Effect of Bacteriophage in Enterotoxigenic Escherichia coli (ETEC) Infected Pigs

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ABSTRACT. We evaluated effect of enterotoxigenic Escherichia coli (ETEC) specific lytic phage CJ12 in ETEC infected pigs. Phage was mixed with feed at a ratio of 1:1,000 (0.1%). One week after initially providing phage mixed feed, pigs were challenged orally with 10^11 CFU of ETEC and body weight, diarrhea score, bacterial CFU and phage PFU in the feces were measured. Pigs of phage treated groups C (10^6 PFU/g) and D (10^8 PFU/g) showed more resistance to diarrhea due to ETEC infection compared to positive control group B on the third day after the initial challenge. Moreover, during the quantitation of ETEC in feces, both groups C and D showed approximately 63.92 and 60.73% reduced ETEC compared to positive control group B. Phages were successfully isolated from feces in both groups C and D during the experiment without any adverse effects, suggesting the possibility of using CJ12 as a feed additive.

KEY WORDS: bacteriophage, ETEC, prophylactic efficacy, swine.


Enterotoxigenic Escherichia coli (ETEC) is a causative agent of diarrhea in neonatal and recently weaned pigs as well as humans [10, 11]. Treatment of ETEC infection could be easily achieved by the administration of antibiotics, but antibiotic abuse may lead to adverse effects [16] such as antimicrobial resistance, which is a major threat to human health through drug resistance gene transfer. The increasing problem of antibiotic resistance has rekindled interest in the development of new methods that could substitute for antibiotics in the prevention of disease as well as therapy. Lytic phages, which can be used for therapy, exhibit a self-replicating virulent infectious cycle result in degradation of host cell DNA, replication, and release of progeny phages [8]. A few studies of phage therapy for ETEC infection in pigs have been conducted, and have demonstrated the efficacy of direct oral administration of phage culture [6, 14]. In this study, ETEC specific bacteriophage was pulverized for field applications and administered to pigs by mixing with feed for one week. We analyzed diarrhea score, bacterial colonization and phage concentration in feces after ETEC challenge.

An ETEC specific lytic phage, CJ12, was evaluated in this study. CJ12 was isolated from the sewage of a pig farm in Korea as described previously [5]. Briefly, sewage was centrifuged at 4,000 g for 15 min and filtered through a sterile 0.45 μm membrane filter. Purified sewage was mixed with ETEC JG280 culture and incubated at 37°C for 18 hr with shaking. Supernatant was harvested by centrifuging at 4,000 g for 15 min, filtered by 0.45 μm filtration and submitted to plaque assay with ETEC JG280 by the soft agar overlay method, as described previously [12]. The plaque was isolated, and this step was repeated for three times with isolated plaque to refine a phage with single plaque formation. Purified phage was propagated using ETEC JG280 culture in LB broth (Difco, Sparks, MD, U.S.A.) and pulverized by a spray drying process. Pulverized phage was dissolved in SM buffer [0.1 M NaCl, 1 mM MgSO4, 0.2 M Tris (pH 7.5), 0.01% gelatin] and the titer was determined by the soft agarose overlay method. The final titer of phage powder was prepared as 10^6 and 10^8 PFU/g for the experiment. The challenge strain used for the experimental infection of pigs was O149:H10:F4 ETEC strain JG280, a hemolytic E. coli with genes for STa, STb, LT and EAST-1. ETEC JG280 was cultured in brain heart infusion broth (BHB, Difco), sedimented by centrifugation at 5,000 g, suspended in 0.01 M phosphate-buffered saline and adjusted spectrophotometrically to an OD 600 of 1.4, equivalent to approximately 10^10 CFU/ml [6].

Twenty-four pigs weaned at 3 weeks of age were used in this experiment. Four groups, each consisting of 6 pigs, were designated as follows: group A was a negative control without phage treatment and ETEC challenge, group B was challenged with ETEC without phage treatment, group C was treated with 10^6 PFU/g of phage and challenged with ETEC, and group D was treated with 10^8 PFU/g of phage and challenged with ETEC. Phages were given orally by mixing with feed at a ratio of 1:1,000 (0.1%) during the experiment for groups C and D. One week after initiating phage mixed feed, the pigs were challenged orally by syringe with 10 ml of ETEC JG280 (10^10 CFU/ml) for three days. After the challenge, body weight, diarrhea score, bacterial CFU and phage PFU were measured in feces. To quantitate ETEC in the feces, real-time PCR with SYBR Green I method was
performed [3] with modification for feces samples, targeting the LT gene with primers LT-RTf (5'-ggcaggcaaaagaga-
atagg-3') and LT-RTr (5'-tccttcacctttcaggtct-3') [9]. Each 0.1 g of feces from uninfected pig was mixed with 10²–10¹¹ CFU of ETEC. The DNA from the feces was extracted as described previously [2] and used as a template for the standard curve. The real-time PCR reaction was performed in total volume of 10 µl containing 5 µl of SYBR Green PCR master mix (Qiagen, Hilden, Germany), 5 pmol of each primer, 3 µl of water and 1 µl of DNA template with 5 min of initial de-
naturation at 95°C, 40 cycles of 15 sec at 95°C denaturation and 40 sec at 60°C annealing and elongation. The standard curve was generated by the cycle threshold (Ct) value and fluorescence based on the threshold, and by Ct value and CFU of bacteria. Quantitation of phage in the feces was performed as described previously with slight modifications [6]. Briefly, 10 g of feces were mixed with 20 ml of SM buffer, the mixture was held for 15 min at room temperature and centrifuged at 4,000 g for 10 min. Supernatant was filtered with 0.45 µm filter, and the filtrate was diluted tenfold in SM buffer. Then, 10 µl of the filtrate dilutions was dropped on the soft agarose overlaid plate and the number of plaques was counted.

This animal experiment was approved by the policies and regulations for the care and use of laboratory animals set of the Laboratory Animal Center, Seoul National University, Seoul, Korea (SNU-110506-8) and statistical significance was determined by Student t-tests using SPSS software (version 12.0). Differences were considered to be significant if P values <0.05 were obtained.

There were no significant differences in weight changes between groups (data not shown). However, analyses of diarrhea scores by a method previously described [4, 5] indicated that pigs of phage treated groups C and D showed greater resistance to ETEC infection than the positive control group B at the third day after challenge (Fig. 1). DNA was extracted each day from 0.1 g feces using the same method as used in standard curve generation, subjected to real-time PCR, and the CT value was substituted (Fig. 2). Both groups C and D showed significant decreases in ETEC, 63.92 and 60.73%, respectively at 4 days post infection. As the ETEC challenge was done for three days, on the first and second days post infection, there was a reduced but not significant decrease of ETEC.

To confirm the activity of orally administered phages, and check whether contamination occurred, the phage concentrations of feces were measured. We detected phage in groups C and D (Fig. 3). At 7 days post phage treatment and 1 day post infection, phage was detected in group D. At 7 days post infection, phage was detected in both groups C and D.

The issue of antibiotic resistance has recently come to the fore, and many studies have shown the therapeutically ef-
ficacy of bacteriophage as a substitute for antibiotics [1, 6, 8, 13, 15]. In addition, in Korea, the addition of antibiotics to animal feed has been prohibited by law since July 2011. Therefore, this experiment was performed using pulverized phage, which could be easily applied to a feed additive.

In this study, we were unable to induce severe diarrhea in both the phage treated group and the positive control group. To establish experimental ETEC infection in pigs, prior oral administration of sodium bicarbonate is often required to address the low pH conditions in the stomach, and florfenicol is also administered prior to ETEC challenge to reduce competing intestinal flora, which may help the ETEC to survive and colonize [4, 6, 7]. However, we did not pretreat the pigs with either sodium bicarbonate or florfenicol, in order to evaluate the effectiveness of the pulverized phage under field conditions. Instead of pretreatment, we infected pigs with high doses of ETEC compared to other studies. Although we were unable to induce severe diarrhea with scores of 3, meaning the frequent passage of watery feces, pigs in the sample exhibited diarrhea scores of 1 or 2, and these pigs were considered to be susceptible to ETEC infection. Based on this criterion, the phage treated group demonstrated a high percentage of resistance to diarrhea due to ETEC infec-
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