საქართველოს მეცნიერებათა ეროვნული აკადემიის მოამბე, ტ. 12, №1, 2018
BULLETIN OF THE GEORGIAN NATIONAL ACADEMY OF SCIENCES, vol. 12, no. 1, 2018

Microbiology

Decontamination Effect of the Eggshells with the Mixture of *Salmonella* and *E.Coli* Specific Phages

Khatuna Makalatia[*], Elene Kakabadze[*], Nata Bakuradze[*], Nino Grdzelishvili[**], Gulnara Natroshvili[**], Nina Chanishvili[*]

** Faculty of Exact and Natural Sciences, Ivane Javakhishvili Tbilisi State University; Department of Research and Development, G. Eliava Institute of Bacteriophage, Microbiology and Virology, Tbilisi, Georgia*
***Department of Research and Development, G. Eliava Institute of Bacteriophage, Microbiology and Virology, Tbilisi, Georgia*

(Presented by Academy Member Tinatin Sadunishvili)

ABSTRACT. Elimination and prevention of food-borne infections are a major focus of Food Safety studies. Development of the new and advanced technologies for prevention and reduction of contamination, associated with different types of food products, is a subject of constant research. The aims of this investigation were: a) to study naturally occurring eggshell contamination, and, b) to investigate an effect of phage treatment on reduction of eggshell contamination caused by *Salmonella enterica* serovars and *Escherichia coli* in a modulated *in vitro* experiment. The results showed that among naturally occurring contamination the fungal species are predominating. This may be explained by an uncontrolled use of antibiotics in chicken farms for prevention of bacterial pollution and spread of diseases. After phage treatment of the artificially contaminated eggshells with one phage clone the total bacterial counts were reduced at least for one log (i.e. 90%) within 15 minutes after the treatment and remained practically unchanged during the next 18 hours. Application of the phage mixture resulted in one log (90%) decrease of bacterial counts after 15 min which continued to decrease within the next 18 h. These results indicated that specific phage preparation, especially composed with multiple phage clones with overlapping host ranges, significantly reduces external contamination of eggs and, therefore, may get an effective practical application in farm production via eliminating contamination and increasing the product quality and safety. To provide more reliable statistical support to this conclusion a large scale decontamination experiment in a natural farm environment is required. *© 2018 Bull. Georg. Natl. Acad. Sci.*

Key words: food safety, food-born Infection, phage treatment, decontamination

Food producers and associated regulatory bodies around the world are concerned about microbial foodborne infections spread through the food chain. Current estimates of foodborne illness in the USA are 76 million cases, 325,000 hospitalizations, and 5,194 deaths from foodborne pathogens per year, in which bacterial pathogens are the leading cause of death, with 72 percent of total foodborne illness deaths attributable to bacteria [1]. Thus, food safety problems are the most important issues in the modern world. Elimination, prevention and control of food-borne

diseases are the concerns of public health, epidemiology and bio-defense services. Meanwhile, long-term storage and prolongation of the food product shelf life is a major priority for world market. Hereby, development of advanced technologies for prevention and reduction of contamination of different kinds of fodder, to meet the requirements of public health and competitive market, is a major challenge in food biotechnology.

Eggs, that are one of the most frequently used highly nutritious product, may be easily contaminated with the pathogens like *Salmonella* spp., *Escherichia coli* (different serotypes), *Pseudomonas* spp., *Streptococcus* spp. *etc* [2].

Eggs can be contaminated in two major ways: via vertical transfer during egg lying process due to poultry internal infections, or as a consequence of eggshell surface contamination related to the external environment. As it has been shown by De Reu, K., *et al* [2] Gram-negative, motile, non-clustering infectious agents, such as *Pseudomonas spp., Alcaligenes* spp. and *S. enteritidis,* may even penetrate through an undamaged egg cuticle within 4-5 days. Furthermore, correlation between eggshell and inner contamination was shown for *Salmonella* spp. [3].

Vučemilo, Marija, *et al*. [3] reported that the eggshell contamination from airborne bacteria and fungi are directly associated with environment and ranges from 2.3×10^3 to 9.6×10^3 CFU/eggshell in aviary and conventional caging systems, which indicates that horizontal eggshell contamination presents the mean of concern [4].

Modern food production industry utilizes number of different decontamination techniques, majority of which are based on nonspecific, expensive and batch limited physical approaches, such as heat and steam treatment, UV irradiation [5, 6]. The alternatives to physical methods, such as chemical treatment of the primary packaging and external surfaces of products, results in increasing resistance of contaminant bacteria and acute deflection of treatment efficiency. Besides that the used chemicals may have a harmful effects on food quality and cause environmental changes [5,7]. Therefore, development of the efficient, affordable, non-damaging, and specific treatment methods for prevention and/or post processing application are required.

Bacteriophage (phage) - based treatment is considered as one of the most valid alternatives for such application. Phages are easy to use, specific agents that can eliminate target pathogen without disrupting the normal microflora [5]. The ability of lytic phages to adapt and evolve together with their host bacteria, makes it possible to get an appropriate mixture for newly emerged serotypes and eliminate bacterial resistance against antibiotics [6].

As for today U.S. Food and Drug Administration (FDA) had already approved the usage of phage-based preparation for ready- to-eat food treatment process. For example in the USA from August 18, 2006 Listeria-specific Bacteriophage Preparation is used on Ready-to-Eat Meat and Poultry Products [8,9]. A number of publications describe effective and rapid phage treatment techniques of food products and packaging supplements against various pathogens. At present, these products are directed against three main foodborne pathogens including *Escherichia coli O157:H7, Salmonella* spp. and *Listeria monocytogenes*. In the future, it is likely that new phage products will be targeted against emerging foodborne pathogens [1,4-7].

At the same time according to the official statistical information published by the Georgian NCDC during the period between 2006 and 2015 the incidences of diarrheal diseases had dramatically increased, however most of them remain unidentified. Among the identified diseases only 1% of the cases are confirmed to be caused by *Salmonella* spp. In 2015 salmonellosis was reported in 100 cases (1% of total identified diarrheal infections) [10]. These data is in contrast with the EU data, where approximately 100,000

human salmonellosis cases (32%) are reported each year, 0.15% of which were followed by fatal outcomes [11]. According to EU data [11] *Salmonella* contamination was rarely found in the table eggs, at levels of 0.3% (single samples) or 1.0% (batch samples). However, eggs and egg products were the most important source of food-borne *Salmonella* outbreaks [11].

According to the study performed by the Georgian Risk Assessment Group aiming monitoring of contamination of the meat products imported into Georgia the highest proportions of *Salmonella*-positive single samples were reported for fresh turkey meat (3.5%) followed by fresh broiler meat (2.2%), pig meat (0.6%) and bovine meat (0.1%) produced in various countries [12].

Altogether 158 samples of frozen chicken meat and minced chicken meat were checked for contamination with Salmonella by three different organizations. Summarizing their results *Salmonella* contamination was detected in 38% -70% cases [12], but data concerning contamination of the local chicken and egg products are unavailable. The low number of registered salmonellosis in Georgia may be explained by the fact that people avoid applying to the doctors and often do not undergo bacteriology examinations. This increases the number of unidentified diarrheal cases. Eggs and chicken meat may be a source of *Salmonella* infection in Georgia as well, however this issue remains unexplored as yet. Although an actual incidence of *Salmonella* infection in Georgia remains unknown, taking into consideration other publications based on well organized epidemic data and results of modern diagnostic methods [13], we may assume that incidence of salmonellosis in Georgia may be as high as in other EU countries and its main source presumably may also be associated with the egg consumptions. An increased rate of patients in the recent years indicates to circulation of shiga-toxin producing *E.coli* in Georgia. Most of patients were rural residents 20 (80%), only 5 (20%) were urban amongst them 11 (44%) patients were children, 14 (56%) were adults [14,15]. All the patients related the onset of the disease with the consumption of unwashed raw fruits or vegetables, unpasteurized dairy products, food from street vendors, soft cheeses made from raw milk and untreated water in areas lacking adequate chlorination [14, 15]. Although this list does not include eggs, it is not excluded that they may also appear as a source of infection.

The aims of our study were: a) to evaluate the level of naturally occurring eggshell contamination existing today on the Georgian market, and, b) to demonstrate an effect of the specific phage treatment on infected eggshell in the modulated environment.

MATERIALS AND METHODS

Samples. Randomly selected twenty fresh eggs samples from different rural farms and two different manufacturing companies were studied for naturally occurring contamination of eggshells. Sixteen heat sterilized eggs were used for experimental contamination of the eggshell followed by phage treatment.

Media and reagents. The following bacteriology media was used for identification and growth of *E.coli* and *Salmonella* spp. and: Growth medium - Luria Bertani Agar (LB), Endo, Salmonella-Shigella (SS); Sabouraud dextrose agar media (HIMEDIA) was used for detection of yeast and mould contamination.

Bacteriophages. Eight bacteriophages clones active against *Salmonella enterica* and *E.coli* were used to compose a mixture designated as 'P1', which was then applied for decontamination of eggshells. A detailed characterization of these phages is given in Table 1.

Bacterial Strains: Eight serotypes of *Salmonella enterica* and three strains of *E.coli*

Table 1. Characterization of bacteriophages used in the study

PHAGE NAME	SOURCE OF ISOLATION	VIRON MORPHOLOGY	GENOME SIZE	BACTERIAL HOST STRAIN	PHAGE STOCK TITER PFU/ML
ΦMRB-2S	Water sample from the river Mtkvari	*Podoviridae*	unknown	*E.coli*	10^8
ΦMRC-2X	Water sample from the river Mtkvari	*Podoviridae*	unknown	*E.coli*	10^8
ΦPIC	Sewage water sample, Tbilisi	*T4 like*	130524bp	*E.coli*	10^8
ΦB1	Water sample from the river Mtkvari	*Felix O1 like*	87638 bp	*Salmonella* Typhimurium	10^8
ΦB3	Water sample from the river Mtkvari	*T5 l like*	83203bp	*Salmonella* Typhimurium	10^8
ΦMG	Sewage water sample, Tbilisi	*T4 like*	170240bp	*Salmonella* Enteritidis	10^8
ΦBS	Water sample from the Black Sea	*Vi01 lke*	158018bp	*Salmonella* Typhimurium	10^8
Φ #5	Water sample from the river Mtkvari	*T5 like*	130524bp	*Salmonella* Typhimurium	10^8

from the Eliava IBMV culture collection initially originated from different farms were used for infecting the eggshells in the model experiments (Table 2).

METHODS:

Surface area of the sample eggs, used in decontamination experiment was calculated using following equation (Equation 1) (Table 3).

$$2\pi a^2 + \pi a\left(\frac{b^2}{\sqrt{b^2 - a^2}}\cos^{-1}\left(\frac{a}{b}\right) + \frac{c^2}{\sqrt{c^2 - a^2}}\cos^{-1}\left(\frac{a}{c}\right)\right)$$

Equation 1. Egg surface area calculation: a - equatorial radius, b - short polar radius, c - long polar radius.

1. Study of naturally occurring eggshell contamination in Georgia.

The fresh eggs were obtained at the local market and immediately transferred to the laboratory in aseptic conditions.

Naturally occurring eggshell contamination of the obtained egg samples were examined according to the method described by Gentry and Quarles [16] with modifications. Twenty eggs from designated source groups were rolled for 2 min on the top of Petri dishes containing each of the following bacteriology media: Luria Bertani Agar (LB), Endo, Salmonella-Shigella (SS) and Sabouraud dextrose agar media *(HIMEDIA)*, ensuring that the whole shell surface area contacted with the agar surfaces. The plates with LB, ENDO and SS agar were incubated for 24h at 37°C and checked for colony formation. The plates with Sabouraud dextrose agar were incubated at 25°C for 5 days for detection of fungal microflora. Duplicates of the tested sample fingerprints were also incubated at 4°C for two weeks to reveal the presence of psychrophilic contaminants on the eggshell surfaces. The smears from the developed colonies

Table 2. Bacterial strains used in the model experiments

NN	Species	Strain ID number	Source	Serotype
1	*Salmonella enterica*	**0016**	Farm House, Germany	Typhimurium
2	*Salmonella enterica*	**001K3**	Farm House, Korea	Enteritidis
3	*Salmonella enterica*	**00121**	Farm House, Germany	Typhimurium
4	*Salmonella enterica*	**00114**	Farm House, Germany	Typhimurium
5	*Salmonella enterica*	**0012**	Farm House, Germany	Typhimurium
6	*Salmonella enterica*	**0011**	Farm House, Germany	Typhimurium
7	*Salmonella enterica*	**0019**	Farm House, Germany	Typhimurium
8	*Salmonella enterica*	**001K4**	Farm House, Korea	Enteritidis
9	*Escherichia coli*	**0B7**	Environmental sample, UK	Not applicable
10	*Escherichia coli*	**0B4**	Environmental sample, UK	Not applicable
11	*Escherichia coli*	**0B8**	Environmental sample, UK	Not applicable

Table 3. Designated name and surface area of in vitro contaminated egg samples

Sample description	Designated name	Surface area
Medium size Heat Sterilized eggs	E1	75.04 cm^2
	E2	76.2 cm^2
	E3	71.25 cm^2
	E4	76.4 cm^2
	E5	72.26 cm^2
	E6	87.92 cm^2
	E7	83.09 cm^2
	E8	85.7 cm^2
	E9	82.9 cm^2
	E10	83.7 cm^2
	E11	83.16 cm^2
	E12	82.7 cm^2
	E13	80.02 cm^2
	E14	84.24 cm^2
	E15	81.3 cm^2
	E16	80.5 cm^2

were Gram stained and examined by light microscopy for basic identification.

2. Phage treatment of experimentally contaminated eggshell. Egg samples were sterilized by boiling for 20 min and were divided into two experimental groups: Gr.1 and Gr. 2 (with 6 eggs in each group, which were numbered as E1-E12) and two control groups: Gr. 4 and Gr. 5 (with two eggs in each group, which were numbered as E13-E16). Eggs from Gr. 1 (E1,E2, E.3, E4, E5 and E.6) were dipped into a bacterial suspension dissolved in saline solution containing a single strain of *Salmonella typhimurium* 0016 (titer 10^8 cfu/ml), the eggs from Gr. 2 (E7, E8, E9, E10, E11 and E12) where dipped into the bacterial suspension, containing a mixture of bacterial strains (*S. typhimurium* 0016, *S. enteritidis* 001K3, *S. typhimurium* 00121, *S. typhimurium* 00114, *S. typhimurium* 0012, *S. typhimurium* 0011, *S. typhimurium* 0019, *S. enterica* 001K4 and *E.coli* 0B7, *E.coli* 0B4, *E.coli* 0B8) dissolved in saline solution (final titer: 10^8 cfu/ml). After 15 minutes of exposure the samples were air dried in a biosafety cabinet (BL2) (Labconco) for 20 min.

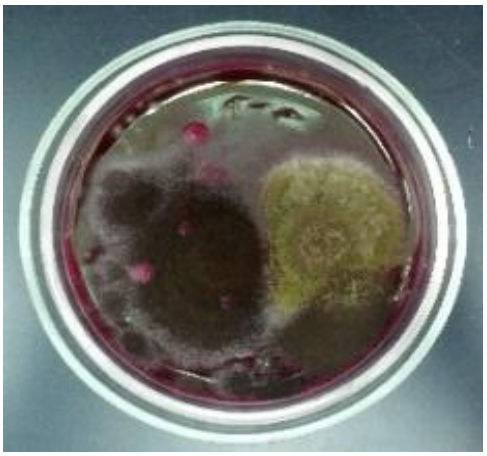

Fig. 1. Egg fingerprint on Endo agar after 5 days of incubation at 25 ^{O}C.

Afterwards 1 ml of single phage lysate (ΦBS-titer 10^8pfu/ml) was evenly sprayed onto the surface of each egg from Gr. 1 and 1ml of the phage mixture P1 was evenly sprayed on the eggs from Gr. 2. Sample fingerprints using sterile moisturized cotton tissue with the known radius were taken from all eggs included into Gr.1 and Gr.2 at the initial point (i.e. at "0" min after phage treatment), then after 15 minutes, 2 h and 18 h of incubation (37°C). Experiment was performed in presence of controls (control for Gr 1: E13, E14; and, control for Gr2: E15, E 16). Resulting bacterial titer was calculated by enumeration of the colony forming units (cfu) per square cm (Fig. 1).

STATISTICAL ANALYSIS

Standard deviation was calculated for two experimental group time series.

RESULTS:

1. Naturally occurring eggshell contamination. In all tested samples the dominating microflora detected on eggshell surfaces was presented by fungi among which the psychrophilic biotypes predominated

2. ***Phage treatment of bacterial eggshell contamination.*** Effects of phage treatment on experimentally contaminated eggshells showed following results: ΦBS bacteriophage application onto the eggs from Group1 resulted in one log (90%) decrease of the total bacterial cell counts, occurring within 15 minutes after the phage application. Decrease in bacterial titer remained stable during the next 18 hours (Fig.2).

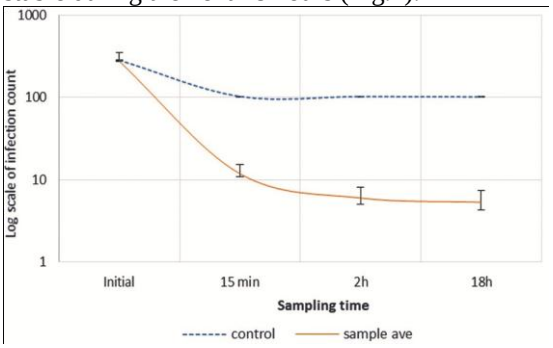

Fig. 2. Results of *Salmonella typhimurium* 0016 contaminated eggshell treatment with ΦBS phage (shown: averaged series with standard deviation error bars).

Application of the phage mixture P1 on the surface of eggs from Gr.2, showed one log (90%) decrease of the total bacterial cell counts already after 15 minutes after the treatment and less intensively continued to decrease during the next 18 hours (Fig.3.)

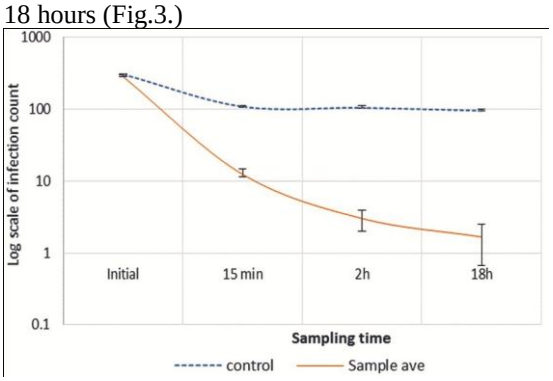

Fig. 3. Results of eggshell treatment with the phage mixture P1. Shell was infected with the mixture of Salmonella and E.coli strains (shown: averaged series with standard deviation error bars).

DISCUSSION AND CONCLUSIONS

With regards of the natural contamination of eggshells our study demonstrated that an external surface microflora of eggs is mainly formed by fungal microbiota. Lack of bacterial microbiota can be

related to an uncontrolled use of antibiotics in the farms widely practiced in Georgia. As is known, even the controlled use of antibiotics leads to enhanced growth of fungal cells in the site of action.

Absence of the regulations and monitoring processes related to the sustainable use of antibiotics as farming supplements may lead to disturbances in bacterial ecology and increasing risk of infectious outbreaks. Problems caused by food-borne multi-drug resistant bacteria in Georgia needs through studies, to find the major causative agents and the best solution for their prevention.

According to the obtained results the phage treatment resulted in a significant decrease of bacterial contamination for one log (90%) on the whole shell surface within the first 15 minutes after phage application which remains stable in case of application of one phage clone and is slightly decreasing during the next 18 hours in case of application of bacteriophage mixture with the overlapping host ranges. This approves the phage decontaminating effect. Therefore, phage application may be suggested for treatment and prevention of eggshell contamination.

Based on the results of the work we can conclude that phages can be considered as an affective alternatives to antibiotic treatment, and, as a prophylactic supplement in food industry. To provide more reliable statistical support to this conclusion a large scale decontamination experiment in a natural farm environment is required.

მიკრობიოლოგია

კვერცხის ნაჭუჭის დეკონტამინაცია *Salmonella* და *E.coli* სპეციფიკური ბაქტერიოფაგების ნაზავით დამუშავების შედეგად

ხ. მაკალათია*, ე. კაკაბაძე*, ნ. ბაკურაძე*, ნ. გრძელიშვილი**, გ. ნატროშვილი**, ნ. ჭანიშვილი*

** ივანე ჯავახიშვილის სახელობის თბილისის სახელმწიფო უნივერსიტეტი, ზუსტ და საბუნებისმეტყველო მეცნიერებათა ფაკულტეტი; გ. ელიავას სახელობის ბაქტერიოფაგიის, მიკრობიოლოგიის და ვირუსოლოგიის ინსტიტუტი, კვლევის და განვითარების დეპარტამენტი, თბილისი, საქართველო*

*** გ. ელიავას სახელობის ბაქტერიოფაგიის, მიკრობიოლოგიის და ვირუსოლოგიის ინსტიტუტი, კვლევის და განვითარების დეპარტამენტი, თბილისი, საქართველო*

(წარმოდგენილია აკადემიის წევრის თ. სადუნიშვილის მიერ)

საკვებთან ასოცირებული ინფექციური დაავადებების პრევენცია და აღკვეთა კვების უსაფრთხოების უმთავრესი საკითხია. სხვადასხვა ტიპის საკვები პროდუქტების ბაქტერიული კონტამინაციის რისკის შემცირებისა და პრევენციის ახალი ტექნოლოგიების კვლევა-განვითარება, საზოგადოებრივი ჯანდაცვის ერთ-ერთი უმთავრესი ამოცანაა. მოცემული კვლევა მიზნად ისახავდა: კვერცხის ნაჭუჭის ზედაპირის ბუნებრივი კონტამინაციის შესწავლას და ***Salmonella enterica*** და ***Escherichia coli***-ის სეროტიპებით ინფიცირებული კვერცხის ზედაპირის სპეციფიკური ფაგების ნაზავით დამუშავების ეფექტის დადგენას მოდელირებულ ***in vitro*** გარემოში. კვლევამ აჩვენა, რომ სპეციფიკური ფაგების ნაზავი ერთი ლოგარითმით (ანუ 90%-ით) ამცირებს კვერცხის გარეგან ინფექციურ დაბინძურებას დამუშავებიდან 15 წუთში, რაც სტაბილური რჩება მომდევნო 18 სთ-ის განმავლობაში.

REFERENCES

1. Logan R., Barrett K. et al. (2017) FoodNet, 2015 Surveillance Report (Final Data), *Foodborne Diseases Active Surveillance Network*. Atlanta, Georgia: U.S. Department of Health and Human Services, CDC.
2. Chemaly M. et al. (2009) Isolation of *Salmonella enterica* in laying-hen flocks and assessment of eggshell contamination in France. *Journal of Food Protection,* 72(10):2071-2077.
3. De Reu K. et al. (2006**)** Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including *Salmonella enteritidis*. *International Journal of Food Microbiology*, **112**(3): 253-260.
4. Vučemilo M., Vinković B., Matković K., Štoković I., Jakšić S., Radović S. & Stubičan Đ. (2010) The influence of housing systems on the air quality and bacterial eggshell contamination of table eggs. *Czech. J. Anim. Sci.* **55**(6): 243-249.

5. Goodridge L. D. & Bisha B. (2011) Phage-based biocontrol strategies to reduce foodborne pathogens in foods. *Bacteriophage*, **1**(3):130-137.
6. Garcia P., Martinez B., Obeso J. M. & Rodriguez A. (2008) Bacteriophages and their application in food safety. *Letters in applied microbiology*, **47**(6):479-485.
7. Whichard J. M., Sriranganathan N. & Pierson F. W. (2003) Suppression of Salmonella growth by wild-type and large-plaque variants of bacteriophage Felix O1 in liquid culture and on chicken frankfurters. *Journal of food protection*, **66**(2):220-225.
8. Leverentz B., Conway W. S., Camp M. J., Janisiewicz W. J., Abuladze T., Yang M. & Sulakvelidze A. (2003) Biocontrol of *Listeria monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Applied and Environmental Microbiology*, **69**(8):4519-4526.
9. Abuladze T. et al., (2008) Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157: H7. *Applied and environmental microbiology* 74, 20: 6230-6238.
10. Department of Health and Human Services, Food and Drug Administration (2016) 21 CFR Part 172, [Docket No. 2002F–0316] (formerly 02F–0316), *Food Additives Permitted for Direct Addition to Food for Human, Consumption; Bacteriophage Preparation, AGENCY: Food and Drug Administration*, HHS.
11. Rizzi Valentina (2016) *Salmonella* monitoring data, food-borne outbreaks and antimicrobial resistance data for 2014 in the European Union, BIOMO team, BIOCONTAM Unit, *EFSA 21st EURL-Salmonella Workshop*, Saint Malo, France.
12. Metreveli M. et al., (2016) Salmonella Risk Assessment in Bird's Frozen and Mechanically Separated Meat, Scientific Risk Assessment Advisory, Council of Risk Assessment Service, Agricultural Research Center.
13. Gentry R. F. & Quarles, C. L., (1972). The measurement of bacterial contamination on egg shells. *Poultry Science*, **51**(3), 930-933.
14. Vashakidze E., Megrelishvili T., Pachkoria E., Tevzadze L., and Lashkarashvili M., (2010) Enterohemorrhagic *E. coli* and hemolytic uremic syndrome in Georgia, *Georgian Med. News*. 186:38-41.
15. Vashakidze E., Megrelishvili T., Pachkoria E., Imnadze P., Canava S., Tevzadze L., Lashkarashvili M. (2010) *E.coli* infectious complicated with HUS in Georgia, *International Journal of Infectious Diseases*, **14**(1):e66 http://doi.org/10.1016/j.ijid.2010.02.1635.
16. Woolston J. et al. (2013) Bacteriophages lytic for *Salmonella* rapidly reduce *Salmonella* contamination on glass and stainless steel surfaces. *Bacteriophage* 3.3 e25697.

Received January, 2018