RESEARCH ARTICLE

Evaluation of the effectiveness of bacteriophage therapy against Salmonella infections in mice

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Farelerde Salmonella enfeksiyonlarına karşı bakteriyofaj tedavisinin etkinliğinin belirlenmesi

Öz

Amaç: Antibiyotiğe dirençli salmonella kaynaklı enfeksiyonlarının artışı dünya genelinde önemli sorunlara neden olmaktadır. Bu çalışmada, alternatif uygulama olarak sığır dışkılarından izole edilen dört ΦSP – 3 litik faj (*Salmonella* Dublin, *S.* Typhimurium, *S.* Anatum, *S.* Kentucky) ile kokteyl hazırlanarak fare modellerinde bakteriyofaj tedavisinin etkinliğinin araştırılması amaçlandı.

Gereç ve Yöntem: Bakteriyofaj tedavi için 4 farklı salmonella bakteriyofaj izolatından kokteyl hazırlandı. Toplam 80 fare (toplam 8 deneme grubu ve her grupta 10 fare) salmonella türleri ile oral yolla çelınç yapıldı. Çelınç sonrası, farelere oral yolla bakteriyofaj kokteyli verildi. Fareler 20 gün boyunca hastalık oluşumu ve ölüm yönünden gözlendi. Aynı zamanda, dışkı ile salmonella türlerinin saçılımı üzerine bakteriyofaj tedavinin etkisini belirlemek için dışkı örnekleri bakteriyolojik olarak incelendi.

Bulgular: *S.* Dublin ve *S.* Typhimurium ile çelınç yapılan 2'şer farede hastalık ve ölüm gözlendi. Ayrıca, sadece ölen 2'şer farenin iç organlarından *S.* Dublin ve *S.* Typhimurium izolatlarının geri izolasyonu yapıldı. *S.* Kentucky ve *S.* Anatum antijenleri ile çelınç yapılan farelerde hastalık ve ölüm vakası gözlenmedi ve farelerin iç organlarından salmonella izolasyonu yapılmadı. Bununla birlikte, tüm gruplardaki farelerin dışkı örneklerinden salmonella türlerinin geri izolasyonları yapıldı.

Öneri: Sığırların dışkısından elde edilen bakteriyofaj kokteyllerinin farelerde mortalite ve morbiditeyi engellediği ve dışkı yoluyla *Salmonella* spp. saçılımını azalttığı belirlendi. Bu sebeple salmonella enfeksiyonlarına karşı korunmada bakteriyofaj terapinin kullanılabileceği kanaatine varıldı.

Anahtar kelimeler: Bakteriyofaj, tedavi, fare, Salmonella

Abstract

Aim: The increase of infections caused by antimicrobial resistant salmonellae has become a serious problem worldwide. In this study, it was aimed to investigate the efficacy of bacteriophage treatment in mouse models by preparing a cocktail with four ΦSP-3 lytic phages (*Salmonella* Dublin, *S.* Typhimurium, *S.* Anatum, *S.* Kentucky) isolated from cattle feces as an alternative application.

Materials and Methods: A total of 80 mice (total 8 experimental groups and each group included 10 mice) were challenged with salmonella strains by oral route. After challenge, bacteriohage coctail to mice were administrated by oral route. Mice were observed for occurence of morbidity and mortality for 20 days. Also, faecal samples were bacteriologically examined to determine the effect of bacteriophage treatment on the spreading of Salmonella species with feces.

Results: The morbidity and mortality were observed in two mice, administered bacteriophage coctail following challenge with *S.* Dublin and *S.* Typhimurium. In addition, re-isolation of *S.* Dublin and *S.* Typhimurium from internal organs in 2 death mice were done. The morbidity and mortality in mice challenged with *S.* Kentucky and *S.* Anatum and administered bacteriophage coctail was not observed and re-isolation from internal organs were not carried out. However, re-isolation from feces of mice in all groups were made.

Conclusion: The findings of present study revealed that bacteriophage cocktails obtained from cattle faeces prevented mortality and morbidity in Salmonella infected mice, and reduced the spread of Salmonella spp. Therefore, bacteriophage therapy could be used for protection against salmonella infections.

Keywords: Bacteriophage, therapy, mice, Salmonella

Introduction

Bacteriophages or phages disrupt bacterial metabolism and cause bacteria to lyse (Sulakvelidze et al 2001). Prior to the discovery of antibiotics, phage therapy had been used to treat infected animals for nearly a hundred years (Chanishvili et al 2001). Phages are rather common in the environment, and it is known that they can infect nearly 4140 bacterial genera (Sulakvelidze et al 2001). Phages are of two main types, which are referred to as lytic and lysogenic bacteriophages (Goode et al 2003). As bacteriophages bind to spesific receptors on bacteria, bacteriophages have no side effects on mammalian cells. This specificity provides bacteriophages an important advantage in the treatment of bacterial infections (Clark and March 2006). The use of phages has also significantly contributed to molecular biology and biotechnology (Bradbury et al 2004). It is estimated that bacteriophages may reach a number of 10^{32} in the environment (Coward et al 2006). In view of their enabling the effective treatment of infectious diseases, antibiotics are considered as the most important discovery in the history of medicine (Banin et al 2017). However, the misuse and overuse of antibiotics for the treatment of bacterial infections caused emergence and dissemination of multidrug resistant (MDR) bacteria. This caused a concern in both veterinary medicine and human medicine (Jensen et al 1998). Therefore, it is needed to develope alternative strategies to combat for the treatment of bacterial infections in humans and animals (Barrow et al 1998).

Although having been used in the past for the treatment of infectious diseases owing to their therapeutic efficacy, phages are known to have lost popularity after the discovery of antibiotics (Kropinski 2006). However, recently, bacteriophage therapy has regained interest as an alternative method and several commercial forms are available on the market for the control and treatment of infectious diseases (Connerton et al 2004). Phage therapy has mostly been tested in animal models for use in public health. Phage therapy also has an important place in the control of zoonotic foodborne pathogens (Abedon et al 2011). Research has been conducted on the impact of phages in reducing the spread of *Escherichia coli* O157:H7, *Salmonella* spp. and *Campylobacter jejuni*, which are part of the microbiota of cattle, poultry and pigs (Barrow et al 1998, Augustine and Bhat 2014, Goode 2003).

Salmonellae, coronaviruses, rotaviruses, enterotoxigenic *E.coli* (ETEC) and Cryptosporidium parvum are main causative agents responsible for infectious diarrhoea in farm animals and the diarrhoea is particularly prevalent during the first 3 months of life in calves (Izzo et al 2011). *Salmonella enterica* subsp. *enterica* has over 2300 serovars and many serovars colonize the digestive of cattle (Fossler et al 2005). The lytic efficiency of a phage cocktail was reported to be high against *S.* Typhimurium and *S.* Enteritidis isolates from various farm animals (Petsong et al 2019).

This study was aimed to investigate the efficacy of bacteriophage treatment in mouse models by preparing a cocktail with four ΦSP-3 lytic phages (*S.* Dublin, *S.* Typhimurium, *S.* Anatum, *S.* Kentucky) isolated from catle feces as an alternative application.

Material and Methods

Preparation of bacteriophage cocktails

Salmonella phages were obtained by a two-step procedure: isolation of Salmonella strains and phage enrichment with host-specific salmonellae by a modified method. Firstly, a total of 40 Salmonella strains were isolated from bovine intestinal contents, as described previously (Hadimli et al 2017), and then Salmonella phages were obtained using a direct procedure and enriched with host-specific salmonellae. Based on the results obtained for multiplicity of infection (MOI), lytic activity, host range, and genotyping of the phages (Sakmanoglu and Hadimli 2020), in total four ΦSP–3 lytic phages were chosen and used to prepare cocktails against experimental model infections of mice caused by *S.* Dublin, *S.* Typhimurium, *S.* Anatum, and *S.* Kentucky.

The experimental mice model for bacteriophage therapy

To determine the effectiveness of the phage treatment, 4 experimental groups, consisting of 20 mice each were formed for *S.* Typhimurium, *S.* Dublin, *S.* Anatum, and *S.* Kentucky. Each group comprised control and phage-treated challenge subgroups as well. A 2-mL lethal dose (LD)50 of 1x107 colony-forming units (CFU) of each of the *S.* Typhimurium, *S.* Dublin, *S.* Anatum, and *S.* Kentucky group was administered by oral route to all four groups (Hadimli et al 2011). Then, 50 mL of each of the four phage cocktails were orally administered to the treatment subgroups at 1, 12, and 24 h. Mice in the control groups were not given the bacteriophage cocktails (Zimecki et al 2009). All mice were checked daily and observed for 20 days. Morbidity and mortality were recorded. Fecal samples were taken from all groups for bacteriological analysis at 2-day intervals. Also, microbiological examinations of the internal organs of mice that died or were euthanized after 20 days were performed (Hadimli et al 2005).

Investigation of the spread of Salmonella spp. in phage-treated mice

To monitor the spread of the indicated agents, faecal samples were collected from all mice groups every two days. *Salmonella* spp. was isolated according to the ISO 6579 standard of the International Standards Organization (ISO). *Salmonella* spp. were isolated following a three-step procedure of pre-enrichment (2.5 g of faeces, added to 22.5 mL of buffered

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peptone water, was incubated at 37 °C for 24 h), selective enrichment (preenrichment culture, added to 1 mL of a sample in Rappaport-Vassiliadis medium, was incubated at 42 °C for 24 h), and isolation (samples, passaged onto xylose lysine deoxycholate (XLD) and/or xylose lysine tergitol-4 (XLT-4) agar, were incubated at 37 °C for 24–48 h). Black colonies on XLT-4 agar or colourless colonies with darker centres on XLD agar were suspected of being salmonellae (Fricker 1987, Nye et al 2003).

Results

In the control groups, morbidity or mortality were detected in almost half of the mice challenged with salmonellae (*S*. Dublin, *S*. Typhimurium, *S*. Anatum, *S*. Kentucky). According to the results of bacteriophage therapy, morbidity and mortality were observed in two mice (20%) from each of the phage-treated challenge groups infected with *S*. Dublin and *S*. Typhimurium (Table 1).

Bacteria were re-isolated from the internal organs of two mice (20%) from each of the two challenge groups infected with *S*. Dublin and *S*. Typhimurium. In the control subgroups, the re-isolation percentages of the strains ranged from 40% to 100%. The highest rate (100%) was achieved with the re-isolation of *S*. Typhimurium from intestinal tissue, whereas the lowest rate (40%) was observed with the isolation of *S*. Kentucky from intestinal tissue (Table 2).

It was determined that the spread of *Salmonella* spp. in the control groups was at significantly higher rates than in the phage-treated challenge subgroups. In the challenged groups, all *Salmonella* spp. strains were isolated from the faeces of mice. However, *S*. Dublin and *S*. Typhimurium were isolated at higher rates (Table 3).

Table 1. Rates of morbidity and mortality in the groups			
Group	Agent	Morbidity	Mortality
Phage	*S*. Dublin	2/10	2/10
	S. Typhimurium	2/10	2/10
	S. Anatum	0/10	0/10
	S. Kentucky	0/10	0/10
Control	*S*. Dublin	6/10	6/10
	S. Typhimurium	5/10	5/10
	S. Anatum	5/10	4/10
	S. Kentucky	5/10	5/10

Table 2. Microbial examination results of experimental models						
Group	Agent	Liver	Spleen	Kidney	Heart	Lung	Intestine
Phage	*S*. Dublin	2/10	2/10	2/10	2/10	2/10	2/10
	S. Typhimurium	1/10	2/10	1/10	1/10	1/10	2/10
	S. Anatum	0/10	0/10	0/10	0/10	0/10	0/10
	S. Kentucky	0/10	0/10	0/10	0/10	0/10	0/10
Control	*S*. Dublin	7/10	7/10	7/10	7/10	7/10	8/10
	S. Typhimurium	9/10	7/10	9/10	9/10	7/10	10/10
	S. Anatum	5/10	5/10	5/10	5/10	5/10	6/10
	S. Kentucky	5/10	4/10	5/10	5/10	5/10	7/10

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Table 3. The spread rates of Salmonella spp. with the mice feces										
	Agent	Sampling (day)								
		4	6	8	10	12	14	16	18	20
Phage	*S.* Dublin	3/8	4/8	2/8	1/8	2/8	2/8	1/8	3/8	2/8
	S. Typhimurium	2/8	3/8	1/8	1/8	1/8	2/8	1/8	1/8	2/8
	S. Anatum	1/10	0/10	1/10	1/10	2/10	2/10	2/10	2/10	1/10
	S. Kentucky	2/10	2/10	0/10	3/10	2/10	1/10	3/10	0/10	1/10
Control	*S.* Dublin	4/5	5/5	4/4	4/4	3/4	3/4	4/4	4/4	4/4
	S. Typhimurium	9/9	5/8	5/7	4/6	4/5	4/5	4/5	4/5	4/5
	S. Anatum	6/10	6/8	7/8	5/7	6/7	5/6	5/6	5/6	5/6
	S. Kentucky	9/10	9/9	8/8	7/8	6/7	6/6	6/6	6/6	6/6

Discussion

Increasing resistance of pathogenic bacteria against antibacterial agents requires the development of alternative strategies to treat infectious diseases. Phage therapy, a previously used method, is a potential alternative (Barrow et al 1998, Jensen et al 1998, Coward et al 2006, Kropinski 2006, Banin et al 2017). In the treatment of chronic infections caused by nosocomial MRSA, experimental phage therapy can be an alternative to antibiotics. In addition, the use of phages can be preferred to antibiotics to decrease treatment-related expenses (Miedzybrodzki et al 2007). Although resistance rarely develops in lytic phages, it may prevent the effectiveness of phages. Also, it has been reported that bacterial resistance may develop against phages (Carlton et al 1999).

Following the discovery of antibiotics, while the use of phages in the treatment of infectious diseases decreased in the western part of the world, it has continued in Eastern countries (Levin and Bull 2008, Kropinski 2006). When compared to antibiotic treatment, the primary advantages of phage therapy are the microbiota remaining undamaged and the specificity to bacterial genera (Fishetti 2008).

To date, phages have been used in different areas for different purposes. The US Food and Drug Administration (FDA) has approved the use of six types of phages for the detection of contamination with *Listeria monocytogenes* in convenience food (Hudson et al 2005). Also, phage therapy has a very significant place in the control of zoonotic pathogens as it effectively decreases possible transmission by food (Augustine and Bhat 2014, Levin and Bull 2004, Fossler et al 2005, Kropinski 2006). There are alternative practices related to bacterial vaccines developed in view of the structure of phages (Barrow et al 1998, Goode et al 2003). Phages have been used for both prophylactic and therapeutic purposes in wound infections of soldiers related to gaseous gangrene (Sulakvelidze et al 2001).

With a view to improve the control and prevention of diseases caused by different bacteria in animals, several studies have been conducted for the investigation of the effectiveness of phages by experimental modelling. Only studies in mice have been evaluated here. Smith and Huggins, (1982) reported that single dose anti K1 phage therapy was more effective than multiple intramuscular doses of tetracycline, ampicillin, trimethoprim, sulfafurazole and chloramphenicol in mice infected with a potential lethal dose of 3×10^8 cfu/ml-1 *E. coli* K1. Biswas et al., (2002) reported that they administered a single intraperitoneal dose and two high doses (10^9 and 10^8 pfu) of lytic ENB6 and C33 phages intraperitoneally to mice infected with vancomycin-resistant *Enterococcus faecium* (VRE), and no death was observed in VRE bacteremic mice. Matsuzaki et al., (2003) reported that bacteremia and death occurred in mice injected with 8×10^8 cfu MRSA intraperitoneally, while deaths were prevented in mice given bacteria and ΦMR11 phage suspension. Boury et al. (2005), tested the ability of a well-known salmonella bacteriophage, Felix 01 and two recently isolated phage (HL03 and HL18) to reduce the *S.* Typhimurium burden in orally challenged, susceptible mice. Felix01 and HL03 were both ineffective when given an hour before or an hour after challenge, but consistently lowered the bacterial burden in mice when given at the same time as the challenge dose. It indicated that bacteriophage-based therapy may be an alternative to antibiotic-based treatments to lower the Salmonella levels. McVay et al., (2007) administered a single dose of 3 different Pseudomonas aeruginosa phage cocktails (each 108 PFU) subcutaneously, intramuscularly and intraperitoneally to mice with burn wounds and infected with lethal dose of P. aeruginosa. Although mortality declined with all administration routes, the best results were achieved with the intraperitoneal route. Zimecki et al., (2009) reported that stated that specific phage administration to immunosuppressive mice infected with S. aureus was highly effective and might provide a potential a potential benefit in immunosuppressive patients exposed to bacterial infections. Dissanayake et al. (2019) reported that a study to investigate efficacy of bacteriophage cocktail to reduce a human patho-

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genic *E. coli* O157:H7 in experimentally infected mice, and determine how bacteriophages impact the normal gut microbiota compared with antibiotic therapy. Finally, it has been stated that bacteriophage cocktail was effective in reducing viable *E. coli* O157:H7 in infected mice with a similar efficacy to ampicillin therapy. Dallal et al. (2019) reported that the animal model showed that mice infected with *S*. Enteritidis developed hepatomegaly and splenomegaly, but did not experience gastrointestinal complications after receiving the bacteriophages. The authors stated that phage SE20 was a promising candidate for controlling salmonellosis caused by *S*. Enteritidis.

In this study, morbidity and mortality were observed in 2 mice each given bacteriophage cocktail and challenged with *S*. Dublin or *S*. Typhimurium. However, no morbidity or mortality was observed in mice challenged with *S*. Kentucky or *S*. Anatum. However, morbidity and mortality were observed in almost half of the mice challenged with salmonella species (*S*. Dublin, *S*. Typhimurium, *S*. Anatum, *S*. Kentucky) in the control group. While re-isolation of Salmonella species from internal organs of mice in control group was high, agents were isolated from internal organs of mice (only 2 mice each died) challenged with *S*. Dublin and *S*. Typhimurium and given bacteriophage cocktail. In the control group, while the re-isolation numbers of salmonella isolates from the fecal samples of mice challenged with salmonella species (*S*. Dublin, *S*. Typhimurium, *S*. Anatum, *S*. Kentucky) were quite high, the re-isolation numbers of the agents from the fecal samples of mice challenged with salmonella strains and given bacteriophage cocktail were relatively low. However, re-isolation was mostly detected in the *S*. Dublin and *S*. Typhimurium subgroups and the least in the *S*. Kentucky subgroup. In mice challenged with *S*. Dublin and *S*. Typhimurium species, 2 death cases, re-isolation of agents from internal organs and re-isolation numbers of agents from fecal samples (*S*. Dublin and *S*. Typhimurium) were determined to be higher than that of other group (*S*. Kentucky and *S*. Anatum). This could be explained by the fact that the bacteriophages found in the bacteriophage cocktail had no host specificity for *S*. Dublin and *S*. Typhimurium serovars. When the results of this study compared with with the results of other researchers working on a similar subject, it was concluded that bacteriophages can be used as an alternative to control infections caused by bacterial agents.

Conclusion

It was considered that bacteriophage therapy was can be useful for protection against salmonella infections.

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Conflict of Interest

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Ethical Approval

This research was approved by the Ethical Committee of Faculty of the Veterinary Medicine, Selçuk University, Konya, Turkey (Ethical Committee No: 2012/008).