Isolation of *E. coli* O157:H7 Bacteriophages of Livestock Waste of Mazandaran Province (Case Study: A Phage Therapy Approach)

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Phage therapy for *E .coli* O157:H7 in animals is considered as a popular research topic. *E .coli* O157: H7 is a pathogenic potential. It can cause hemolytic uremic syndrome (HUS). Considering this matter, the present research aims at analyzing the isolation of *E. coli* O157:H7 bacteriophages of livestock waste of Mazandaran province using a phage therapy approach. The separation and culture processes of *E .coli* O157:H7 from livestock wastewater have been analyzed in this research. Also, differential tests were used to identify *E. coli* O157:H7. First, the Lytic bacteriophages of *E .coli* O157:H7 were isolated from livestock wastewater. Then, both identification and susceptibility tests for bacteria *E. coli* O157:H7 were done and phages were isolated using various methods. Finally, phage therapy to animal model was conducted. In this study, I have used two methods including disk diffusion and overlay tests. This test can be used for three strains standards including Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E.coli* (ETEC), Enteroinvasive *E. coli* (EIEC), and wild *E. coli* O157:H7. According to the results, 45 samples had lytic phages vs. four different *E .coli* strains and only 19 samples had significant effect on *E. coli* O157: H7. Also, it can be concluded that isolated phages affect bacteria in a significant manner (p<0.05). In the current research, the isolated lytic phages removed the infection of *E.coli* O157: H7 of five groups of mice. As a result, the lytic bacteriophages have the ability to replace antibiotics.

Keywords: *E .coli* O157:H7, livestock, Mazandaran, mouse, phage therapy.

The World Health Organization (WHO) has declared antimicrobial resistance as one of the greatest threats to human health. There is urgent need for developing the antimicrobial agents; however, few are in the pharmaceutical pipeline, and the number of new antibacterial drugs was approved for marketing in the United States continue to drop (World Health Organization, 2001). Also, the Infectious Disease Society of America (IDSA) confirmed that the world is in the midst of an emerging crisis of antibiotic resistance for microbial pathogens (Boucher et al., 2009). Hence, *E. coli* O157:H7 has become a worldwide threat to public health and is one of today's most troubling food-borne pathogens. Human illnesses with *E. coli* O157:H7 range from self-limited watery diarrhea and hemorrhagic colitis to life-threatening manifestations such as the hemolytic uremic syndrome and the thrombotic thrombocytopenic purpura. Cattle and sheep, important domestic ruminants, are the primary reservoirs for this human pathogen and are the most common sources for foodborne, waterborne, and direct-animal-contact infections. Successful strategies to control or reduce the carriage and prevalence of *E. coli* O157:H7 in live ruminants would reduce the risk of human exposure to this pathogen (Sheng et al., 2006). Antibiotics have been a panacea in animal husbandry as well as in human therapy for decades. The huge amount of antibiotics was used to induce

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the growth and protect the health. It led to the evolution of bacteria that are resistant to the drug's effects. Today, many researchers are working with bacteriophages (phages) as an alternative to antibiotics in the control of pathogens for human therapy as well as prevention, control, and therapy in animal agriculture. Phage therapy and control have fulfilled their promise or potential yet, largely due to several key obstacles to their performance. Current strategies were employed to combat the rise of multi drug resistance organisms, focus on a more judicious use of antibiotics, and methods to restore the indigenous Microbiota (Knoll et al., 2014). However, *E. coli* O157:H7 has become a worldwide public health concern since it was first identified as a human pathogen in 1982. This pathogen has a very low infectious dose (approximately 10 cells) in humans, and symptoms of infection range from watery diarrhea to hemorrhagic colitis and hemolytic uremic syndrome, and in some cases, death (Rivas et al., 2010). Since ancient times, there have been documented reports of river waters having the ability to cure infectious diseases such as leprosy (Keen, 2012). However in 1896, the British bacteriologist Ernest-Hankin reported antibacterial activity against Vibrio cholera which he observed in the Ganges and Jumna rivers in India. He suggested that an unidentified substance was responsible for this phenomenon and for limiting the outbreak of cholera epidemics. Two years later, Gamaleya, the Russian bacteriologist, observed a similar phenomenon while working with Bacillus subtilis (Adhya et al., 2006). Phages are naturally occurring predators of bacteria. They can be effective antibacterial agents due to their specific role against particular bacterial species and their lack of impact on the other Microflora (Jassim et al., 2014). Bacteriophages are very specific to their hosts, so this minimizes the chance of secondary infections, but antibiotics do target both pathogens and normal flora of patients, which can cause the secondary infections or sometimes super infections. Also, bacteriophages replicate at the site of infection where they are mostly needed to lyse the pathogens, but antibiotics travel throughout the body and do not concentrate on the site of infection. No side effects have been reported during or after phage application, but resistant bacteria, allergies (sometimes even fatal anaphylactic reaction), and secondary infections are the common side effects of antibiotics treatment .Lastly, bacteriophages are environmentally friendly and are based on natural selection, isolating and identifying bacteria in a very rapid process compared to new antibiotic development, which may take several years, may cost millions of dollars for clinical trials, and may also not be very cost effective (Weber-D¹browska et al., 2001). Effective elimination of pathogenic bacteria from gastrointestinal diseases using phage preparation has been demonstrated in multiple experiments that are focused on the therapeutic use of phages (Marinelli et al., 2012). The therapeutic effect of the phages can be limited to a decrease in the pathogen's population down to a point at which the immune system can effectively control its reproduction. Several current strategies to combat livestock-associated pathogens such as toxicogenic *E. coli*, Campylobacter, and Salmonella are direct extensions of "classical" phage therapy approaches in that they focus on targeting the bacteria in animals before slaughter (Sheng et al., 2006). The prevalence of *E. coli* O157:H7 in cattle has been the subject of much research, with most studies concluding that at any given time the majority of cattle are negative for *E. coli* O157:H7 (La Ragione et al., 2009). So, some studies have cited sheep products as important sources of *E. coli* O157:H7. Phages specific for *E. coli* O157:H7 have previously been isolated from human fecal materials or animal manures from bovine, ovine, swine, and chicken, lake or pond water, and sewage (Tomat et al., 2014; Shahrbabak et al., 2013). The clinical syndromes, pathogenic characteristics, the pathobiology of these organisms and the toxins they produce are reviewed in Kruger and Lucchesi (Krüger et al., 2015). The aim of this study was to isolation of *E. coli* O157:H7 bacteriophages of Livestock waste of Mazandaran province; a phage therapy approach.

MATERIAL AND METHODS

For preparing wild phages and *E. coli* O157:H7, 70 and 30 samples were respectively collected from many Stockyards in Tonekabon that located in north of Iran. For this, wastewater contains all kinds of microbes like *E. coli* O157:H7and phages harvested by sterile dishes.

Then, three standard strains Enteroinvasive *E. coli* (EIEC ATCC 43893), Enteropathogenic *E. coli* (EPEC ATCC 43887), and Enterotoxigenic *E. coli* (ETEC ATCC 35401) provided from Pasteur Institute of Iran were used to evaluate the phages.

Isolation of bacteria from livestock wastewater

For isolation of *E. coli* O157:H7, 1 ml of wastewater was solved in 9ml of physiological serum, then a serial dilution (10^{-7} to 10^{-1}) was prepared, then concentrations of 10^{-5} and 10^{-4} were selected for culturing. It should be noted that for ensuring the accuracy of special antiserum, we tested it by three standards strain and wild *E. coli* O157:H7, the results indicated agglutination with *E. coli* O157:H7. It means that our antiserum act in an appropriate manner (Wagner, 1992).

Isolation of special bacteriophages from livestock wastewater

For isolation of special phages, 15 ml wastewater was prepared and was centrifuged for 25 min in 3500×g, and then supernatant was filtered via 0.45µm. Then 8.5 ml of filtered liquid was harvested to mix by 10 ml of Nutrient broth media. Then 100µl from *E. coli* O157:H7 was added to it, and was incubated at 37°c in 150 rpm for 18 hours, in shaking station to airing. Then, it was centrifuged again in 10000×g for 15 min, and then it was filtered through 0.45µm. At last supernatant was gathered for assessing phage activity by two methods: diffusion disks and overlay methods (Ferens et al., 2011).

Differential tests for confirmation of *E. coli* O157:H7

These tests included: growth on Eosin Methylene Blue, Triple Sugar Iron agar, Simmons Citrate, Sorbitol MacConkey agar, SIM media, gram staining, oxidase, catalase, urease ,H_2S and CO_2 production, glucose and lactose fermentation , IMVIC, , MRVP, and special antiserum for strain *E. coli* O157:H7 (World Health Organization, 2001).

Agglutination test by special antiserum for *E. coli* O157:H7

In this test, the colonies which were isolated from Sorbitol MacConkey agar media were suspected to *E. coli* O157:H7 (colorless colonies/ sorbitol-negative). They were selected for agglutination test by *E. coli* O157:H7 special antiserum. In this method, at first, we pilled one droplet normal saline (as control) in right side of slide and one droplet antiserum in left side. Then,

we added some *E. coli* O157:H7 colonies to each droplet. Then we mixed them completely. Then we shook slide for 60 second slowly for observing agglutination process. Formation of agglutination means that existence of *E. coli* O157:H7.

Detecting *E. coli* O157:H7 and *E. coli* O157:H7 Special Bacteriophages

Finally, the strain of *E. coli* O157:H7 was detected by using of Sorbitol MacConkey agar and special antiserum. As well, the effective bacteriophages vs. *E. coli* O157:H7 was detected by disk diffusion and Overlay tests. All isolated phage samples were tested on three *E. coli* standard strains (ETEC, EPEC, and EIEC) and wild *E. coli* O157:H7.

Effect of different antibiotics on *E. coli* O157:H7

Disks diffusion test contain Tetracycline, Ampicillin and Gentamycin are used to evaluate the sensitivity of wild *E. coli* O157:H7. For this purpose, wild *E. coli* O157:H7 bacteria were cultured in Mueller-Hinton media and relevant antibiotics placed on the media and for 48 hours were incubated at 37°C and then the plates were examined.

Disk diffusion test contain specific bacteriophage

In this method, fresh disks were put into final supernatants for 24 hours, and then were incubated at 37°C. To determine the effect of isolated phages, the disks were dried and were put on Nutrient agar media (that newly cultured by *E. coli* O157:H7) by 2cm distance, then were incubated for 24 hours at 37°C (Payne et al., 2003).

Overlay

Overlay culture was used for this test. First, we cultured *E. coli* O157:H7 on Nutrient agar. Then 400 µl final filtered liquid was piled on that cultured media. Then, 3 ml of Nutrient agar as semi-solid (0.75%) was added to it, then plates were incubated for 18-24 hours, at 37°C (Ibid).

Animal stage

For evaluation the effect of phages on bacteria, we divided all mice to six groups, a control group as well as five treated groups. Treated groups include: 1-5. So, groups 1-4 each groups were collected from a different region in north of Iran. Also, fifth group was a mixed of groups 1-4. The isolated lytic phages were those four samples with maximum effect vs. *E. coli* O157:H7 out of initial samples, so these samples had been detected

by their diameter in disk diffusion method. These samples were tested on male 6-weeks old mice for 14 days on six groups of by three repeats. The treating of animals lasted 2 weeks. In the first day, all mice (control and others) received 100 µl of wild *E. coli* O157:H7 liquid (1×10^7 cfu /ml), then after 2 hours, all mice except control group received 100 µl phages by two methods: intraperitoneal injection and gavaging, 2-times by 3 hours interval (1×10^8 pfu /ml). For preventing digestion of phages in mice gastric acidity, we used calcium carbonate (0.25%) during gavaging. From first day to fourteen, the mice received phages by the same manner.

At day 14, 1 gr stool of mice was collected for testing the existence of *E. coli* O157:H7. The dilutions of stools (10^{-3} and 10^{-4}) were prepared and were cultured on EMB, then they were tested by: gram staining, oxidase, urease, IMVIC, MRVP, growth on the Simmons Citrate, Triple Sugar Iron agar, SIM, Sorbitol MacConkey agar, media and special antiserum to evaluate the existence of *E. coli* O157:H7. Also, the overlay method tested on stools of mice, for evaluating existence of phages (Chapman et al., 2000; Thorpe et al., 2002).

RESULTS

Culture mediums and Biochemical tests

The isolated bacteria from wastewater was cultured on Eosin Methylene Blue media, indicated green metal shining in Eosin Methylene Blue that is a feature of *E. coli* O157:H7.

In catalase test, from biochemical tests, the samples produced the bulb of air that is an indication of positive-catalase feature. Also, produced blue from oxidase test showed that the samples contained the negative-oxidase bacteria. Indeed, in microscopic observation, the shape of the bacterial sample was negative-gram bacillus. In Triple Sugar Iron agar media, the isolated bacteria changed color of media to yellow (acid-acid) due to fermenting of glucose and lactose as well as gas formation, but no H_2S produced (Figure 1). In SIM media, formation of red ring on surface was observed, that is an indicator of positive-Indole feature of bacteria (using Kovacs reagent). The movement of bacteria observed (it means it have been a flagellated bacteria). Also, no black color was observed that means that no H_2S produced, it

means the bacteria are H_2S-Negative (Figure 1). In IMVIC test, regard to MR test (by methyl red indicator), constant red color on the surface is an indicator of acid formation due to lowering pH under 4.4, so the test is positive. In VP test (by Voges- Proskauer indicator) red color during 15 minute after adding the indicators is an indicator of positive feature, because the existence of di-acetyl is an indicator of acetoin but the isolated bacteria did not red color, that means the bacteria is positive-MR. In citrate test (Simons citrate media), the bacteria did not change the media color and no growth was observed, it means that they were not able to use citrate as carbon source (negative-citrate); so we know that formation of blue color after 24-48 hours is an indication of citrate consumption and positive featuring. So biochemical results validated that the samples contained *E. coli*; following table is showed the results of biochemical test on sample (Table1).

Special antibody test

Colonies isolated from Sorbitol MacConkey agar media was agglutinated, that agglutination is an indication of existence of *E. coli* O157:H7 (Figure 2).

Growth on Sorbitol MacConkey agar media

The isolated bacteria produced colorless colonies on Sorbitol Macconkey agar that is a feature of *E. coli* O157:H7. Because *E. coli* O157:H7 is not able to fermentation of sorbitol in Sorbitol MacConkey agar, so it produces the colorless colonies, therefore, it can be distinguish from other strains of *E. coli* that produce pink colonies.

Phages stage

For isolation of *E. coli* O157:H7 special phages, the number of 70 samples were gathered from livestock wastewater in Tonekabon city. In this study we used two methods disk diffusion and overlay tests. In this test were used three strains standard: EPEC, ETEC and EIEC and wild *E. coli* O157:H7. Forty five from 70 samples had lytic phages vs. four different *E. coli* strains, only 19 samples had significant effect on *E. coli* O157: H7 (Table2).

Disk diffusion test

This test was done for all 70 samples and against all three standard strains as well as for wild *E. coli* O157:H7. The results from disk diffusion test revealed that the diameter of halos formed by phages was between 10-11 mm (Figure 3). Nineteen

Table 1. The results of biochemical tests and growth on SIM and TSI media

Movement	SIM			TSI		
	H_2S Formation	Indole-formation		Acid-acid acid	Gas formation	H_2S
+	-	+		+	+	-
		IMVIC			Other biochemical	
Citrate	Indole	MR	VP	Urea hydrolysis	catalase	Oxidase
-	+	+	-	-	+	-

Table 2. The results of phages isolation from all samples

Number of Samples	Type of phages
11	Lytic phages effective on all four strains
5	Lytic phages effective on EPEC
10	Lytic phages effective on EPEC and ETEC
19	Lytic and special phages effective on *E. coli* O157:H7

Table 3. Evaluation of antibiotics on isolated *E. coli* O157:H7

Antibiotics	Effects	Diameter
Tetracycline	No sensitive	-
Ampicillin	Sensitive	14mm
Gentamycin	Sensitive	15mm

samples had lytic and special effect against *E. coli* O157:H7, but no effect on other strains, so these phages are special for *E. coli* O157:H7.

Overlay method

The results in this method indicated some plaques were formed due to interaction of bacteriophages and *E. coli* O157:H7 on Nutrient agar media. These plaques formed due to inhibitory effect of bacteriophages on *E. coli* O157:H7. It means that the samples had enough special bacteriophages against *E. coli* O157:H7 (Figure 4).

Effect of different antibiotics on *E. coli* O157:H7

Disks diffusion test contain Tetracycline, Ampicillin and Gentamycin are used to evaluate the sensitivity of wild *E. coli* O157:H7. This test indicated that isolated *E. coli* O157:H7 had different sensitivity to different antibiotics. This test indicated that wild *E. coli* O157:H7 was resistant to Tetracycline but sensitive to isolated special phages and some antibiotics such as Ampicillin and Gentamycin, in this study (Table 3).
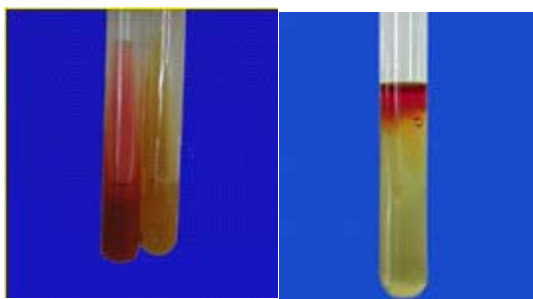
The results of phage therapy

Five phage samples have included most effective among all of them against *E. coli* O157:H7 (based upon halos diameter in diffusion disk method and number of plaque in overlay) were selected for phage therapy stage. This stage lasted for 14 days, so at fourteenth day the excretion of animal gathered for assessing whether have any *E. coli* O157:H7, as well as in the same day, we tested existence of bacteriophages from excretion of mice (by disk diffusion and overlay methods). In this stage, in the control group (only received bacteria) showed low-movement as well as diarrhea after 5th days, then inflammation in tail and face and severe diarrhea after 8th days, so at days 10, 12 and 13th, we observed death of mice. In five different groups of experiment, for example first and fourth groups, one of the animal's death at day 12th due to diarrhea, vomiting and inflammation-Bleeding in tail and face area. In the other groups (2, 3 and especially mixed phage group), no death is observed (due to special antibacterial effect of isolated phages). So according these results, we can say that the phages could survive infected mice by *E. coli* O157:H7 from given death, significantly ($p < 0.05\%$).

**Approval test for existence of *E. coli* O157:H7 in excretion of mice**

Finally, we tested the excretion of all mice to ensure all of them received either *E. coli* O157:H7

or bacteriophage. The results from culture of excretion of mice on plates showed some plaques on overlay of *E. coli* O157:H7. These results indicated that there were some phages in excretion of 5 groups of mice under phage therapy. Recent studies have shown high efficacy of using phages against *E. coli* O157:H7. Use of *E. coli* O157:H7 special phages resulted in significant and log-unit reduction in *E. coli* O157:H7 numbers. In this way, we observed live *E. coli* O157:H7 from excretion of control group, so it would be expected; and special phages vs. *E. coli* O157:H7 from groups 1, 2, 3, 4 and 5.

Fig. 1. Triple Sugar Iron agar media (A), SIM media (B)
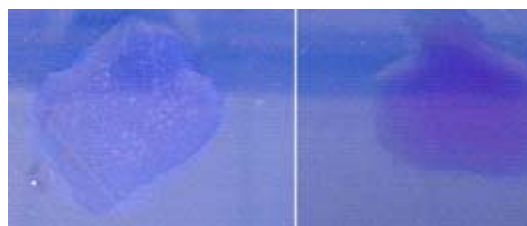
Fig. 2. The results of special antiserum, (A) antiserum (agglutination), (B) control, normal saline (no reaction)

Fig. 3. Evaluation of phage's effect on *E. coli* O157:H7 using disk diffusion method

Fig. 4. The effect of Phage on *E. coli* O157:H7 using overlay method and plaque formation

DISCUSSION

This study successfully demonstrated that special bacteriophages reduced inoculated *E. coli* O157:H7 levels in animal model. One of the few published studies that have used animal system to evaluate the activity of phage against *E. coli* O157:H7 is a study by Bach et al (2009) also, *E. coli* O157:H7 was found to survive the animal model for over 24 h, whereas Bach et al (Ibid) reported a natural decline of *E. coli* O157:H7 in a continuous system over a longer incubation time (192 h). Indeed, *E. coli* O157:H7 in the present study were also found to be recovered in the rumen but were found to decline over time (92 h). Shedding of *E. coli* O157:H7 also appeared to vary among animals in the trials, but the numbers of *E. coli*

O157:H7 shed usually decreased over time, and most animals remained *E. coli* O157:H7 enrichment positive through to the end time of the trials. Other researchers have reported heterogeneity of *E. coli* O157:H7 carriage and excretion rates among animals. The factors that contribute to the persistence of *E. coli* O157:H7 in animal model are unclear, but it has been suggested that the model may be a transient site for *E. coli* O157:H7 and that the microorganism primarily colonizes at the recto-anal junction (RAJ) of the animal (Naylor et al., 2003). It was previously reported that the application of phage directly to the RAJ, as well as the addition of the phage to the drinking water, significantly reduced *E. coli* O157:H7 populations but did not eliminate the organism in cattle (Walker et al., 2010). It was encouraging to find that the

phages in the present study were recoverable from the feces of the yearling animals, as it demonstrated that the phages can survive transit through the GITs of these animals. It may be possible in the future to incorporate the phages into protective delivery systems, such as microencapsulation, which could aid in increasing the protection of the phages from inhibition and successfully transferring higher numbers of phages to target areas within the GIT, such as the RAJ (Bach et al., 2009). So, we can say, bacteriophage therapy has a potential for many applications, most importantly for the prevention and treatment of infections due to multiple drug resistant organisms (Merabishvili et al., 2012). 45 samples from 70 were contained lytic phages. Among these, 19 samples had specific lytic activity against *E. coli* O157:H7. The phages in this study had isolated from wastewater. Finally it was observed that 11 samples were active against all four strains. Plaque formation and antibiogram test by overlay method and disks was detected, respectively. Five samples effect on EPEC, so had specific phages vs. bacteria, and ten samples on both ETEC and EIEC. At last, 19 samples were active against *E. coli* O157:H7. Among these methods, overlay used for rapid detection, but antibiogram was the best method. Capparelli et al investigated on lytic bacteriophages isolated from cow manure and its impact on bacteria *E. coli* O157:H7 in mice. Phages acquire stability in the circulatory system of mice within 38 days. After infecting mice with bacteria (1×10^7 cfu/ml) dose, bacteria were gone from the animal within 48 hours, so about 60% of the mice survived and 40% died. The mortality will occur due to the release of Shiga toxin and rapid lysis of bacteria by phages (Capparelli et al., 2006). Tanji et al (2005) evaluated the combination effect of three bacteriophage sp15-sp21-sp22 on *E. coli* O157:H7 in the digestive tract. Addition of one or two phages reduced the accumulation of bacteria under anaerobic conditions. Reduction in concentration of bacteria was observed after adding phage (1×10^8 pfu /ml). Repeat in addition of phage cocktail *E. coli* was effective in reducing the phage cocktail. Existence of bacteria in the gastrointestinal tract of mice was decreased when the excretion was full of phages. Repeating administration of phage (sp15-sp21-sp22) was useful for purifying *E. coli* O157:H7 from manure and digestive tract of mice (Tanji et al., 2005). In

presence study, we repeat the processes of feeding in rats; it resulted in clearing phages from the digestive tract of mice. Also phage suspension (25.0% calcium carbonate) could maintain the pH of the stomach in favorite manner for phage therapy. The main differences of our work with Tanji et al (2005) was that we administrated phages on the same day that the bacteria entered into mice but they long it two days more. In this study, to evaluate the effect of specific lytic phages isolated from sewage, an animal model was used. Six Groups in 3 repeat selected in similar conditions. The results showed that four specific lytic phage effect on *E. coli* O157:H7 but one group was not. In mixed group, there were not any significant differences, but in the control group, on the second day after gavage diarrhea was observed that resulted in a general weakness, loss of mobility and weakening of the mice. The results showed the effectiveness of the phage about lack or inhibition of growth of bacteria that can confirm the presence of phage effect in the treatment of *E. coli* O157:H7.

CONCLUSION

Bacteriophages are a diverse group of viruses which are easily manipulated, and therefore have potential uses in biotechnology, research, and therapeutics. It is a fact that, now, from years ago, we have reached a critical point in treating infectious diseases: new drugs are not being developed at anywhere near the pace necessary to keep ahead of the natural ability of bacteria to evolve and defend themselves against antibiotics. The result is that some of our most powerful drugs are becoming useless. Finally, it can be said that the isolated phages was highly specific for *E. coli* O157:H7. Several suggestions are shared in order to point a direction for overcoming common obstacles in applied phage technology. The key to successful use of phages in modern scientific, farm, food processing and clinical applications is to understand the common obstacles as well as the best practices and to develop answers that work in harmony with nature.

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