h intervals beginning 24 h after challenge had significantly improved mean weight change, mean duration of diarrhea, mean diarrhea score, and mean composite diarrhea score (GLM, $P < 0.0001$). Also, the average percentage of the challenge ETEC JG280 shed in

![Image](https://via.placeholder.com/150)

Table 1

Diarrheal disease in pigs treated with florfenicol by the I.M. route for 2 days and with sodium bicarbonate orally just prior to oral challenge with O149 ETEC strain JG280 (Trial 2).

<table>
<thead>
<tr>
<th>Pig #</th>
<th>Weight change (kg)</th>
<th>Duration of diarrhea (days)</th>
<th>Mean diarrhea score</th>
<th>Composite diarrhea score</th>
<th>% of total E. coli that was JG280</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−1.77</td>
<td>3</td>
<td>3</td>
<td>9</td>
<td>100</td>
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<td>−1.38</td>
<td>6</td>
<td>3</td>
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<td>100</td>
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<td>1.5</td>
<td>3</td>
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</tr>
<tr>
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<td>9</td>
<td>100</td>
</tr>
<tr>
<td>Mean</td>
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<td>3.6</td>
<td>2.21</td>
<td>8.6</td>
<td>77.32</td>
</tr>
</tbody>
</table>

*a* Mean diarrhea score = sum of daily diarrhea score/number of days with diarrhea.

*b* Composite diarrhea score = average diarrhea score times duration of diarrhea.

*c* Died on day 3 and assigned the maximum score 18.

The mixture of phages was effective in significantly reducing the mean duration of diarrhea, mean diarrhea score (GLM, $P < 0.0001$) but not a shorter mean duration of diarrhea (Fig. 1). The mean percentage excretion of the challenge ETEC within all phage-treated groups (range of group means, 2.75–37.26%) was significantly lower (ANOVA, $P < 0.0001$ for GJ1–GJ5 and 0.0002 for phage GJ6) than that of the infected but untreated controls (group mean, 83.31%). Phages that were lytic for the challenge ETEC were not detected in fecal samples from any of the pigs that had received phages. The challenge bacteria that were recovered from the feces of the pigs that received phages remained susceptible to the phages that had been administered.

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feces over days 2–6 was significantly lower (ANOVA, \( P < 0.0001 \)) in the treated pigs (mean = 20.97 ± 2.34%) than in the controls (mean = 79.81 ± 7.32). Mean total fecal \( E. coli \) counts of the treated and control groups were very similar on day 0, and increased similarly in both groups on day 1, 24 h after challenge (Fig. 4). Thereafter, the total \( E. coli \) counts of the treated pigs fell to day 0 levels by days 3, 4, 5 and 6, but in the untreated control pigs remained significantly higher than day 0 levels (ANOVA, \( P = 0.35, 0.49, 0.14 \) and 0.12 for the treated group and <0.0001 for the untreated group, respectively). The phages were detected in the feces of the treated pigs for 1–5 days, at progressively declining mean titres ranging from \( 10^{11} \) PFU/g on day 2 to \( 10^{3} \) PFU/g on day 5 (Fig. 5).

4. Discussion

This is the first report on the use of phages for the prevention and treatment of experimental \( E. coli \)-induced diarrhea in weaned pigs, although similar studies have been reported for neonatal pigs (Smith and Huggins, 1983; Smith et al., 1987a). The successes reported in the present study were similar to those reported previously for neonatal pigs. Because both the challenge ETEC and the phages may be susceptible to low pH in the stomach and upper small intestine, the model of experimental ETEC diarrhea was modified by the pre-challenge oral administration of sodium bicarbonate. We also treated the pigs with florfenicol prior to the challenge in an attempt to reduce competing intestinal flora and enhance the chances for the challenge ETEC to colonize. Cox et al. (1991) and Van der Stede et al. (2003) have used this protocol in several studies of experimental ETEC diarrhea in weaned pigs. With this protocol, all six phages were detected in the feces of pigs that had received them. Administration of the phages shortly after feeding also is another strategy for protecting them from exposure to low pH in the stomach (Brussow, 2005).

The combination of phages tested for prophylaxis included GJ1 and GJ2, selected based on their performance against O149:H10 ETEC, and GJ7, which was active primarily against O149:H43 ETEC (Jamalludeen et al., 2007). The latter was expected to have only a moderate effect on the O149:H10 challenge ETEC, but was included as it may be valuable in field tests in which some O149:H43 ETEC are expected to be present. Using the modified protocol, the cocktail of phages proved to be effective. However, although phages were detected in the feces for 6 days, at titres as high as \( 10^{11} \) PFU/g on days 1 and 2, reflecting replication of the phages, the results were not as impressive as those with the individual phages. In part this perhaps reflects the lower phage dose used in Trial 3 (\( 10^{6} \) PFU of each phage), compared to Trial 1 (\( 10^{10} \) PFU), and greater survival of the challenge strain with the modified protocol. The advantage in using more than one phage is that if resistant mutants arise to one phage other phages to which the strain is susceptible would be available. This approach was successfully used with ETEC diarrhea in calves (Smith et al., 1987a), Salmonella Enteritidis PT4 in broilers’ caeca (Fiorentin et al., 2005), and Salmonella from poultry carcass rinses (Higgins et al., 2005).
A mixture of phages GJ1 and GJ6 was selected to determine effectiveness in a therapeutic mode. Previous studies (Jamalludeen et al., 2007) showed that an O149:H10:F4 ETEC mutant resistant to phage GJ1 was still susceptible to phage GJ6 and a mutant resistant to phage GJ6 was still susceptible to phage GJ1. The findings that there were significant differences in diarrhea, weight change, and excretion of the challenge ETEC in phage-treated pigs compared with the control pigs indicate that this combination of phages is suitable for further tests to optimize doses, preservation and storage, and evaluate efficacy in field studies.

After the onset of diarrhea the opportunity for phage to contact ETEC may be more limited, as most ETEC will be associated with the epithelial lining of the small intestine (Moon et al., 1977; Kasman, 2005) and rapid excretion of the fluid intestinal contents may promote removal of the phage particles. For the therapeutic application, the phages were therefore administered in three doses over an 18-h period to reduce the chances that the entire phage inoculum would be rapidly washed through the intestine. The dose of phage was reduced compared to the dose of approximately 10^9 PFU that was used in the prophylactic mode because the expectation was that during ETEC diarrhea there would be a heavy growth of the challenge E. coli strain in the intestine of the pigs, and this would permit considerable phage replication. The data on monitoring of total E. coli in the feces are interpreted to mean that phage-treatment had a minimal effect on the total E. coli fecal flora. Similarly, there are reports that there was no decline in commensal E. coli gut biota in mice that were orally administered a four-phage cocktail (Chibani-Chennoufi et al., 2004) or in human volunteers orally exposed to phage T4 (Bruttin and Brussow, 2005).

In conclusion, all six phages that were tested individually showed significant prophylactic activity against diarrhea and shedding of the challenge ETEC following experimental oral infection of pigs with an O149:H10:F4 ETEC. A combination of three phases also moderated the course of diarrhea. In the therapeutic mode, a combination of two phages significantly reduced the development of diarrhea and the number of challenge bacteria in feces without an apparent reduction in the normal E. coli flora.

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