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Use of a Bacteriophage Cocktail to Reduce *Salmonella* Colonization in Experimentally Infected Chickens

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ABSTRACT

Antimicrobial-resistant *Salmonella* is dangerous for animal health with possible transmission from animals to humans. The bacteriophages may be a safe, effective alternative of antibiotics for the treatment and prevention of Salmonella colonization in poultry. The main goal of this work is to study the efficacy of Salmonella phage cocktail in elimination, reduction and prevention of colonization in poultry of the chicken infectious model. Four groups of experimental animals (n=24) were enrolled in this experiment. Chickens of three groups were challenged orally with a single dose of salmonella (10^6 CFU/chicken). Group I - received orally 1 ml salmonella phage cocktail 10^9 PFU per chicken on the day before challenge, immediately after the bacterial challenge and 1 treatment per day the next 8 days. Group 2 – treated with the same dose of cocktail for the next 10 days post-challenge. Group 3 - treated with dialysis buffer for 10 days post-challenge (control group 1) and Group 4 - non-infected chickens (control). In each group, 4 chickens were euthanized on days 1, 3, 5, 7, 10 and 21. The cecum of chickens was checked for *Salmonella* quantification. The experiment results show that the use of phage cocktail before infection (group 1) significantly reduced colonization of *Salmonella* and show complete and irreversible elimination of the pathogen after 5 days post-challenge. The complete elimination of pathogen was reached on the 7th day of treatment in group 2.

Keywords: Bacteriophage cocktail, *Salmonella*, Antibiotic resistance, Elimination, Poultry, Infection.

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Introduction

Salmonella species induce bacterial illness and are also one of the leading causes of hospitalization among all the foodborne bacterial pathogens [1, 2].

CDC estimates *Salmonella* causes about 1.2 million illnesses, 23,000 hospitalizations, and 450 deaths in the United States every year. Among the illnesses acquired in the United States, CDC estimates that food is the source for about 1 million illnesses, 19,000 hospitalizations, and 380 deaths [3].

Salmonella can be found in different types of food, ranging from poultry, pig and bovine products and meat to vegetables, fish or other fishery products. Most *Salmonella* associated infection occurs after ingestion of contaminated foods - mainly meat, poultry, eggs, milk, and vegetables.

Salmonella bacteria are a major problem in the poultry industry. This is largely the result of the entry of these bacteria into the human food chain through poultry. In chicks *Salmonella* colonizes the gastrointestinal tract but does not cause clinical disease. Infected chicks are able to shed pathogen into the environment for extended periods of time increasing the risk for environmental contamination, spread of the organism within the flock and contamination of the food supply [4,5].

Human *S. Enteritidis* cases are most commonly associated with the consumption of contaminated eggs and poultry meat. Reduction of the number of S. Enteritidis-contaminated eggs or egg contamination in flocks of laying hens is a main target for reduction of human salmonellosis. Food can become contaminated with pathogens at every stage from "field to fork." Being free of pathogens or lightly contaminated at a farm, food might arrive heavily contaminated by the time it reaches the consumer. Controlling pathogens on the farm is a complex process, and for many years the main line of defense in disease prevention and treatment has been antibiotics, but important public health concern is the emergence of antibiotic resistant strains of *Salmonella* [6.7].

Bacteriophages (phages) are the natural enemies of bacteria and have proven to be a valuable natural weapon to fight against disease. Phages show great promise as alternatives to traditional antimicrobials in the control of pathogens. The extreme specificity of phages makes them ideal candidates for applications designed to increase food safety during production process (including the quick and specific identification of unwanted viable pathogens in food), and for decontaminating food surfaces and equipment surfaces in food-processing facilities [8-11].

The aim of this study was to test the efficacy of application of Salmonella phage cocktail for elimination, reduction and prevention of colonization in poultry of the chicken infectious model.

Methods

Bacteriophages: *Salmonella* phages Sal.phi13, Sal.phi18, and vB.Stm 21 were used in this experiment. Data about the cocktail's phages: phage morphology, host range, restriction analysis of gemone, in vitro efficiency have already been documented [12].

Propagation of the phages in the liquid medium

To amplify the phages, 10 ml of overnight culture of host strains (10^9 CFU/ml) and 1 ml of corresponding phage were added to 500 ml of Luria-Bertani (LB) broth and incubated in the shaker at 37°C for 6 hrs. After that, 10 ml of chloroform was added to the cell-phage lysate to release any progeny phage, which remained associated with the host cells. Then, the suspension was incubated for additional 10 min in the shaker at 37°C. To remove bacterial debris, the suspension was centrifuged at 5,000 x g for 15 min; the supernatant was carefully separated and filtered through 0.22-µm Millipore filters. The phage lysates were stored at 4°C.

Preparation of concentrated phage stocks (Bilayer agar method)

100µl of the host bacterium culture grown overnight and 1 ml of phage (10^3PFU/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 37°C. 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.

Preparation of phage cocktail. The preparation was carried out individually for all three phages and the cocktail was mixed according to the adequate concentration in the proportion 1:1:1 of each phage.

Animals. One hundred tventy laying hens, aged 3 weeks, were obtained from two commercial grower salmonella-free poultry farms near Tbilisi. The hens were not vaccinated against Salmonella spp.

Bacterial inoculum.The *Salmonella* strain used in this experiment was a poultry isolate of *Salmonella tiphimurium.* A stock culture was prepared in sterile phosphate buffered saline (PBS) and was used for inoculation at a dose of 10^6 CFU/chicken.

Experimental design

Chickens under the experiment (96 chicks) were randomly distributed into four groups, each group contained 24 chickens (n=24). Each group of birds was placed individually, provided feed and water and libitum. Chickens of three groups (group 1-3) were challenged orally at day two with a single dose of salmonella (10^6 CFU/chicken). Chickens of group 4 was left as a negative control and not inoculated with phage cocktail or *Salmonella*.

All chicks of group 1 were orally treated with the *Salmonella* phage cocktail 10^9 PFU per chicken before challenge, immediately after the bacterial challenge and the next 8 days 1 treatment per day. Chicks of group 2 – were treated with the same dose of cocktail the next 10 days after challenge, 1 treat-

ment per day. Chicks of group 3 – were treated with dialysis buffer the next 10 days after challenge, 1 treatment per day (positive control group).

Sampling

In each group, 4 chickens were euthanized on days 1, 3, 5, 7, 10 and 21 post challenge. The cecum of chickens was checked for *Salmonella* quantification. The cecum from each chicken was removed aseptically. Each sample was transferred to a sterile plastic bag and transported at approximately 4 ± 2°C. Laboratory testing of all samples took place within 24 h after sampling.

Bacteriology

The homogenized cecum samples (0.25 g each) were diluted in 2.25 mL PBS (pH 7.0), decimal dilutions were prepared in PBS and spread onto XLT- plates to enumerate Salmonella. The plates were incubated at 41°C for 24 h, and the number S. typhimurium cells per g of cecal contents determined. The isolates were biochemically identified as Salmonella (methyl red, citrate utilization, triple sugar iron and catalase tests positive & negative to urease and indole tests).

Statistical Analyses

The mean and SEM for each cytokine/chemokine were calculated at each time and statistical analyses performed (Student's t-test). For all analyses, significance was considered if $P \leq 0.05$.

Results

For formulating phage cocktail 3 phages with wide, complementary, not-fully-overlapping host ranges were selected. *Salmonella* phages Sal.phi13, Sal.phi18, and vB.Stm 21 were mixed in the proportion 1:1: 1 (Fig 1).

We have studied the efficacy of this phage cocktail in the chicken model. 3 groups of chicken were infected by oral inoculation of the same dose *S. Typhimurium* (10^6 CFU/chicken). Chickens of groups 1 and 2 treated with the bacteriophage cocktail 10^9 PFU per chicken using different treatment schedules.

Significant reduction in *Salmonella* concentration (on day 5) and total elimination (on day 7) was reached when chickens were treated with bacteriophage cocktail 1 day after bacterial infection and then again next 9 days (group 2). On day 5 *Salmonella* concentration in cecum of chickens group 2 was decreased significantly compared to the control group (1.6 log 10 vs 6.6 log 10) (Fig.2). From day 7 to the end of the experiment (day 21) all the chicks were cleared for Salmonella.

Treatment of chickens with the *Salmonella* phage cocktail before bacterial challenge immediately after the challenge and the next 8 days caused a significant delay in bacterial colonization of cecum (1.6 log10 Vs 3.0 log10 on day 1 after challenge), and total elimination of *Salmonella* on day 5 (Fig 3). From day 7 to the end of the experiment all the chicks were cleared for *Salmonella.* In chickens of group 4 (negative control group) no pathogens were isolated during the whole course of experiment (Fig 4).

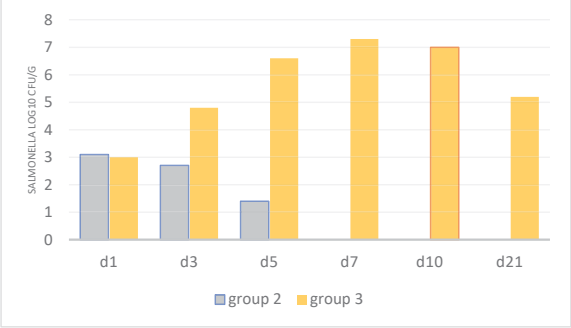

Fig. 2. *Concentration of S. Typhimurium in the cecum of chickens post bacterial challenge. Group 2-chickens treated with salmonella phage cocktail 10 days post infection, Group 3 – untreated chickens.*

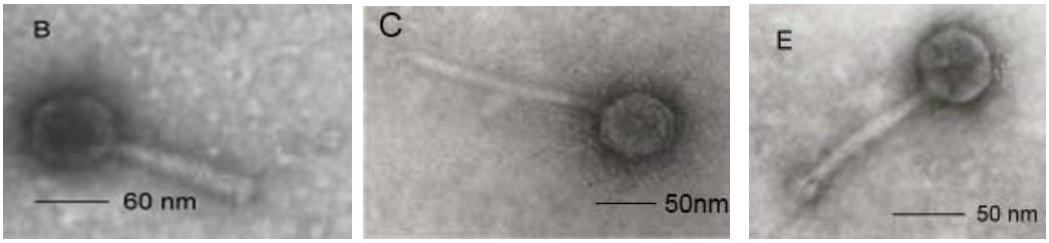

Fig. 1. *B- Sal.phi13, C- Sal.phi18, and E- vB_Stm 21 phages micrograph*

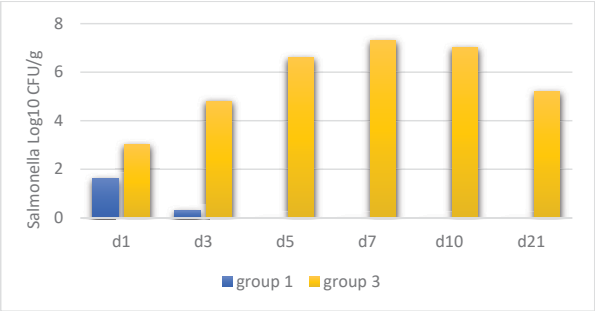

Fig. 3. *Concentration of S. Typhimurium in the cecum of chickens post bacterial challenge. Group 1-chickens treated with salmonella phage cocktail day before bacterial challenge, immediately after challenge and the next 8 days. Group 3 – untreated chickens.*

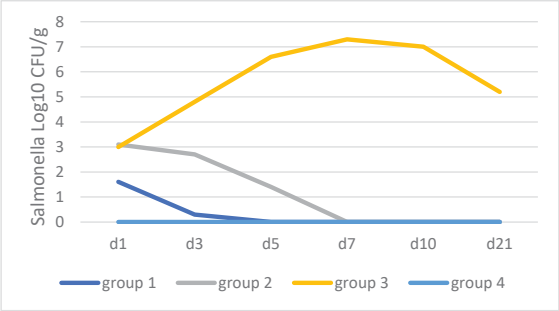

Fig. 4. *Concentration of S. Typhimurium in the cecum of chickens. Group 1-chickens treated with salmonella phage cocktail day before bacterial challenge, immediately after challenge and the next 8 days: Group 2-chickens treated with Salmonella phage cocktail 10 days post infection, Group 3 – infected but untreated chickens (positive control): group 4 (negative control, uninfected group).*

Discussion

According to the Center for Disease Control and Prevention (CDC) and the European Food Safety Authority (EFSA), *Salmonella* spp. are among the most important human pathogenic bacteria that frequently cause foodborne diseases worldwide. It was analyzed outbreaks reported to the United States' Foodborne Disease Outbreak Surveillance System from 1998 to 2012 in which the implicated food or ingredient could be assigned to one food category. Out of 1114 outbreaks, poultry was associated with 279 (25%), accounting for the highest number of outbreaks, illnesses and hospitalizations, and the second highest number of deaths. Out of the 149 poultry-associated outbreaks caused by a confirmed pathogen, *Salmonella* enterica (43%) was the most common pathogen [13, 14].

Antibiotic resistance is of great public health concern because the antibiotic-resistant bacteria associated with the animals may be pathogenic to human, easily transmitted to human via food chains, and widely disseminated in the environment via animal wastes. The routine employment of antibiotics, for prevention and growth promotion purposes in livestock farming, selects for antibiotic resistance among commensal and pathogenic bacteria. Owing to the fact that most of these antibiotics are not fully metabolized but released into the environment as waste products, antibiotic resistance has an ecological impact, since these waste products still have the potential to influence the bacteria population and promote antibiotic resistance [15].

Over the past 50 years, as poultry farms became larger and more concentrated, farmers began using antibiotics to prevent disease and speed growth in broiler chickens. The industry today remains dependent on their widespread use, and antibiotics are frequently given to birds that are not sick. When antibiotics are routinely given to entire flocks, resistant bacteria are likely to survive and proliferate. These resistant bacteria can even share resistance genes with other bacteria. Antibiotics misuse by poultry farmers has resulted in multidrug resistance and impeded efficiency of antibiotic treatments in the industry. The ability of pathogens to colonize in the gut increases after antibiotic administration because of a loss of resident microflora. (www.nrdc.org/sites/default/files/poultry-industry-antibiotic-stewardship-IB.pdf).

Salmonella spp. is one of the most common microbial contaminants in the poultry industry. Due to the common foodborne illness cases caused by *Salmonella*, prevention of *Salmonella* colonization in the gastrointestinal tract (GIT) of chickens is necessary. There is great potential for the use of phages as natural antibacterial remedy to control food pathogens at the pre- and postharvest stages of production [15-17].

Reduction of pathogen colonization in animals during primary production (*phage therapy*) is a strategy followed in primary production just before slaughter or during animal growth to reduce the probability of cross-contamination with the animal feces during food processing [18].

Previous studies have shown that phages significantly reduce the colonization level of *Salmonella* spp. in the avian gut. The results of our experiment

are in approximate agreement with other experimental studies [19-25]. Bardina at al. have investigated that frequent treatment of the chicken with bacteriophages, and especially prior to colonization of the intestinal tract by *Salmonella*, is required to achieve effective bacterial reduction over time. The best results, defined as a reduction of *Salmonella* concentration in the chicken cecum, were obtained when the bacteriophage cocktail was administered 1 day before or just after bacterial infection and then again on different days post-infection [26]. The study of Nabil N. at al. has shown that the effectiveness of bacteriophage treatments on Salmonella colonization in cecum of infected chicks was increased after five doses of phage treatment. At day 3 post-infection (dpi), cecal contents showed a marginal decrease in Salmonella loads with more reduction at 5 dpi. From 7 dpi to the end of the experiment at 15 dpi, all the chicks were cleared for *Salmonella* [27].

Our study has demonstrated that bacteriophages can be used for reduction and elimination of the cecal colonization of *Salmonella* in poultry. In this study, we have demonstrated that administration of salmonella phage cocktail to chickens before oral *Salmonella* infections followed by 9 consecutive phage treatments after the bacterial challenge as well as treatment with the same doses of phage cocktail (10 days post infection) were very effective. Further research should be done to investigate the possible use of our phage cocktail for prevention of salmonella colonization in farm level as our study showed that 1 day phage treatment before experimental infection decreased bacterial load from $\log_{10}$ 3.1 (control group) to $\log_{10}$ 1.6, no bacteria was detected in chickens of both groups on day 7 post-infection and all chickens were free of salmonella during 10 days after last administration of phage cocktail (21 day post-infection). This indicated that treatment with phage cocktail leads to complete elimination of *salmonella* contamination.

Conclusion

Our results demonstrate efficacy of used phage preparation in reducing *Salmonella* colonization in chickens and possibility of using this preparation as alternative to antibiotics for the reduction of *Salmonella* infection in poultry.

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