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Bacteriophage preparation for Treatment and Prevention of Salmonella Infection in Poultry

T. Gabisonia[a], M. Loladze[a], N.Chakhunashvili[b], N. Tamarashvili[b]*, M. Nadiradze[b], T. Katamadze[b], T. Eliava[b], T. Kalandarishvili[b], M. Alibegashvili[b]

[a]Petre Shotadze Tbilisi Medical Academy, 51/2, Ketevan Tsamebuli Ave., Tbilisi, 0144, Georgia

[b]G. Eliava Institute of Bacteriophages, Microbiology and Virology 3, Gotua str., Tbilisi, 0160, Georgia

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ABSTRACT

Enteric Salmonella infection is a global problem both in man and animals, and has been attributed to be the most important bacterial etiology for enteric infections worldwide with massive outbreaks occurring in recent years. Food animals are the primary reservoir for human non-typhoid Salmonella infections. Poultry products are considered one of the major sources of Salmonella infections. The development of alternative anti- microbial remedies has become one of the highest priorities of modern medicine and biotechnology. One of such alternatives might be bacteriophages as a prospective biocontrol method against contaminations caused by antimicrobial resistant pathogens. The aim of this study was to develop bacteriophage-based product that can be used to control salmonella contamination on farm level. 31 *S. typhimurium* strains were isolated from 200 poultry meat and eggs. All of the isolates showed high resistance to antibiotics used in the test. For formulating polyvalent phage preparation 3 phages out of 8 with wide, complementary, not-fully-overlapping host ranges were selected. Lytic activity and host specificity of each individual phage was compared with that of the phage cocktail. It was observed that the cocktail was superior to the individual phages. .Salmonella phage cocktail was effective against 46 out of 45 (98%) tested *Salmonella* strains in in vitro experiments.

Keywords: Salmonella, Bacteriophage, Antibiotic resistance, Treatment, Prevention, Chikens

*Corresponding author: Natia Tamarashvili: E-mail address: .ntamarashvili@gmail.com

Introduction

Food animals are the primary reservoir for human non-typhoid *Salmonella* infections [1]. According to the Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA) 75% of the annual cases of human salmonellosis are due to the consumption of contaminated poultry, beef, and egg product. In general, poultry products are considered one of the major sources of Salmonella infections [2, 3]. In fact, several Salmonella strains persistently colonize chickens but without causing any signs of illness [4]. The ceca of chickens are the major sites of Salmonella colonization, and chickens can become asymptomatic carriers of Salmonella. This lack of clinical symptoms facilitates the dissemination of Salmonella within flocks. *Salmonella* can be transmitted into foods at different stages of the food production chain. Reduction of pathogens colonization in animals during primary production is very important strategy to reduce the probability of cross-contamination with the animal feces during food processing. Controlling of food-born pathogens at the firm level will reduce their occurrence in the environment and food line and dissemination. Consequently, it could help prevent

the occurrence of food pathogens in animal, food, humans and environment [5].

Recent years show a dramatic rise both in terms of incidence and severity of human salmonellosis cases. The continuing emergence of Salmonella strains that are resistant to antimicrobials is also a cause of increasing concern [6].

Existence of such pathogens is problematic not only for animal health, but also because of possible transmission of antibiotic resistant bacteria from animals to humans through the food supply. Antibiotic-resistant bacteria from poultry houses contribute to the spread of antibiotic-resistant bacteria in the air, water, and soil so antibiotic-resistant bacteria from poultry production can contribute to rising rates of antibiotic resistance [7, 8].

The development of alternative anti- microbial remedies has become one of the highest priorities of modern medicine and biotechnology. One of such alternatives might be bacteriophages – bacterial viruses that infect and replicate within bacterial cells causing irreversible cell death [9].

Recently, there has been an increased interest in the application of bacteriophage as an alternative antimicrobial chemotherapy in various fields including human infections, food safety, agriculture, and veterinary applications [10, 11]. This interest is enhanced by a number of positive properties of bacteriophages. These include the possibility of high specificity towards the pathogenic agents while sparing the regular flora of the human, their lytic capability against antibiotic-resistant strains and, most important, their safety for the animals and environment. They have been identified as a prospective alternative biocontrol measure for infections and contaminations caused by antimicrobial resistant pathogens. The use of bacteriophages may be a safe and effective alternative to antibiotics for the treatment and prevention of Salmonella infection in poultry [12].

The main goal of this work is the development of bacteriophage-based commercial product that can be used to control salmonella contamination at the farm level.

Materials and Methods

Isolation and identification of Salmonella strains: For isolation of salmonella strains total 200 samples of poultry meat and eggs were purchased at the farmer markets and supermarkets in Tbilisi area. Fresh samples were placed to sterile sampling tubes and transported to the laboratory. For primary enrichment, 25g of each sample was incubated in 225 mL of buffered peptone water (BPW) during 24-48 hours at 37°C. For secondary enrichment, 1 mL of the first enriched broth was added to 10 mL of Hajna tetrathionate broth and incubated for 22-26 hours at 42°C. After incubation, broth was inoculated onto desoxycholate hydrogen sulphide lactose agar and brilliant green agar, both supplemented with 20 mg/L of novobiocin sodium, and incubated at 37°C for 18 h. Suspected colonies were isolated for biochemical and microbiological studies.

Bacterial strains

For investigation of bacteriophage host range, apart from new isolated salmonella strains 15 clinical S. typhimurium strains from laboratory collection were used. For assessment of lytic activity of the selective phages toward essential bacteria characteristic of GI tract 8 probiotic strains from institute collection were also enrolled in this study.

Antimicrobial susceptibility testing. All the S. typhimurium isolates were exposed to different antibiotics for its antimicrobial susceptibility and drug resistance pattern determination using disk diffusion assay following the guidelines of clinical and laboratory standard institute [13]. Pre-incubated 24 h cultures of S. typhimurium were swabbed over Brain Heart agar. After placing the antibiotic discs aseptically, the plates were incubated at 37oC for 18-24 h and zone of inhibition were measured subsequently. 8 different antimicrobial agents, most widely used in clinics and also used in this study were: Ampicillin (10 μg), Streptomycin (10 μg), Ciprofloxacin (5 μg), Trimethoprim (5 μg), Chloramphenicol (30 μg), Tetracycline (30 μg), Gentamicin (10 μg), Kanamycin (30 μg). Each strain was identified susceptible (S), intermediate (I), or resistant (R) in accordance with the National Committee for Clinical Laboratory Standards (NCCLS) guidelines.

Characterization of selected bacteriophages
Spotting assay

The host bacteria were grown overnight and then 100 µl of cell suspension was added to 5 ml of soft LB agar (0.6% agar), which had been pre-heated to 42°C in a water bath. Resultant mixture was gently vortexed, poured over LB agar plates (1.5% agar), and allowed to solidify at room temperature during 30 min to produce bacterial lawns. Then, 10 µl of phage stock dilutions (10-fold serial dilutions in SM buffer) were spotted onto the upper agar layer, and the plates were dried at room temperature for 30 min. Then, the plates were incubated overnight at 37°C, and inspected for single plaques or bacterial growth inhibition zones after 24 hours.

Preparation of concentrated phage stocks (Bilayer agar method)

100µl of the host bacterium culture grown overnight and 1 ml of phage (103PFU/ml) were mixed; then 3 ml of molten soft-agar (0.7%) was added to each tube and the mixture was gently vortexed and poured over LB agar plates (1.5% agar). The Petri dishes were incubated at 37oC. After 18-20 hours incubation, 3 ml of broth was spread over the agar and left for 15-20 min. Using spreading rod or spatula, the soft-agar with broth were scraped I and transferred to a centrifuge tube, and centrifuged at 6000 g for 45 min. The supernatant was filtered through 0.22 nm filter, transferred into the sterile vial and titrated.

Bacteriophage host range and selection of the most efficient phage

The phages were investigated for host range specificity and lysis efficiency against Salmonella strains. Each strain was inoculated on Tryptic Soy Agar (TSA) and 10 µl of phages (1 × 104 - 1 × 107 PFU m^{l}-1) was dropped over the plate with inoculated culture. The plates then were incubated during18 hrs at 370C and the presence of plaques was observed.

Characterization of phage genomes Restriction analysis

For investigation of phages genome, phage DNA was isolated though standard Phenol–Chloroform extraction method from high-titer phage lysates. Phage nucleic acids were purified in a three-stage organic extraction using equal volumes of buffered phenol, then phenol-chloroform (1:1), and finally chloroform. At each stage the aqueous and organic phages were mixed by gentle inversion for 3 min, followed by centrifugation at 13,500 × g for 5 min at room temperature. DNA was precipitated by adding sodium acetate to a final concentration of 0.3 M and adding an equal volume of 100% ethanol, until the phage DNA had just precipitated. The precipitated DNA was collected by centrifugation at 15,000 × g for 15 min at 4°C, washed with 70% ethanol, and air dried before being resuspended in 10 mM Tris-HCl (pH 7.5) and stored at −20°C. The restriction patterns of phages DNA were determined with restriction endonucleases HindIII, EcoRI from Sigma-Aldrich Inc. according to the manufacturer's instructions. Incubation time was 2 h at 37oC. Fragments were separated on a 1% agarose gel at 70 V for 2 h and stained with ethidium bromide (1 µg/ml).

Results

Isolation and identification of S. *typhimurium* strains

A total of 200 samples of poultry and eggs were taken from several markets in Tbilisi area. The samples were analyzed for the presence of Salmonella using standard biochemical and serologic methods. A total of 31 S. *typhimurium* strains were isolated.

Antimicrobial susceptibility testing

The next step of our work was testing antibiotic susceptibility of isolated Salmonella strains. 7 different antibiotics widely used in clinics and veterinary were used in this study.

The study revealed that the majority of salmonella cultures were characterized by high levels of antibiotic resistance. Multiresistance was revealed when strains were resistant to four and more antibiotics (Table 1).

Table 1. *Antibiotic resistance of isolated S. typhimurium strains*

		Resistant strains[a]		Multiresistant strains[b]	
Serotypes	Number of strains	n	%	n	%
S.typhimurium	46	46	100	35	76

[a] Isolates resistant to one and more antibiotics
[b] Isolates resistant to four and more antibiotics

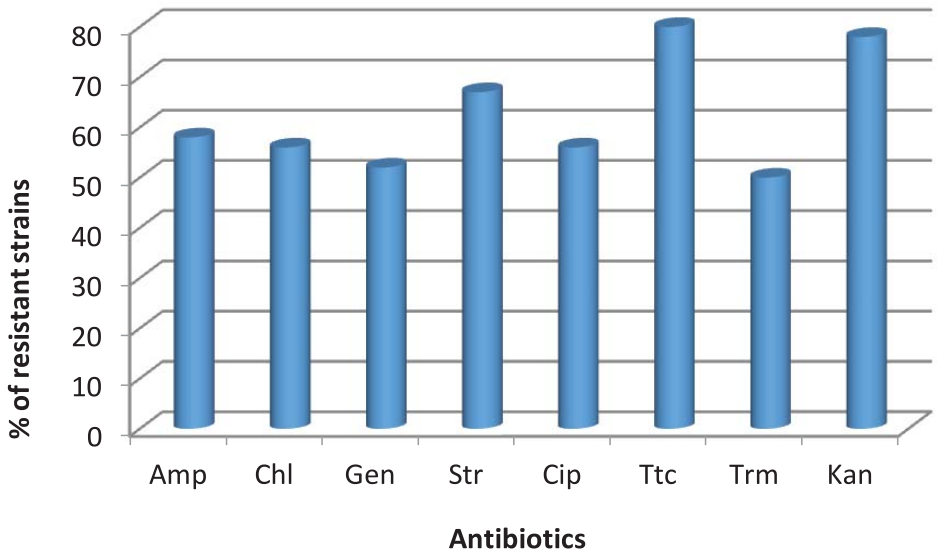

Fig. 1. *S. typhimurium antibiotic susceptibility test*

As follows from the Table 1, investigated *Salmonella* isolates were resistant to 4 from 7 examined antibiotics. S. *typhimurium* strains revealed high resistance to Tetracycline (80%), Kanamycin (78%), Streptomycin (67%). Actually, all newly isolated strains were characterized with high to moderate level of antibiotic resistance.

Selection and study of effective bacteriophages against Salmonella

The aim of our work was selection and study effective bacteriophages against salmonella strains. Our purpose was to create an effective, non-overlapping phage cocktail against salmonella. We considered that it would also be important to select bacteriophages with different genotypes.

A host specificity of Salmonella phages

In the test, we used 46 S. typhimurium strains to determine the range of host cells susceptible for previously selected 8 salmonella bacteriophages. Sal.phi13, Sal.phi18 and vB_stm 21 were characterized by the wide range of host cells. From the results obtained, Sal.phi13 phage lysed 36 out of 46 strains (82.6%). The activity of Sal.phi18 was high compared to the Sal.phi13 phage. The Sal. phi18 lysed 43 out of 46 strains (93.4%) and vB_ Stm phage lysed 35 out of 46 strains (76%) (Table 2).

Table 2. *A host specificity of Salmonella phages*

Tested *S.typhimurium* phages	A total *S.typhimurium* strains	Lysed *S. typhimurium* strains	Lysed *S. typhimurium* strains, %
vB_Stm 17	46	29	63
vB_Stm 18		30	64
vB_Stm 21		35	76
vB_Stm 29		24	52
Sal.phi 13		36	82,6
Sal.phi 14		32	69,5
Sal.phi 16		31	67,4
Sal.phi 18		43	93,4

Three lytic salmonella phages, *Sal.phi13, Sal. phi18,* and *vB_Stm 21,* which belong to two families, were selected to compose the phage cocktail. Each of these phages was shown lytic and highly specific toward *Salmonella typhimurium* strains. These three phages have been isolated from individual cell-phage lysates, purified, and stock suspensions of approximately equal concentration of the phages were prepared. The phages were mixed in the proportion 1:1:1 and lytic activity, and host specificity of each individual phage was compared with that of the phages cocktail (Fig 2). It was observed that the phage cocktail possessed broader host specificity within *S. typhimurium* serotype than each of three phages alone. The cocktail composed of these phages was shown lytic toward 65 out of 66 tested *S. typhimurium* strains (98%).

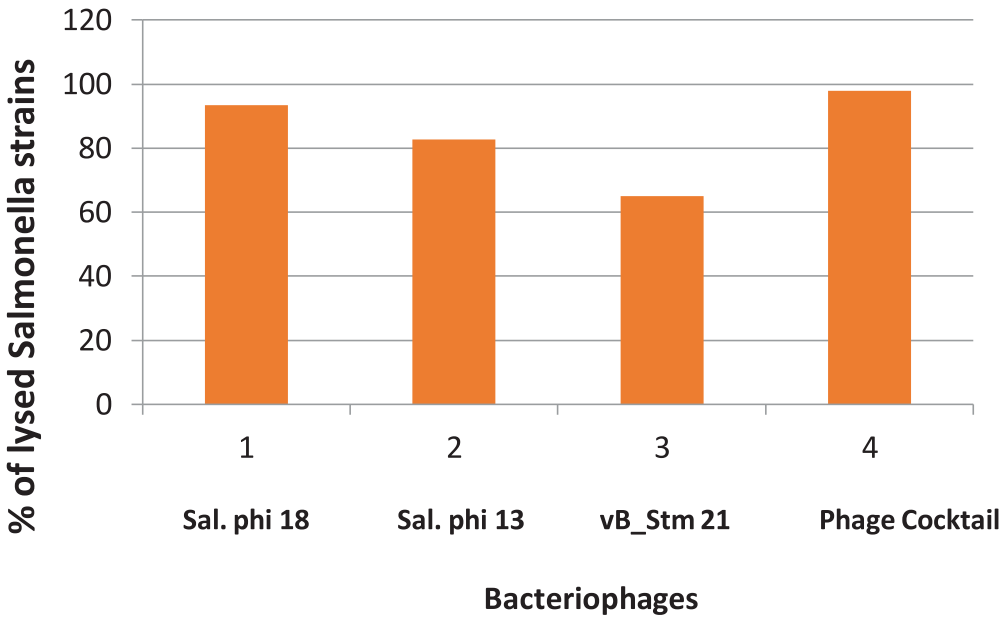

Fig. 2. *Comparison of host specificity of single Salmonella phages and their combination within S. typhimurium serotype*

Figure 2 demonstrates that a host specificity of *Salmonella* cocktail was noticeably wider than that of the individual *Salmonella* phages. It can be easily explained by the fact that host specificities of the individual phages were not completely overlapping, and thus, each phage in the cocktail contributed its activity toward species for which other phages were not lytic. We are going to study the efficacy of *Salmonella* phage cocktail preparation in elimination, reduction and prevention of colonization of poultry in the chicken infectious model.

Morphological characteristics of selected Salmonella phages

Electron microscopy study of pre-selected salmonella phages allowed classifying them (Fig. 3, Table 3). Phages Sal. phi 13 and vB_Stm 21 had a tubular tail with helical symmetry and the diameter of 10-20 nm. The tail length of phages was in the range from 80 to 90 nm, and the head diameter of 50-60nm. These phages were referred to *Myoviridae* family. Phage Sal. phi 18 had relatively thin, long, non-contractile, and flexible tail with an isometric head. The tail length of phage Sal. phi 18 was of 140 nm, and its head diameter was 45 nm. It was referred to *Siphoviridae* family.

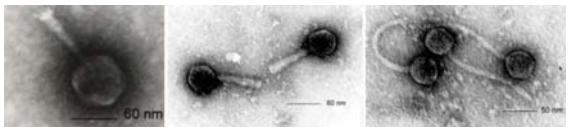

Fig. 3. *vB_Stm 21, Sal.phi13, Sal.phi18 phages electron micrograph*

Table 3. *Morphological characteristics of selected Salmonella phages*

Phage	Head diameter nm	Head length nm	Tail diameter nm	Tail length nm	Family
vB_Stm 21	50	50	15	90	*Myoviridae*
Sal.phi13	60	60	15	80	*Myoviridae*
Sal.phi18	45	45	10	140	*Siphoviridae*

Characterization of phage genomes

Restriction analysis

Bacteriophages vB_Stm 21, Sal.phi 13 and Sal. phi 18 showed distinct restriction endonuclease patterns after digestion with EcoRI and HindIII endonucleases (Fig. 4, 5, 6, 7).

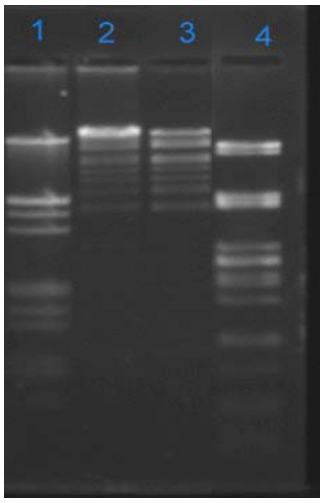

Fig. 4. *Digestion of the Sal. phi 13 and Sal. phi 18 phage DNA with HindIII endonuclease. 1. λ DNA (HindIII digest), 2. Sal. phi 13, 3. Sal. phi 18, 4. λ DNA*

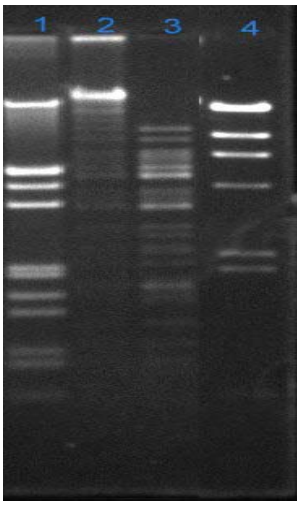

Fig. 5. *Digestion of the Sal. phi 13 and Sal. phi 18 phage DNA with EcoR1 endonuclease. 1. λ DNA (EcoR1 digest), 2. Sal. phi 13, 3. Sal. phi 18, 4. λ DNA*

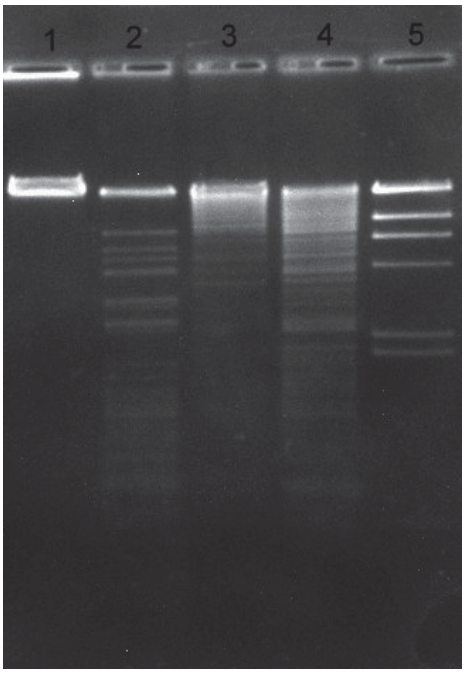

Fig. 6. *Digestion of the vB_Stm phage DNA with HindIII endonuclease. 2. vB_Stm 21, 5. λ DNA (HindIII digest)*

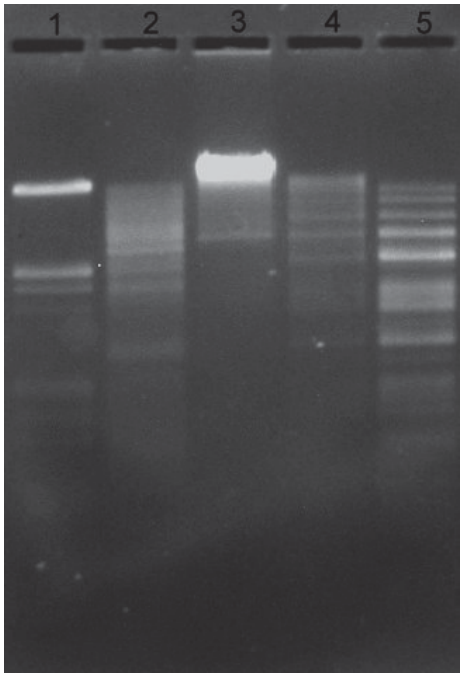

Fig. 7. *Digestion of the vB_stm 21 phage DNA with EcoR1 endonuclease. 1. λ DNA (EcoR1 digest), 4. vB_Stm 21*

Lytic activity of the selected Salmonella phages toward essential bacteria characteristic of GI tract

We examined lytic activity of the Sal. phi 13, Sal. phi 18 and vB_Stm 21 phages toward essential bacteria characteristic of GI tract. Results presented in the Table 4 demonstrates that no lytic activity of examined phages was observed toward essential microorganisms characteristic of GI tract.

Table 4. *Lytic activity of Sal.phi13, Sal.phi18, and vB_ Stm 21 phages toward essential bacteric characteristic of GI tract*

Phages	*vB_Stm 21*	*Sal phi 13*	*Sal phi 18*
Bacterial strains	Lysis with phages		
Bifidobacterium bifidum Ac-1246	-	-	-
Bifidobacterium longum Ac-1252	-	-	-
Bifidobacterium adolescentis Ac-	-	-	-
Lactobacillus acidophilus B-2585	-	-	-
Lactobacillus fermentum B 7580	-	-	-
Lactobacillus plantarum B 7583	-	-	-
Escherichia coli M17 B 8208	-	-	-
Enterococcus faecium B 4054	-	-	-

Discussion

Bacterial antibiotic resistance is an increasingly important problem in farm animal and human medicine. Although resistance in bacterial species responsible for human infections is mainly caused by indiscriminant use of antibiotic in humans, for a range of bacteria, farm-animal use of antibiotics is a significant contributor, and for some infections is the main source of resistance development. In food animal production antimicrobial resistance is increasing in both zoonotic and commensal bacteria. This has raised concerns about the risks of transmission of resistant zoonotic bacteria and resistance genes from food animals to humans and the consequences for health care and public health. Also, results obtained from our work showed high level of antibiotic resistance of the studied S. *typhimurium* strains.

Considering the above-mentioned problems, interest in the use of bacteriophage preparations as an alternative to antibiotic treatments has been steadily increasing worldwide. This interest is enhanced by a number of positive properties of bacteriophages. These include i) high specificity towards the pathogenic agents, which means that the regular human microflora is spared, ii) ability to lyse antibiotic-resistant strains and, iii) safety for the animals and environment, and iv) virtually unlimited number of phages, which should allow facile isolation of phages specific for any given bacteria, including isolation (or selection) of phages capable of lysing bacteria that acquired resistance to a phage(s) used in the initial round of therapy.

These properties give bacteriophages or phage-derived products wide potential to be used: a) as possible alternatives to antibiotics for the treatment of bacterial diseases in humans and animals b) to minimize the pathogen loads in food products of animal and plant origin -biocontrol of microorganisms in food c) an alternative to industrial disinfectants and sanitizers.

Using bacteriophage preparation as a phage cocktail composed of two or more individual bacteriophages enhances the efficiency of the preparation activity. Formulation of phages into cocktails increases their potential to be used presumptively, that is, prior to identification of pathogens (e.g., in terms of phage susceptibility), and the more phages are included, the greater the potential that there will be sustainable levels of medical as well as commercial demand for a given formulation. Perspective of using bacteriophage endolysins instead, or along with intact bacteriophages increases a potential value of bacteriophages as natural alternative to antibiotics.

Therapeutic use of phage cocktails, attracts more and more attention. According to authors [14, 15] the primary motivation for the use of cocktails is their broader spectra of activity in comparison to individual phage isolates: they can impact either more bacterial types or achieve effectiveness under a greater diversity of conditions. The combining of phages can also facilitate better targeting of multiple strains making up individual bacterial species or covering multiple species that might be responsible for similar disease states, in general providing, relative to individual phage isolates, a greater potential for presumptive or empirical treatment. Also, it is expected that cocktails consisted of several genetically distinct phages has lower chance to induce microbial resistance. Our study demonstrates the lytic activity of selected bacteriophages against multidrug-resistant S. Typhimurium strains, including antibiotic-induced resistant and clinically isolated strains. The three bacteriophages used in this study, were chosen from our collection of Salmonella bacteriophage based on their broad host range, different restriction patterns with EcoRI and HindIII and their morphological characteristics. It was observed that the phage cocktail possessed broader host specificity within S. typhimurium serotype than each of three phages alone (Fig. 2).

Advantages of phage therapy over the use of chemical antibiotics can be framed in terms of phage properties that can contribute substantially to phage therapy utility. These include: i) effective against multidrug-resistant pathogenic bacteria; (ii) substitution of the normal microbial flora does not occur because the phages kill only the targeted pathogenic bacteria; (iii) respond quickly to the appearance of phage-resistant bacterial mutants because the frequency of phage mutation is significantly higher than that of bacteria [16, 17]. Also, according to our results (Table 4) no lytic activity of examined phages was observed toward essential microorganisms characteristic of GI tract. Though more essential microorganisms must be tested, the data presented in the Table 4 are of a