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Decontamination of major *Salmonella* serovars derived from poultry farms on eggs using *Salmonella* phage cocktail

Wattana Pelyuntha[1], Ruttayaporn Ngasaman[2], Mingkwan Yingkajorn[3], Kridda Chukiatsiri[4], and Kitiya Vongkamjan[1,*]

[1]Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Chatuchak, Bangkok, Thailand
[2]Faculty of Veterinary Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand
[3]Division of Pathology, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand
[4]Faculty of Animal Science and Technology, Maejo University, Chiang Mai, Thailand
[*]Corresponding author: kitiyavongkamjan.a@ku.th

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Abstract

Salmonella contamination in human food can lead to the serious human disease of salmonellosis, if contaminated food is consumed. Both eggs and egg products have been often linked to the occurrence of *Salmonella* along with product recalls due to *Salmonella* contamination. Overall, *Salmonella* is present on the external shells surface and internally. Many strategies have been established to decontaminate *Salmonella,* for increasing the safety of eggs and egg products; especially the use of bacteriophages (phages) as a biocontrol agent. In this study, three phages; namely: vB_SenS_WPX1, vB_SenS_WPX2, and vB_SenS_WPX3 were tested for their lytic activity against eight major *Salmonella* serovars, which were recovered from poultry farms. Three phages showed robust lytic activity against five serovars tested; including, Corvalis, Kentucky, Saintpaul, Schwarzengrund, and Typhimurium. Phages vB_SenS_WPX1 and vB_SenS_WPX2 were effective against *S.* Mbandaka; whereas, *S.* Agona and *S.* Albany were most susceptible to phage vB_SenS_WPX3. Three phages were further developed, as a phage cocktail, and showed an ability to reduce *Salmonella* count by 4 log colony-forming unit (CFU)/mL at 6 h post-phage treatment. The cocktail could immediately decrease the *Salmonella* count adhered on eggshells by 3.8 log CFU/egg after 1 day of storage, and further prevent the penetration ability of *Salmonella* into egg yolks and the white albumen. The results suggest that a developed phage cocktail could be a potential, alternative strategy for decontaminating *Salmonella* on eggs, so as to increase the safety of the food.

Keywords: Biocontrol, Eggs, Food safety, Phage cocktail, *Salmonella*

1. Introduction

Eggs are a rich source of nutrients that provide a high quality of protein, polyunsaturated fatty acids, vitamins, and minerals [1]. Nowadays, the demand of egg consumption is continually increasing, leading to consumer awareness of food safety [2,3]. Pathogen infections in eggs can occur via two different routes; including vertical and horizontal transmission. Through horizontal transmission, eggs are usually contaminated from feces released via the cloaca, and other environmental vectors in animal farms; whereas, bacteria surviving in the reproductive tissues is an important factor related to vertical transmission [4]. *Salmonella* is the most reported bacteria responsible for foodborne illness outbreaks linked to eggs and egg products worldwide [5]. Dirty and cracked eggs may carry harmful *Salmonella* on their shells as well as internally. In the United States, there are approximately 48 million annual cases of foodborne illness; resulting in 128,000 hospitalizations and 3,000 deaths [6]. *Salmonella* can cause a foodborne illness called “salmonellosis”, which resulted in 420 deaths occurring due to non-typhoidal *Salmonella* annually in the United States [7].

To prevent *Salmonella* in food production chains, several measures have been used for controlling this foodborne pathogen. Although, food hygiene practices can decrease the spread of contamination, long-term

preventive measures and food safety protection at the first step of production are further required [8,9]. The use of effective, alternative approaches; including, antibacterial peptides, chemical agents, disinfectants, vaccinations, and effective microflora have been introduced in food production chains [10]. However, these increase several problems within food industries. For example, the use of antibiotics in animal cultivation could increase the number of antibiotic-resistant bacteria, and subsequently resistance in humans. Additionally, this would require expensive vaccinations and treatments, create toxicity and residue from the chemicals used, and cause difficulty and complexity of administrations [11].

Bacteriophages (phages) are the viruses that specifically infect bacteria. Phages have harmless effects on human and animal health, but are effective in reducing pathogens. In addition, phages are easy to administer [12,13]. Recently, phages have been applied as antibacterial agents within the food industry to increase food safety, and in food animal production to prevent zoonotic diseases [14,15]. However, the use of phages in food industries is limited, due to high specificity on pathogens and potential development of phage resistance in bacteria [13]. The development of different phages, in combination as a phage cocktail, could overcome these problems. Phage cocktails not only potentially avoid phage resistance, as compared to the use of a single phage, they also allow for the treatment of pathogens simultaneously [16,17]. The objectives of this study were: (i) to investigate the ability of a phage cocktail against major *Salmonella* serovars, derived from poultry farms, via lytic ability and (ii) to evaluate the *Salmonella* phage cocktails effectiveness on the reduction of *Salmonella in-vitro* and on eggs.

2. Materials and methods

2.1 Bacterial culture and conditions

Eight major *Salmonella* serovars; including, Agona, Albany, Corvalis F3-W5-S2, Kentucky S1H28, Mbandaka H17D2, Saintpaul H13, Schwarzengrund H2, and Typhimurium F1-W1-C2, from our collection at the Department of Biotechnology, Kasetsart University, Bangkok, Thailand were recruited in this study. These bacteria were previously isolated from cloacal swabs and environmental samples (bedding and boot cover swabs) collected from broiler farms located in Thailand in a previous study [18]. All serovars were activated in soyabean casein digest medium (tryptic soy broth; TSB) at 37℃ for 18 h for this study.

2.2 Salmonella phage lysate preparation

Three potential *Salmonella* phages, namely: vB_SenS_WPX1, vB_SenS_WPX2, and vB_SenS_WPX3, in our collection were selected for this study; based on their previous characterization. *S.* Anatum A4-525 was used as a propagating host for phages vB_SenS_WPX1 and vB_SenS_WPX2; whereas, *S.* Kentucky S1H28 was a propagating host for phage vB_SenS_WPX3. All phages were propagated using a double-layer agar assay according to the protocol of Pelyuntha et al. (2021) [18]. A ten-fold serial dilution using salt magnesium (SM) buffer was used to obtain appropriate concentrations of phage stock, and then overlaid with a natural host to obtain the semi-confluent lysis plates. Phages were collected by scrapping the top-layer of the agar, suspended using a SM buffer, and then centrifuged at 15,000 g for 15 min at 4℃. Filtrates were collected after being filtered through 0.20 µm syringe filters, and then stored at 4℃ until analysis. Phage titers were enumerated by observing plaques presented on each plate of each given dilution [18].

Table 1 Characterization of *Salmonella* phages used in this study.

Description	*Salmonella* phage		
	vB_SenS_WPX1	vB_SenS_WPX2	vB_SenS_WPX3
Host of isolation	*S.* Anatum A4-525	*S.* Anatum A4-525	*S.* Kentucky S1H28
Source of isolation	Wastewater[1]	Wastewater[2]	Fresh water[2]
Plaque morphotype (mm)	2.0	1.0	0.2
Classification			
Order	*Caudovirales*	*Caudovirales*	*Caudovirales*
Family	*Siphoviridae*	*Siphoviridae*	*Siphoviridae*.

[1] Wastewater collected from the wastewater treatment station of Songklanagarind hospital, Prince of Songkla University, Hat Yai, Songkhla, Thailand. [2]Fresh water was collected from a reservoir of the Veterinarian teaching hospital, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

2.3 Determination of the lysis ability of Salmonella phages

Lysis profiles of three phages were investigated, by dropping 20 µL of lysate (8 log plaque-forming unit (PFU)/mL) on lawn cultures of each *Salmonella* serovar; as listed above and according to the protocol of Pelyuntha et al. (2021) [18]. All plates were observed for the appearance of the lysis zone after growth at 37°C for 18 h.

2.4 Phage cocktail development and in-vitro efficiency evaluation

Each phage was mixed in equivalent quantities to obtain a working cocktail stock. A developed phage cocktail was assessed for its efficiency on 8 *Salmonella* serovars. A 20 mL of bacterial strain suspension (final concentration of 4 log colony-forming unit (CFU)/mL) was combined with a 20 mL developed phage cocktail (final concentration of 7 log PFU/mL) to achieve an effective multiplicity of infection (MOI) of 1000. These were then incubated at 37°C for 18 h in a shaking incubator, at a shaking speed of 220 rpm. Each culture of the *Salmonella* strains, with a phage cocktail omitted, was kept as a control. Cell reduction of *Salmonella* in the control and treatments was evaluated every 6 h by a spread plate on tryptic soy agar (TSA) plates [18,19].

2.5 Effectiveness of a phage cocktail on Salmonella reduction on eggs

Freshly laid eggs (size 3, approximately 68 cm^2) were purchased from supermarkets. The eggs were washed three times with sterile deionized (DI) water at room temperature (25°C), to remove dust and visible dirt, then allowed to dry under a laminar flow cabinet for 15 min. To prepare the *Salmonella* mixture, eight tested serovars were grown in 5 mL TSB at 37°C overnight. One milliliter of each culture was mixed and diluted with Phosphate buffer solution (PBS) to obtain the final concentration of 8 log PFU/mL, using 0.5 McFarland standard. Eggs were swabbed with the culture mixture to obtain the final concentration of bacteria on the eggshell at 4 log CFU/egg, and allowed to dry for 30 min at room temperature. Each inoculated egg was sprayed with a suspension of phage cocktail (7 log PFU/mL), at 1 mL per egg (MOI 1000). This MOI was selected based on the efficacy of this phage cocktail from our previous study [18]. The treated eggs were dried in a laminar flow for 1 h, then transferred to sterile zip-lock plastic bags, and kept at room temperature for 5 days. This was to represent the storage conditions where temperature abuse at room temperature may be the cause of the short shelf-life for eggs. PBS was sprayed on the eggs as a control. Viable *Salmonella* on the eggshells were enumerated on day 0, 1, 3, and 5 of storage by a swab test. Cotton swabs were mixed with 9 mL of buffered peptone water (BPW), then diluted with the same diluent to yield appropriated dilutions, and spreaded on Xylose Lysine Decarboxylate (XLD) agar. A typical *Salmonella* colony was observed and counted after incubation at 37°C for 24 h. Moreover, egg yolks and their white albumens were also investigated for the number of penetrating *Salmonella*, by being spreading on the XLD agar on day 0 to 5 [18].

2.6 Statistical analysis

Data were analyzed using the SPSS statistics software (version 22, SPSS Inc., Chicago, IL, USA). The reduction of *Salmonella* during each sampling period was employed to variance analysis, followed by the Tukey's range test. An independent-samples t-test was used to calculate the significant difference between the control and each phage cocktail treatment within the same sampling period. A *p*-value of 0.05 was considered as statistically significance.

3. Results

3.1 Determination of lysis ability of Salmonella phages

The lysis profiles of three selected phages are shown in Table 2. Three phages showed strong lysis on *S.* Corvalis, *S.* Kentucky, *S.* Saintpaul, *S.* Schwarzengrund, and *S.* Typhimurium. Only phage vB_SenS_WPX3 could lyse *S.* Agona and *S.* Albany strongly, while the other two phages were able to lyse *S.* Mbandaka.

3.2 Phage cocktail development and in-vitro efficiency evaluation

A phage cocktail treatment significantly reduced the population of *Salmonella* by 100%, as indicated by 3 to 4 log-unit reduction ($p < 0.05$) for *S.* Corvalis, *S.* Kentucky and *S.* Saintpaul at 6 h post-phage treatment (Table 3). A reduction of *S.* Schwarzengrund and *S.* Typhimurium by 100% ($p < 0.05$) was observed at 12 h post-phage treatment. However, a phage cocktail could not completely reduce the population of *S.* Agona, *S.* Albany, and *S.*

Mbandaka. A reduction was only observed by 0.3 to 2.2 log units (4-28% reduction) after 6 h of phage treatment, which continually raised until the investigation was concluded; whereas, other serovars were completely eliminated after 6 to 12 h of phage treatment.

3.3 Effectiveness of a phage cocktail on Salmonella reduction on eggs

The results showed no significant difference in *Salmonella* counts between the control eggs (3.8±0.4 log CFU/egg) and the phage cocktail-treated eggs (3.8±0.2 log CFU/egg) at day 0 of storage ($p > 0.05$) (Table 4). The *Salmonella* count in the control eggs increased to 5.3±0.1 log CFU/egg at day 2, then slightly decreased at day 3 (4.4±0.1 log CFU/egg) and 5 (4.4±0.3 log CFU/egg). In the phage cocktail treatment group, *Salmonella* cells were not detected on the eggs at day 1, nor up to 5 days of storage. In addition, *Salmonella* cells could not be detected in the egg yolks or their white albumen among the phage cocktail-treated eggs; whereas, the control group showed 2.3±0.0 log CFU/mL of penetrating *Salmonella* cells; found in the eggs on day 5.

Table 2 Lysis ability of three potential phages on major *Salmonella* serovars derived from poultry farms.

Salmonella serovars	*Salmonella* phages		
	vB_SenS_WPX1	vB_SenS_WPX2	vB_SenS_WPX3
Agona H3D6	-	-	+
Albany H32	-	-	+
Corvalis F3-W5-S2	+	+	+
Kentucky S1H28	+	+	+
Mbandaka H17D2	+	+	-
Saintpaul H13	+	+	+
Schwarzengrund H2	+	+	+
Typhimurium F1-W1-C2	+	+	+

Lysis ability: + represents lysis and – represents no lysis.

Table 3 Efficacy of a phage cocktail on *Salmonella* serovars derived from poultry farms.

Salmonella	Time (h)	Bacterial count [A] (log CFU/mL)		% Reduction
		Control (no phage)	Phage cocktail	
Agona H3D6	0	3.8±0.2^{a}	3.9±0.2^{a}	-
	6	7.1±0.1^{b}	7.4±0.1^{b}	0
	12	9.0±0.0^{b}	8.9±0.1^{b}	1
	18	9.2±0.2^{b}	9.5±0.1^{b}	0
Albany H32	0	4.0±0.1^{a}	4.1±0.2^{a}	-
	6	7.9±0.0^{b}	5.7±0.0^{b*}	28
	12	8.1±0.1^{b}	7.0±0.2^{bc}	14
	18	9.4±0.1^{b}	9.0±0.0^{c}	4
Corvalis F3-W5-S2	0	3.3±0.0^{a}	3.3±0.1	-
	6	7.7±0.2^{b}	nd	100
	12	9.3±0.1^{b}	nd	100
	18	9.5±0.2^{b}	nd	100
Kentucky S1H28	0	3.9±0.2^{a}	3.9±0.1	-
	6	7.9±0.1^{b}	nd	100
	12	8.3±0.1^{bc}	nd	100
	18	9.7±0.0^{c}	nd	100
Mbandaka H17D2	0	3.4±0.1^{a}	4.0±0.2^{a}	-
	6	7.7±0.1^{b}	7.4±0.1^{b}	4
	12	9.3±0.0^{b}	9.4±0.4^{b}	0
	18	9.1±0.1^{b}	9.4±0.3^{b}	0
Saintpaul H13	0	3.8±0.1^{a}	3.7±0.1	-
	6	7.8±0.2^{b}	nd	100
	12	8.4±0.1^{b}	nd	100
	18	9.6±0.2^{b}	nd	100
Schwarzengrund H2	0	4.0±0.5^{a}	4.0±0.2^{a}	-
	6	7.5±0.2^{b}	3.3±0.2^{b*}	56
	12	8.3±0.0^{c}	nd	100
	18	9.5±0.0^{d}	nd	100
Typhimurium F1-W1-C2	0	3.9±0.4^{a}	3.9±0.5^{a}	-
	6	7.4±0.3^{b}	3.9±0.3^{a*}	47
	12	9.2±0.0^{c}	nd	100
	18	9.3±0.1^{d}	nd	100

[A]All values are provided as mean ± SD of three replicates. The lowercase letters ($^{a, b, c, d}$) for phage cocktail or control and those connected by the same letter are not significantly different ($p > 0.05$). The *asterisk* (*) defines the significant difference ($p < 0.05$) of *Salmonella* counts between the phage treatment and control during the same sampling time. "nd" refers to no *Salmonella* cell detected. The percentage of bacterial reduction at 0 h was normalized to 0.0.

Table 4 Effectiveness of a phage cocktail on *Salmonella* reduction on eggs

Time (days)	*Salmonella* count (log CFU/egg)			
	Eggshell		Egg yolk & White albumin	
	Control (no phage)	Phage cocktail	Control (no phage)	Phage cocktail
0	3.8±0.4[a]	3.8±0.2	nd	nd
1	5.3±0.1[c]	nd	nd	nd
3	4.4±0.1[b]	nd	nd	nd
5	4.4±0.3[b]	nd	2.3±0.0	nd

All values are provided as mean ± standard deviation in triplicate. The lowercase letters ([a, b, c]), in the same column connected with the different letters, indicate the significant difference ($p < 0.05$). "nd" refers to zero *Salmonella* cell detected.

4. Discussion

Eggs possess three physical barriers: the egg cuticle, the crystalline eggshell, and the shell membranes. The cuticle of eggs is a proteinaceous layer, with hydrophobic properties covering the eggshell and the pore openings. The shell membranes are comprised of 3 layers different: the inner membrane, outer membrane, and limiting membrane. Shell membranes are associated with the bacterial defense system of eggs. Several antibacterial proteins located in the eggshell and shell membranes have been identified; such as, ovocalyxin-36, ovotransferrin, lysozyme, and gallinacins (β-defensins). These proteins possess antibacterial activity against a wide range of both Gram-positive and Gram-negative bacteria [20]. Infection with *S.* Enteritidis or interaction with purified lipopolysaccharides can increase the expression of antibacterial proteins; especially gallinacins. Moreover, proteinase-inhibiting proteins have also been identified; such as, ovomucoid, ovoinhibitor, cystatin, and ovostatin. These proteins are important to inhibit the tryptic digestion activity of egg proteins, caused by microorganisms. In addition, they also protect the antimicrobial activity of albumin proteins [20,21]. *Salmonella* can easily pass through the eggshell; especially serovar Enteritidis. This serovar can survive and/or grow in the internal egg constituents; whereas, other serovars including Typhimurium and Hadar are only found on the eggshell [20,22]. *Salmonella* can survive effectively inside of the egg contents by using their virulence factors to escape the bacterial defense system of eggs. Moreover, the *rfbH* gene, responsible for lipopolysaccharide o-antigen synthesis, plays a key role during the growth of *S.* Enteritidis in whole eggs; whereas, the Δ*rfbH* mutant strain was immediately killed according to a previous study [23]. Distinctive genes responsible for cell wall formation and metabolism also support *Salmonella* adaptation during survival in the eggs albumen as well as which the siderophore can effectively uptake iron during survival in egg whites [24-26].

The natural contamination of eggshells caused by external surface microflora; such as, bacteria and fungi [27]. Sanitizers; including, quaternary ammonium compound (QAC, pH 7.5), sodium carbonate (Na_2CO_3, pH 12), and sodium hypochlorite (NaOCl, 100 ppm, pH 7.5), are commonly used as chemical cleansers for egg washing along with warm water (41-44℃) [28,29]. However, organic substances in washing water can be combined with chlorine, resulting in the formation of trihalomethanes and other organochlorine compounds; which are potentially carcinogenic [30]. In addition, chlorine is slightly active against pathogen loads on the eggs surface [31]. Hence, safe and effective approaches should be established.

Several studies have shown that the use of phage and phage cocktail for effectively reducing the number of pathogenic bacteria in food surfaces. Phages have gained additional interest for use in controlling foodborne pathogens; especially *Salmonella* [16,32]. For example, the treatment of *Salmonella* phages Eϕ151 and Tϕ7 could reduce the number of *Salmonella* presented on skin surfaces of chickens. These phages significantly reduce the median of *S.* Enteritidis and *S.* Typhimurium, by 1.38 and 1.83 log MPN per chicken skin section when compared to the control ($p < 0.0001$) [33]. Moreover, a phage cocktail of UAB_Phi_20, UAB_Phi_78, and UAB_Phi_87 significant reduced *S.* Enteritidis and *S.* Typhimurium on pig skin, by 2 and >4 log CFU/cm^2 [34].

Additionally, phages and phage cocktails were studied for their potential application against contaminated bacteria presenting on egg surfaces. The application of ΦBS phage onto the eggs resulted in 1 log reduction (90%) of *S.* Typhimurium within 15 min after application [35]. However, a phage cocktail of UAB_Phi_20, UAB_Phi_78, and UAB_Phi_87 (MOI 1000) slightly decreased *S.* Enteritidis and *S.* Typhimurium (0.9 log unit reduction) in fresh eggs. The variations of *Salmonella* concentrations of the eggshells can be attributed to the characteristics of the eggshell surface [34]. A phage cocktail of F1055S and F12013S (2×10^6 PFU/mL) were also applied by aerosol spraying on the fertilized eggs. The treatment with this cocktail can reduce the disease symptoms (arthritis and pasting) in the hatched chicks. The authors suggest that the application of phage-aerosol sprays during the transfer of the eggs from incubators to hatchers also reduces the horizontal transmission of *Salmonella* in poultry [36]. In our study, our phages and phage cocktails were shown to be more effective than above reports. This might be due to the high specificity and % lytic ability of phages on targeted bacteria. A phage cocktail composed of vB_SenS_WPX1, vB_SenS_WPX2, and vB_SenS_WPX3 was shown to be very

effective against major *Salmonella* serovars derived from poultry farms, as indicated by the ability to reduce the *Salmonella* count by 3 to 4 log CFU/mL at 6 h *in-vitro*, and 3.8 log CFU/egg at 24 h post-phage treatment. The phage cocktail also could prevent the penetration of *S.* Enteritidis into egg whites and egg yolks. Therefore, the phage cocktail of vB_SenS_WPX1, vB_SenS_WPX2, and vB_SenS_WPX3 was shown to be a promising candidate as a biocontrol agent to control *Salmonella* in eggs and eggshells. This cocktail can be applied to other foods, along with food contact surfaces, for potentially reducing *Salmonella* contamination and salmonellosis.

5. Conclusion

The presence of *Salmonella* in human food is a global issue for human health, and requires urgent, effective and alternative approaches for control; such as, phage cocktail treatments. In this study, three *Salmonella* phages showed high efficacy against major *Salmonella* serovars that have caused serious issues to poultry and egg production. Our phages might be recommended due to their specificity and bacteriostatic activity on targeted bacteria. There has also been no report concerning the harmfulness of phages on human and animal health. Therefore, the phage cocktail studied herein showed to be a promising tool for use to control the contamination of *Salmonella* on egg surfaces. However, additional information on safety and stability of phage cocktail applications for eggs and egg products is still required.

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7. Conflict of interest

The authors declare no conflict of interest.

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