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Typing of ΦSP–3 lytic *Salmonella* bacteriophages obtained from various fecal sources

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Abstract: Although several reports are available on both ΦSP–1 and ΦSP–3 lytic *Salmonella* bacteriophages obtained from poultry, further research is required to study the effectiveness of ΦSP–3 type on serovars isolated from other sources. In the present study, we aimed to isolate bacteriophages from 8 serovars previously obtained from 869 fecal samples (calf, dairy cow, buffalo, and camel), genotype the bacteriophages, and detect the cross-lytic activities of the bacteriophages on *Salmonella enterica* subsp. *enterica* serovar Kentucky, *S.*Anatum, and *S.*Muenchen. A total of 16 bacteriophages were detected as ΦSP–3 type via PCR. The Hunter-Gaston Discriminatory Index of SDS-PAGE was calculated to be 0.825. Determination of multiplicity of infection (MOI) values were different for each bacteriophage according to the cross-lytic activity assessment. The MOI of the most effective *S.* Kentucky bacteriophage was 79.11 µg/mL for 2.5×10^4 cells, whereas that of the most ineffective *S.*Muenchen bacteriophage was 1.142 µg/µL for 2.5×10^4 cells. In conclusion, it was assumed that owing to the high and cross-lytic activity of the *S.* Kentucky bacteriophage, it has a larger host range, which differs in the lytic activities of each bacteriophage, despite being the same serovar, and that calf feces is the most important source for obtaining *Salmonella* bacteriophages.

Key words: Bacteriophage, concentration, feces, polymerase chain reaction, *Salmonella*

1. Introduction

Salmonella is an important pathogen which causes major foodborne bacterial outbreaks in the population [1]. The most significant symptom is infectious diarrhea caused by *Salmonella*, enterotoxigenic *Escherichia coli* (ETEC), coronavirus, rotavirus, and *Cryptosporidium parvum* [2]. *Salmonella enterica* subsp. *enterica* colonizes the digestive tract of cattle and calves, and this infection is particularly prevalent in the first 3 months of age [3]. *Salmonella* infection is also observed in buffaloes and camels [4]. The major source of infection is asymptomatic adult animals; up to 5% of recovered animals may transfer this organism with their feces. *S. enterica* subsp. *enterica* comprises more than 2600 serovars, which may cause systemic infections characterized by diarrhea, septicemia, and various clinical symptoms [5]. It is essential to know the serovar types that are commonly isolated from the target region, as these serovars can vary depending on the geographical region and different sources [6]. In previous studies, it has been reported that different serovars were isolated from humans and animals [7–12]. Phages have been isolated from various sources [13–18], and can be displayed in the infectious cycle as different types. Virulent phages replicate exclusively using lytic cycles [19]. Lytic bacteriophages are highly bactericidal, possessing several characteristics known as autodosing and specificity. Such specificity also limits the ability of nontargeted bacterial cells to select specific phage resistance mechanisms, in contrast to antibiotics [20,21]. Therefore, it is presumed that bacteriophages can be used alone or in combination with antibiotics to treat bacterial infections without negative effects on human or animal cells [22]. Tailed bacteriophages, named Caudovirales, have a common origin and constitute an order with 3 families and 3 types of lysogeny: phages lambda, Mu, and P1 [23]. *Salmonella* bacteriophages are defined in the National Center for Biotechnology Information (NCBI) Gen Bank [24,25].

In the present study, we aimed to obtain bacteriophages from calf, dairy cow, buffalo, and camel feces to genotype them based on their protein profiles, and to detect the cross-lytic activities of phages for *S.* Kentucky, *S.*Anatum, and *S.*Muenchen serovars.

2. Materials and methods

2.1. Bacterial strains used in the study

All feces included in this study were collected from clinically or subclinically infected animals. This research was approved by the Ethics Committee of the Faculty of Veterinary Medicine at the Selçuk University in Konya,

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Turkey. These samples were sent to the laboratory under cold-chain conditions. A total of 869 fecal samples (437 calves, 287 dairy cows, 100 buffalo, and 45 camels) were collected from 21 dairy farms located in 5 regions (Aegean, Black Sea, central Anatolia, Marmara, southeastern Anatolia) in Turkey between April 2012 and March 2014. In a previous study, a total of 40 (4.60%) *Salmonella* strains were obtained from (38.09%) of the 21 farms [9]. (Table 1).

2.2. Characterization, concentration, and isolation of bacteriophages

For the bacteriophage positive control, the ΦSP–3 lytic phage was isolated from the intestinal contents of broiler chickens, as described previously [26, 27]. The *Salmonella* phages were obtained through 2 methods: a direct procedure and phage enrichment with a host-specific *Salmonella* bacteria by a modified double-layered agarose method. For direct isolation, aliquots of samples were mixed at a 1:10 ratio with salt magnesium (SM) buffer (50 mM Tris-HCl [pH: 7.5], 0.1 M NaCl, 8 mM $MgSO_4$), then it was filtrated followed by a two-step process through a 0.45-mm bottle top filter and a 0. 2-mm syringe-attached filter. This filtrate of 4 mL of 0.7% nutrient agar (NA) was tempered to 55°C and the mixture was poured into Petri dishes with a bottom layer of 1.5% TSA, followed by incubation at 37°C. For phage enrichment, aliquots of the same samples used for direct isolation were mixed at a 1:10 ratio with NA broth followed by addition of 1 mL of the host strain. It was incubated at 37°C for 16 h, then treated with the modified double-layered agarose method [28] (Table 1). The culture supernatants were concentrated at 7500× g for 15 min using an ultrafiltration unit with a MWCO of 10 kDaVivaspin® 20 (Sartorius, Göttingen, Germany) [24]. The presence of bacteriophages was investigated using the freeze-drying method for scanning electron microscopy (SEM) [29]. Protein amounts of the concentrated lysates were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Determination of multiplicity of infection (MOI)

Determination of multiplicity of infection (MOI) was defined as the ratio of virus particles to the potential host cells. Briefly, the host strain in the early phase was cultured in NA broth at 30 °C. The concentrated phage stocks were serially diluted two-fold (2^1, 2^2, 2^3, 2^4, 2^5, 2^6, and 2^7), and 2 mg/mL standardized protein and sterile distilled water were prepared for the control. To determine the phage titer, triplicate samples were collected from each MOI set [30].

2.4. Characterization of bacteriophage lysis profiles of different hosts

The lysis activity of each of the 16 bacteriophage isolates was evaluated in both the specific host and other hosts. The cross-lytic activity of phages was determined as it was presumed that the structure of the isolates might change due to differences in the serotype, source, region, and year. Initially, the lysis activity of bacteriophages was standardized in their host, and then their activities were determined in other hosts. For this purpose, a modified double-layered agarose method was used, as described previously [31].

2.5. Bacteriophage typing

Phage DNA extraction was carried out using the Phage DNA Isolation Kit (Norgen Biotek, Thorold, ON, Canada), as previously described [31]. To identify the conserved stretches of the major capsid genes and ΦSP-1 with ΦSP-3 types of *Salmonella*phages, 5 primers were used in this study. The presence of major capsid genes was investigated by PCR as described previously [32]. In the present study, specific primers were used for identifying ΦSP1 and ΦSP3 phages via PCR., We designed specific primers using the NCBI primer program according to the data for ΦSP-1 (GenBank Accession number JQ638925.1) and ΦSP-3 (GenBank Accession number JQ638926.1) in NCBI. The specificity of the primers was confirmed using the Basic Local Alignment Search Tool (BLAST) program. All PCRs were prepared with 5 µL 5× FIREPol®Master Mix (Solis Biodyne, Tartu, Estonia), DNA template (100 ng/µL), 20 pmol of each primer (SP1F 5'CGCTGCAAACTATCAGGCAC'3, SP1R 5'CTTGACCGTACCAACCCAGG'3, SP3F 5'AACACTCAAACCTGCACCGT'3, SP3R 5'GGTGTTGAGATCCTGCGCT'3), and 1 µL water (negative control). The PCR was performed with an initial denaturation at 94°C for 5 min, followed by 30 cycles (94°C for 1 min, 60°C for 1 min, 72°C for 1 min), and a final extension at 72°C for 10 min. Amplification products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. A 1-kb DNA ladder (Thermo Scientific, SM0313) and 100 bp (Thermo Scientific, 15628019) were used for comparison of DNA sizes.

The protein profiles of the concentrated positive control and 16 bacteriophages' structural proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [33]. Protein Ladder (Thermo Scientific, 26634) was used to determine the molecular weights of the sample bands [34].

3. Results

3.1. Lytic activity of bacteriophages

For calculating the MOI, 2.5×10^4 bacterial cells were detected for each of the 16 bacteriophages and positive control, whereas the effective protein amount of bacteriophage changed according to lytic titers. The MOI of the most effective *S.* Kentucky bacteriophage was 79.11 µg/mL, whereas that of the most ineffective *S.* Muenchen bacteriophage was 1.142 µg/µL. It was determined that there might be differences in the lytic activities of each of

Table 1. Phages types obtained from Salmonella serovars and serotype isolated from different regions of Turkey.

Farm	Year	Source	Region	Sample	Farm	Number of phage isolates obtained on host strains representing serovar and serotypes (%)								Total phage isolates
						Kentucky (8,20:i:z6)	Typhimurium (1,4,[5],12:i:1,2)	Anatum (3,10,[15],[15,34]: e,h:1,6)	Muenchen (6,8:d:1,2)	Enteritidis (1, 9,12: g,m:-)	Abony (1,4,[5],12,27: b:e,n,x)	Gaminara (16:d:1,7)	Muenster (3,10[15][15,34]: e,h:1,5)	
1	2012	Calf	M	34	0	0	0	0	0	0	0	0	0	0
2	2012	Dairy cow	C	23	0	0	0	0	0	0	0	0	0	0
3	2012	Calf	C	115	19	19	0	0	0	0	0	0	0	13/19
4	2012	Dairy cow	C	35	1	0	0	1	0	0	0	0	0	1/1
5	2012	Dairy cow	C	63	0	0	0	0	0	0	0	0	0	0
6	2012	Dairy cow	C	12	0	0	0	0	0	0	0	0	0	0
7	2012	Dairy cow	C	16	0	0	0	0	0	0	0	0	0	0
8	2013	Calf	C	40	0	0	0	0	0	0	0	0	0	0
9	2013	Dairy cow	C	16	0	0	0	0	0	0	0	0	0	1/3
10	2013	Dairy cow	C	30	0	0	0	0	0	0	0	0	0	0
11	2013	Dairy cow	C	57	3	3	0	0	0	0	0	0	0	0
12	2013	Calf	S	24	5	0	0	0	5	0	0	0	0	1/5
13	2013	Dairy cow	C	22	0	0	0	0	0	0	0	0	0	0
14	2013	Calf	C	25	0	0	0	0	0	0	0	0	0	0
15	2013	Buffalo	C	100	1	1	0	0	0	0	0	0	0	0
16	2013	Calf	C	28	3	0	0	3	0	0	0	0	0	0
17	2013	Dairy cow	B	13	0	0	0	0	0	0	0	0	0	0
18	2013	Calf	C	102	7	0	1	0	0	1	0	4	1	0
19	2014	Calf	C	30	0	0	0	0	0	0	0	0	0	0
20	2014	Camel	A	45	1	0	0	0	0	0	1	0	0	0
21	2014	Calf	C	39	0	0	0	0	0	0	0	4	0	0
Total				869	40	23	1	4	5	1	1	4	1	16

M: Mediterranean; C: Central Anatolia Region; B: Blacksea; A: Aegean

the 14 *S.* Kentucky bacteriophages, despite them having the same serovar. These calves had diarrhea as a clinical finding, in contrast to the dairy cows. According to the cross-lytic activity of the phages, the most effective phages were 2*S.* Kentucky phages obtained from calf feces; their activity was 100% effective against all phage hosts. *S.* Muenchen phage activity (18.75%) was lower than that of other phages' activity (Tables 1 and 2).

3.2. Bacteriophage typing

The presence and morphology of the positive control were investigated via SEM (Figure 1). The 16 phage and positive control DNA extractions were determined to be ΦSP3 because products of 163 bp were obtained via PCR. According to the SDS-PAGE results, all 17 *Salmonella* bacteriophages exhibited between ~10 kDa and 70kDa. The 2 main groups were determined by cluster analysis. Group I comprised the positive control and 11 isolates (1, 4, 6, and 8–15), with a similarity of group of at least 50%. High percentages of phages were distinguished based on the origin of the serovar that were found in the same cluster (subgroup A of cluster I), 75%, (n = 6) for *S.* Kentucky phages obtained from calf feces, and 12.5% (n = 1) for *S.* Muenchenphages obtained from calf feces (Figure 2). The phages 2, 3, 7, 16 were placed in group A of cluster II, which had a similarity of group of at least 60%, but only *S.*Muenchen phage 5 was placed in group B of cluster II. Jaccard's coefficient of similarity was determined to be 0.825 by unweighted pair group method with arithmetic mean, and Hunter-Gaston discriminatory index was calculated to be 0.825.

4. Discussion

In general, studies on the distribution of *Salmonella* infection have been reported in calves, dairy cows, and poultry samples [8]. There are many factors such as acquisition of immunity to the predominant serovars, genetic adaptation to hosts of specific serovars, and management interventions [35,36]. Numerous studies are available about the use of bacteriophages against infections caused by different bacteria, such as therapeutic applications, activity, and protection [37]; however, broad-host-range bacteriophages are the major limitation for phage therapy because of their cross-interaction and further lysis of the microbiota [38]. There is a need for the determination of bacteriophage lysis profiles for determining the bacteriophage–host relationship because it provides useful information for phage-based control of *Salmonella* serovars predominant in different sources. It was reported that bacteriophages present the highest lysis ability against *S.* Enteritidis and *S.* Typhimurium obtained from various farm animals [39]. Dueñas et al. [40] obtained 45 phages; the majority were isolated with the *S.* Enteritidis host (64.4%), followed by *S.* Heidelberg (20%), *S.*Typhimurium (8.9%), and *S.* Infantis (6.7%).

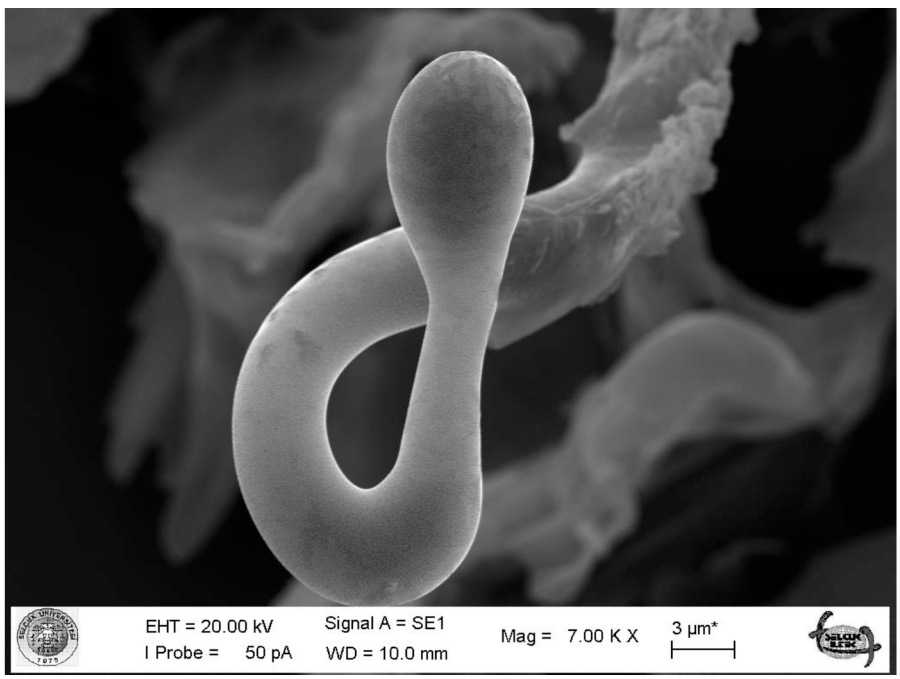

Figure 1. The investigating of positive control by scanning electron microscope (SEM). EHT: extra high tension, Mag, Magnification; WD, Waddel Diameter; SE1: Secondary Electron; Signal A, Emitted Electrons

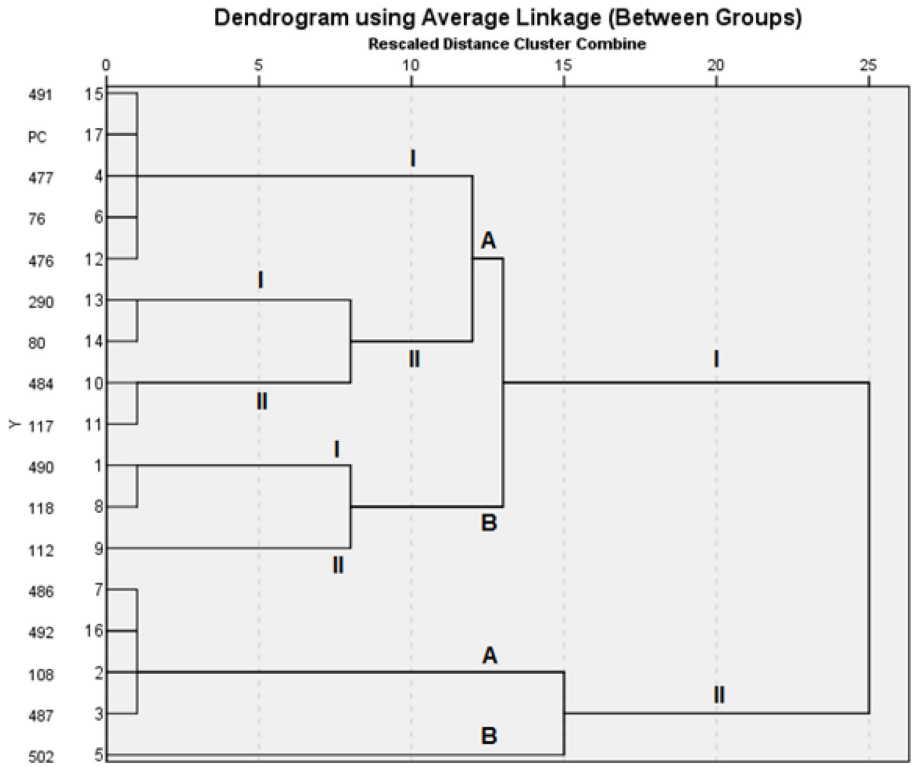

Figure 2. Similarity analysis of the profiles produced by SDS-PAGE cluster analysis. It was shown in the X (rescaled distance cluster combine) and Y (the numbers representing bacteriophages) axis. *S.* Anatum, 290; *S.* Muenchen, 502; *S.* Kentucky, 118-76-477-486-492-108-117-490-112-484-80-476-491-487; 1–16, Bacteriophage isolates: 17, PC, Positive Control.

In this study, the diversity and presence of *Salmonella* bacteriophages were investigated in fecal samples collected from calves, dairy cows, buffalos, and camels in farms with unknown history of *Salmonella* isolation. Bacteriophages were obtained from calves and dairy cows in contrast to those from buffalo and camel farms because it was presumed that *Salmonella* infection has rarely occurred in buffalo or camel farms in Turkey [9].

Salmonella bacteriophages are generally host-specific or possess only 1 serovar; however, there are several reports on phages that productively infect a range of bacterial species, crossing the genus barrier [4]. It was determined that the lytic activity rate of ΦSP3 bacteriophages was higher in their host, and the lytic activities and cross-activity of *S.* Kentucky ΦSP3 bacteriophages were higher than those of the other serovars in contrast to the *S.* Muenchen bacteriophage. In addition, these *S.* Kentucky bacteriophages, which have the highest lytic activity (2, 3, 7, and 16 with sample numbers), were detected as group A of cluster II, although only the S. Muenchen bacteriophage phage was found in group B of cluster II (Figure 2). In particular, it was determined that *S.* Kentucky was the dominant serovar in our country (Table 1), and it was presumed that *S.* Kentucky might prevent the growth of *S.* Enteritidis, *S.* Gaminara, *S.* Typhimurium, and *S.* Muester through its bacteriophage (Table 2). There can be differences in the bacteriophage host range depending on the prevention of adsorption by modification of the resistant bacterial system, bacterial receptor mutations [41]. It was assumed that there might also be differences in phage type and effectiveness due to shifts in the predominant serovars. In general, bacteriophages have been characterized using transmission electron microscopy (TEM) over the years according to ΦSP1 and ΦSP3 types; however, any structural details are not shown by TEM, although this technique is commonly used for enumerating viruses from the environment [24,42]. Therefore, it has been assumed that molecular techniques have been useful in the typing of bacteriophages.

In conclusion, the *S.* Kentucky bacteriophage has a larger host range owing to its high lytic and cross-lytic activity. It was determined that there might be differences in the lytic activities of each bacteriophage, despite having the same serovar, and that calf feces are the most important sources for obtaining *Salmonella* bacteriophages. There is insufficient data about the typing and efficiency of lytic bacteriophages in Turkey. These results may be used in the control of *Salmonella* infection in farm animals.

Table 2. The determining of the lysis activity of each one of sixteen bacteriophage isolates by both in their host and in other hosts.

Culture → Phage titers ↓	118	290	76	502	477	486	492	108	117	490	112	484	80	476	491	487	MOI**	Lytic activity rate (%)
118*	1/128 (2^7)	1/16 (2^4)	1/64 (2^6)	1/32 (2^5)	-	1/32 (2^5)	1/64 (2^6)	1/64 (2^6)	-	1/32 (2^5)	1/32 (2^5)	-	-	1/64 (2^6)	1/64 (2^6)	-	142.65 µg/µL 1/14.02 ($2^{3.81}$)	11/16 (68.75%)
290*	1/32 (2^5)	1/128 (2^7)	1/32 (2^5)	1/16 (2^4)	1/4 (2^2)	1/32 (2^5)	1/32 (2^5)	1/16 (2^4)	-	1/8 (2^3)	-	-	-	1/32 (2^5)	1/8 (2^3)	-	250 µg/µL 1/8 (2^3)	11/16 (68.75%)
76*	-	-	1/64 (2^6)	-	-	-	-	1/32 (2^5)	-	-	1/64 (2^6)	1/16 (2^4)	-	-	-	-	809.7 µg/µL 1/ 2.47 ($2^{1.31}$)	4/16 (25%)
502*	-	-	-	1/32 (2^5)	-	-	1/8 (2^3)	-	-	1/32 (2^5)	-	-	-	-	-	-	1.142 µg/µL 1/ 1.75 ($2^{0.81}$)	3/16 (18.75%)
477*	1/16 (2^4)	-	-	-	1/32 (2^5)	-	-	-	1/8 (2^3)	-	1/32 (2^5)	1/8 (2^3)	1/8 (2^3)	-	-	-	743.49 µg/µL 1/ 2.69 ($2^{1.43}$)	6/16 (37.5%)
486*	1/32 (2^5)	1/16 (2^4)	1/32 (2^5)	1/32 (2^5)	1/16 (2^4)	1/64 (2^6)	1/64 (2^6)	1/16 (2^4)	1/8 (2^3)	1/32 (2^5)	1/16 (2^4)	1/64 (2^6)	-	1/16 (2^4)	1/32 (2^5)	1/16 (2^4)	79.11 µg/µL 1/ 25.28 ($2^{4.66}$)	15/16 (93.75%)
492*	-	-	-	-	1/16 (2^4)	-	1/32 (2^5)	-	1/64 (2^6)	-	-	-	1/16 (2^4)	-	-	-	884.95 µg/µL 1/ 2.26 ($2^{1.18}$)	4/16 (25%)
108*	1/32 (2^5)	1/16 (2^4)	1/32 (2^5)	1/8 (2^3)	1/32 (2^5)	1/8 (2^3)	1/32 (2^5)	1/64 (2^6)	1/16 (2^4)	1/32 (2^5)	1/16 (2^4)	1/64 (2^6)	1/16 (2^4)	1/16 (2^4)	1/32 (2^5)	1/16 (2^4)	105.15 µg/µL 1/19.02 ($2^{4.25}$)	16/16 (100%)
117*	1/32 (2^5)	¼ (2^2)	1/64 (2^6)	1/16 (2^4)	-	1/8 (2^3)	-	-	1/32 (2^5)	-	-	1/16 (2^4)	1/32 (2^5)	-	1/64 (2^6)	1/16 (2^4)	297.61 µg/µL 1/ 6.72 ($2^{2.75}$)	10/16 (62.5%)
490*	1/8 (2^3)	-	-	-	-	-	1/64 (2^6)	1/16 (2^4)	-	1/32 (2^5)	-	-	-	-	1/16 (2^4)	-	775.19 µg/µL 1/ 2.58 ($2^{1.37}$)	5/16 (31.25%)
112*	-	-	-	-	1/32 (2^5)	-	-	-	1/16 (2^4)	-	1/64 (2^6)	-	-	-	-	1/32 (2^5)	843.88 µg/µL 1/2.37 ($2^{1.25}$)	4/16 (25%)
484*	-	1/8 (2^3)	-	-	-	-	1/16 (2^4)	-	1/16 (2^4)	-	-	1/32 (2^5)	-	-	-	-	1000 µg/µL 1/ 2 (2^1)	4/16 (25%)
80*	-	-	-	-	1/32 (2^5)	-	1/8 (2^3)	-	-	-	-	1/8 (2^3)	1/32 (2^5)	-	-	-	1000 µg/µL 1/ 2 (2^1)	4/16 (25%)
476*	1/16 (2^4)	1/4 (2^2)	1/16 (2^4)	1/16 (2^4)	-	1/8 (2^3)	1/16 (2^4)	1/32 (2^5)	-	1/32 (2^5)	-	-	-	1/32 (2^5)	1/16 (2^4)	1/32 (2^5)	285.3 µg/µL 1/ 7.01 ($2^{2.81}$)	11/16 (68.75%)
491*	1/16 (2^4)	-	1/64 (2^6)	-	1/16 (2^4)	1/64 (2^6)	1/64 (2^6)	1/16 (2^4)	-	1/32 (2^5)	1/16 (2^4)	1/16 (2^4)	1/32 (2^5)	1/16 (2^4)	1/32 (2^5)	1/8 (2^3)	148.69 µg/µL 1/13.45 ($2^{3.75}$)	13/16 (81.25%)
487*	1/32 (2^5)	1/16 (2^4)	1/32 (2^5)	1/8 (2^3)	1/8 (2^3)	1/16 (2^4)	1/16 (2^4)	1/16 (2^4)	1/64 (2^6)	1/16 (2^4)	1/8 (2^3)	1/16 (2^4)	1/32 (2^5)	1/16 (2^4)	1/32 (2^5)	1/32 (2^5)	105.15 µg/µL 1/19.02 ($2^{4.25}$)	16/16 (100%)
Positive control	1/32 (2^5)	-	1/16 (2^4)	-	-	1/32 (2^5)	-	1/32 (2^5)	1/64 (2^6)	-	1/8 (2^3)	1/32 (2^5)	1/16 (2^4)	-	1/64 (2^6)	1/32 (2^5)	250 µg/µL 1/8 (2^3)	16/16 (62.5%)
Negative control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0/16 (0%)

*S. Anatum; 290, S. Muenchen, 502; S. Kentucky, 118-76-477-486-492-108-117-490-112-484-80-476-491-487

** For 16 bacteriophages, the 2 mg/mL standardised protein were used for calculating of multiplicity of infection (MOI).

MOI = 2000 µg/µL
the average lytic value

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