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Isolation and application of bacteriophages to reduce *Salmonella* contamination in raw chicken meat

Hoang Minh Duc[1], Hoang Minh Son[1], Ken-ichi Honjoh, Takahisa Miyamoto[*]

Laboratory of Food Hygienic Chemistry, Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, 6-10-1, Hakozaki, Higashi-ku, Fukuoka, 812-8581, Japan

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ABSTRACT

Chicken meats are considered as main sources associated with *Salmonella* infections in humans. In this study, lytic phages against *Salmonella* were isolated and examined for their efficacy to control *Salmonella*. Eighteen lytic phages were isolated from raw chicken skin and gizzard. Five phages belonging to *Myoviridae* and *Siphoviridae* families were characterized and selected for bacterial challenge tests. The treatment of raw chicken breast samples contaminated with *S.* Enteritidis and *S.* Typhimurium at 8 °C by the cocktail of five phages significantly reduced ($P < 0.05$) viable counts by 1.41 and 1.86 log CFU/piece, respectively. When incubated at 25 °C, the highest reductions of viable counts of *S.* Enteritidis and *S.* Typhimurium in the phage-treated samples were 3.06 and 2.21 log CFU/piece, respectively ($P < 0.05$). These data suggested that the phages isolated from raw chicken meats are potential agents for controlling *Salmonella* in raw meats.

1. Introduction

Salmonella spp. is one of the most important foodborne pathogens worldwide. In the United States, more than 40,000 cases of salmonellosis were annually reported (Finstad, O'Bryan, Marcy, Crandall, & Ricke, 2012). In Europe, *Salmonella* is responsible for more than 94,625 cases in 2015 (Authority & Control, European Centre for Disease Prevention and, 2016). In Japan, the second most common foodborne pathogen is *Salmonella* (Kumagai et al., 2015), causing 2551 cases of illnesses in 2008 (Ishihara et al., 2009; Kumagai et al., 2015). More than 2500 serovars of *Salmonella* have been identified but some of these serovars, especially *S. enterica* serovar Enteritidis (*S.* Enteritidis) and *S. enterica* serovar Typhimurium (*S.* Typhimurium) are frequently associated with human infection (Hendriksen et al., 2011). *Salmonella* is found in intestinal tract of warm-blood animals, especially chicken, which consequently allows them to be easily transferred to meat during slaughtering and processing (Antunes, Mourao, Campos, & Peixe, 2016). Chicken meats have been considered as the main sources of *Salmonella* infections in humans (Authority & Control, European Centre for Disease Prevention and, 2016; Doi et al., 2003; Noda et al., 2015). Thus, reducing *Salmonella* contamination in chicken meats is necessary to prevent human salmonellosis.

Various methods either based on chemical, physical, or biological agents have been developed for the treatment of bacterial contamination in foods. However, these methods still represent some major disadvantages such as harmfulness or toxicity to the foods, altering the quality, or high cost for practical applications (Aymerich, Picouet, & Monfort, 2008; Chen et al., 2012; Grant & Parveen, 2017; Troy, Ojha, Kerry, & Tiwari, 2016). Lytic phages or bacteriophages are bacterial viruses that infect their hosts and were used increasingly as alternative tools to disinfect pathogenic bacteria in foods (Guenther, Huwyler, Richard, & Loessner, 2009; Pang, Lambertini, Buchanan, Schaffner, & Pradhan, 2017; M.; Sharma, Patel, Conway, Ferguson, & Sulakvelidze, 2009). Using phage-based methods for treatment of bacterial contamination has some obvious advantages over conventional ones. Firstly, phages only kill their host bacteria even antibiotics resistant ones thus they are harmless to others, especially beneficial microorganisms which also present in the foods and human intestinal tract (Loc-Carrillo & Abedon, 2011). Secondly, the addition of the lytic phages to the foods is unlikely to induce negative impacts to food properties (Perera, Abuladze, Li, Woolston, & Sulakvelidze, 2015). In addition, the phages are abundant living organisms in natural environment, self-replicating, and self-limiting, which show great chances for their successful isolation, economic and environmental benefits (Hagens & Loessner, 2010; Loc-Carrillo & Abedon, 2011). Some commercial phage products including ListShield™, Listex P-100™, EcoShield™, SalmoFresh™, and Salmonelex™ have been officially approved by the United States Food and Drug Administration (FDA) and the United

[*] Corresponding author.
E-mail addresses: hmduc@agr.kyushu-u.ac.jp (H.M. Duc), sonbiotechn@gmail.com (H.M. Son), tmiyamot@agr.kyushu-u.ac.jp (T. Miyamoto).
[1] These authors contributed equally to this work.

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States Department of Agriculture (USDA) for use in foods to control foodborne pathogens (Atterbury, 2009; Garcia, Martinez, Obeso, & Rodriguez, 2008; Hagens & Loessner, 2010).

Various phages infecting different foodborne pathogens have been isolated from environmental samples such as municipal waste water (Higgins et al., 2005; Hong, Pan, & Ebner, 2014), sewage (Carey-Smith, Billington, Cornelius, Hudson, & Heinemann, 2006; Hooton, Atterbury, & Connerton, 2011; Hudson et al., 2013; Pereira et al., 2016), human and animal feces (Bao et al., 2015; Bigot et al., 2011; Bigwood, Hudson, Billington, Carey-Smith, & Heinemann, 2008; Hungaro, Mendonça, Gouvêa, Vanetti, & Pinto, 2013; O'Flynn, Ross, Fitzgerald, & Coffey, 2004; Tomat, Migliore, Aquili, Quiberoni, & Balague, 2013; Viazis, Akhtar, Feirtag, Brabban, & Diez-Gonzalez, 2011a). In these reports, the isolated phages have also been demonstrated to be active against their hosts either in the broth or food environment. However, the safety of them when used in foods for human consumption is a matter of concern. For this reason, the isolation of phages directly from meat samples could be a good choice for ensuring the safety of phages because meats carrying phages are daily consumed by humans without causing serious health problems. Despite the usefulness of the isolation of phages from food samples, it has been rarely reported in literature (Atterbury, Connerton, Dodd, Rees, & Connerton, 2003; Firlieyanti, Connerton, & Connerton, 2016; Hoang Minh, Hoang Minh, Honjoh, & Miyamoto, 2016). The aim of this study was to isolate lytic phages against *Salmonella* from raw chicken meat products and examine their efficacy in reducing *S.* Enteritidis and *S.* Typhimurium *in vitro* and in chicken breast.

2. Materials and methods

2.1. Bacterial strains

Twenty-two *Salmonella* serovars used in this study were listed in Table 1. *Salmonella* Enteritidis NBRC3313 and *Salmonella* Typhimurium NBRC12529 obtained from National Institute of Technology and Evaluation (NITE), Biological Research Center (NBRC), Chiba, Japan were used as hosts for phage isolation. Other *Salmonella* serovars were isolated in our laboratory. To prepare bacterial inoculum, frozen stock was streaked on Tryptic Soy Agar (TSA; Becton, Dickinson and Company) and incubated at 37 °C for 24 h. A single colony was inoculated into 5 ml Luria Bertani broth (LB; Becton, Dickinson and Company) and incubated overnight at 37 °C. The bacterial cultures were serially diluted in Phosphate Buffered Saline (PBS) to obtain desired concentrations for each experiment.

Table 1
Lytic range of isolated phages.

Serovar designation	Serovar	Phages				
		SEG5	SES8	STG2	STG5	STS9
S78	*S.* Enteritidis NBRC 33113	+	+	+	+	+
S79	*S.* Typhimurium NBRC 12529	+	-	+	+	+
S96	*S.* Typhimurium	-	-	-	-	+
S97	*S.* Arahus	-	-	-	+	-
S98	*S.* Agona	+	-	+	-	-
S99	*S.* Anatum	-	-	-	-	-
S100	*S.* Braenderup	-	-	-	-	-
S101	*S.* Derby	+	-	+	-	-
S103	*S.* Enteritidis	+	+	+	+	+
S104	*S.* Hadar	-	-	+	-	-
S106	*S.* Typhi	±	-	+	-	±
S107	*S.* Heidelberg	+	-	+	-	±
S108	*S.* Istanbul	+	-	+	+	±
S109	*S.* Infantis	+	-	-	-	-
S110	*S.* Litchfield	+	-	+	+	-
S111	*S.* London	+	-	-	+	-
S112	*S.* Montevideo	+	-	-	-	-
S113	*S.* Muenster	+	-	-	+	-
S114	*S.* Schwarzengrund	+	-	+	+	+
S115	*S.* Stanley	±	-	+	+	+
S116	*S.* Sofia	-	-	-	-	+
S117	*S.* Thompson	+	-	-	-	-
Total infected strains (%)		16/22 (73)	2/22 (9)	12/22 (55)	10/22 (46)	10/22 (46)

(+), clear lysis; (±), turbid; (−), no lysis.

2.2. Phage isolation, purification and propagation

Forty-one raw chicken meat samples (12 gizzard, 11 liver, and 18 skin samples) purchased from local supermarkets in Fukuoka, Japan were used as the primary sources for phage isolation. Each meat sample (50 g) was inoculated with overnight culture (100 µl) of *S.* Enteritidis and *S.* Typhimurium, homogenized with LB broth (100 ml) supplemented with 10 mM $CaCl_2$ in a sterile stomacher bag, and incubated overnight at 37 °C. After incubation, the culture (10 ml) was centrifuged at 12,000 × *g* for 5 min at 4 °C, and the supernatant was filtrated through a 0.45 µm-pore size membrane filter (Merck Millipore, Ireland) to yield crude phage lysate.

To isolate lytic phage, the crude phage lysate (100 µl) was serially diluted in Saline Magnesium (SM) buffer (0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl, 0.008 M $MgSO_4$, 0.01% gelatin), mixed with 100 µl of each host strains in 4 ml molten agar (LB broth, agar powder 0.4% [w/v]). The mixture was then poured on TSA. The double layer agar plates were incubated overnight at 37 °C. Following incubation, a single plaque was picked up and re-suspended in SM buffer to generate phage suspension. The suspension was serially diluted with SM buffer, mixed with its respective host, poured on TSA plates and incubated overnight at 37 °C for producing a single plaque and these processes were repeated at least three times to ensure the purity of the phages.

In order to produce high titer stocks for further experiments, the isolated phages were propagated with their original hosts. Briefly, phage suspension (1 ml) was mixed with host culture (30 ml) at exponential phase and incubated overnight at 37 °C. After incubation, the mixture was centrifuged at 12,000 × *g* for 5 min at 4 °C and the supernatant was filtrated through 0.22 µm-pore size membrane filter (Merck Millipore, Ireland) to obtain phage stock. The phage stock was then serially diluted in SM buffer. The dilution (100 µl) was mixed with bacterial host (100 µl) in molten agar (4 ml). The mixture was poured on TSA plate and incubated at 37 °C overnight. The phage titer of the stock was determined as the numbers of plaques per milliliter (PFU/ml).

2.3. Characterization of isolated phages

2.3.1. Phage host range

The host range of each isolated phages was examined on 22 *Salmonella* serovars listed in Table 1. Aliquot of phage stock (10 µl) was dropped on the surface of double layer agar inoculated with 100 µl of each bacterial culture followed by the incubation at 37 °C for 24 h. The presence of a clear zone at the application points indicated strong lytic activity while the absence of this zone was regarded as no lytic ability.

2.3.2. Phage morphology

For morphological observation, the purified stocks of five phages (STG2, SEG5, STG5, SES8, and STS9) were dropped onto carbon-coated grid, negatively stained with 2% uranyl acetate, and visualized by using the transmission electron microscope (TEM) (Hitachi H-7650, Hitachi, Japan) operating at a voltage of 80 kV. The phage pictures were taken at a magnification of 150,000. The morphological characteristics were used to classify phages into families according to the phage typing instructions (Ackermann, 2009).