



Control of Antibiotic-Resistant *Salmonella enterica* Serovar Typhi in Water and Milk Using Phage Cocktail

Noha K. El-DougDoug⁽¹⁾, Mervet G. Hassan⁽¹⁾, E. Elashkar⁽²⁾, A.I. Ahmed⁽²⁾, M.M. Hazaa^{(1)#}

⁽¹⁾Botany and Microbiology Department, Faculty of Science, Benha University, Benha, Egypt; ⁽²⁾Benha Fever Hospital, Benha, Egypt.



CrossMark

THE RISE of clinical concern about antibiotic-resistant *Salmonella enterica* serovar Typhi is mainly attributed to the frequent contamination of food and drinking water. The aim of this study is to assess the sensitivity of *S. Typhi* to antibiotics as well as isolation of lytic bacteriophages specific to the bacterium and investigate their ability to enhance the safety of tap water and milk. *S. Typhi* clinical isolates recovered from infected blood specimens were included in this study. The bacterial isolates were identified by conventional biochemical tests; Vitek analyzer in addition to Widal test to determine the serotypes of *Salmonella* isolates. Four lytic bacteriophages specific to *S. Typhi* were isolated and characterized using TEM and designated as vb_StyM,EN-1, vb_StyM,EN-2, vb_StyS,EN-3 and vb_StyP,EN-4. The results showed resistance of *S. Typhi* isolates to various antibiotics. Phage cocktail was prepared and applied for controlling *S. Typhi* growth in milk and drinking water at 4°C and room temperature. After treatment, complete eradication of bacterial growth was observed in broth medium at 4°C and room temperature after 24hrs. In addition to that, phage cocktail showed complete inhibition of *S. Typhi* growth in treated tap water at 25°C after 24hrs and in milk at 4°C. A significant reduction; nearly five log reductions was observed in skim milk while one log reduction was obtained in full fat milk at 25°C. These results indicate that *S. Typhi* is resistant to some antibiotics and bacteriophages could be promising antimicrobial alternatives.

Keywords: *Salmonella* Typhi, Phage cocktail, Antibiotic resistance, Bio-control, Full fat and skim milk.

Introduction

Foodborne illnesses are serious threat to public health and economy (Anany et al., 2018; El-DougDoug et al., 2019). Globally, *Salmonella enterica* is one of the leading causes of foodborne diseases and mortality all over the world (Scallan et al., 2015; Marus et al., 2019). *S. enterica* serotype Typhi is a typhoidal *Salmonella* that has been attributed to food and water outbreaks (CDC, 2008). Typhoid fever is associated with the consumption of food and water contaminated with *S. Typhi* (Jam, 2003; CDC, 2010). Typhoid fever is responsible for a major public health problem in many developing countries as an estimation of around 15 million people get sick from typhoid

and up to 120 000 people die every year due to the disease (Cooke & Wain, 2004; Steele et al., 2016; Rasul et al., 2017; WHO, 2018).

Drug resistance has emerged as a serious issue facing the control and management of infection; furthermore the development of multi-drug resistance has great implications for therapy (Clarke, 2003). Recent trends, tend to develop potential ways to eradicate microbial contamination, improve food safety and public health, but still challenges to find an alternative to antibiotic for therapy exist (El-DougDoug et al., 2019).

Corresponding author email: drmhazaa@gmail.com, Noha K. El-DougDoug email: nohaeldougDoug@gmail.com

Received 19/2/2019; Accepted 8/9/2019

DOI: 10.21608/ejbo.2019.7596.1284

Edited by: Dr.

©2020 National Information and Documentation Center (NIDOC)

Bacteriophage is considered as a natural and promising tool for safety and preservation of food (Anany et al., 2014; Moye et al., 2018). Bacteriophages are viruses that have ability to infect and replicate within bacterial pathogen in a strain-specific manner (Beke et al., 2016). The ubiquitous and specificity of bacteriophages makes them ideal for the bio-control of pathogenic bacteria in food and environmental samples (Zhan et al., 2015; Anany et al., 2018; El-DougDoug et al., 2019).

S. Typhi caused outbreaks that may be related to contaminate tap water with sewage, or milk and other foods that is a threat of the human health (Kaur et al., 2018; Liu et al., 2018). Bacteriophages have a bio-control application advantages over a chemical and conventional preservation of food (Huang et al., 2018).

Therefore, the present investigation was conducted to isolate *S. Typhi* from clinical cases and use lytic bacteriophages to control antibiotic-resistant *S. Typhi* in water and milk.

Materials and Methods

Sampling

Laboratory work was conducted at Benha Fever Hospital, Benha city and Microbiological lab, Faculty of science, Benha university, Egypt. A total of 233 sera specimens were screened for typhoid fever infection. Clinical isolates of *S. enterica* were detected from aseptically collected blood specimens with suspected enteric fever. The specimens investigated in this study were available from microbiology analysis lab in the Benha fever hospital.

Isolation of Salmonella spp.

Isolation and identification of *Salmonella* isolates was performed by standard microbiological techniques. Clinical specimens were enriched in blood culture bottles (5ml). All bottles were incubated at 37°C then a loopful of the blood culture was sub-cultured each two days on blood agar, macconkey agar and selective medium; *Salmonella Shigella* agar (SS agar). The inoculated plates were incubated at 37°C for 18-24hrs. Once a positive culture bottle was detected, Gram staining was prepared from colonies grown on SS agar plate. Negative bottles were incubated for further 2 days and then incubated again up to 7 days before they were reported as negative samples.

Identification of Salmonella spp.

The positive colonies were sub-cultured three successive times for purification on SS agar medium. *Salmonella* isolates were identified by colonial morphology, gram's staining and other conventional biochemical tests (Bergey's manual 2009). In addition, clinical isolates were identified using the VITEK® 2 GN system (Version 08.01) (Andrews et al., 2014) and the serotypes of *S. enterica* isolates was identified serologically using Widal test (Mukesh et al., 2018). Widal test was performed as described by (Widal, 1896) that based on revealing the presence of antibody (agglutinin) in the infected serum, against the flagellar (H) and somatic (O) antigens of *S. Typhi*.

Antibiotic sensitivity test

The antimicrobial susceptibility of *Salmonella* isolates were tested by modified Kirby-Bauer disk diffusion method on Muller Hinton Agar as per CLSI recommendations. (CLSI, 2019). The antibiotics tested in this study include Ampicillin (10µg), Amoxicillin (25µg), Tetracycline (30µg), Chloramphenicol (30µg), Ceftriaxone (30µg), Cefadroxil (30µg), Co-trimoxazole (25µg), Ciprofloxacin (5µg), Levofloxacin (5µg) and Nalidixic acid (30µg). Interpretation the results of antibiotic susceptibility tests was made according to standard interpretative zone diameters suggested in CLSI guidelines (CLSI, 2019). The bacterial response to antibiotic was interpreted as: R: Resistant, I: Intermediate and S: Sensitive.

Isolation of bacteriophages.

Different samples of sewage water collected from Virology Lab, Department of Microbiology, Faculty of Agriculture, Ain Shams University were examined for isolation of bacteriophages. Each sample (about 10ml) was enriched in an equal volume of blood culture broth medium and 100µl of an overnight culture of *S. Typhi* isolates were added. The mixture was incubated for 24-48hrs. with shaking at 37°C. After that, the mixture was centrifuged at 4,000×g for 20min at 4°C. The supernatant was filtered through a 0.45µm sterile filter. The spot test technique was performed to detect the presence of lytic phages (Sambrook et al., 1989).

Purification of phages

The soft agar overlay method (plaque assay) was used for purification the isolated phages (Sambrook et al., 1989). The plaque assay was performed by addition 100µl of overnight

culture of *S. Typhi* to 100µl of phage lysate. The mixture was incubated for 10-15min at 37°C for attachment. Then, 4ml of semisolid media were added, mixed and poured on agar plate. The plates were incubated at 37°C for 24hrs. The individual plaques were selected based on their morphologies and sizes. They were picked from the plates and placed separately in 300µl CM phage buffer. The plaque assay for individual plaques were repeated three times for highly purification, Then the phage lysate was stored at 4°C before propagation.

Propagation of bacteriophage.

The propagation of isolated phages was processed using the overlay (soft agar) method (Sambrook et al., 1989). Then, 4ml of CM buffer was added onto each plates and phage lysate was scraped off from the upper layer of soft agar. The suspensions containing phage lysate were put on ice for 15min. The phage lysate was centrifuged at 4,000×g for 20min at 4°C and filtered using a 0.45µm membrane filter. The propagated phage lysate was stored at 4°C.

Titration of bacteriophages

The phage lysates were diluted in CM buffer. The titers of the propagated phages were determined by spotting 10µl from decimal dilutions of phage suspension onto soft overlay prepared as described previously. The plates were incubated upright for 16-20hrs at 37°C. The titers of the phages were determined using the following equation:

$$\text{Phage titer (PFU/ml)} = \frac{\text{Number of plaques}}{\text{Volume spotted} \times \text{Dilution factor}}$$

Characterization of S. Typhi phages

Morphology of the isolated phages using TEM.

The isolated *S. Typhi* phages were examined using transmission electron microscopy (TEM). The phage sample was prepared as described in (Ackermann, 2012) and examined by TEM (JEOL-JSM-5500LV, the Regional Center of Mycology and Biotechnology, Cairo, Egypt) using high vacuum mode at an acceleration voltage of 80 kV .

Stability of phages.

pH stability: the stability of isolated *S. Typhi* phages was tested at different pH values (5.0, 7.0 and 9.0) for 1, 3, 5 and 24hrs by adding 100µl of the phage lysate to 900µl of CM buffer and

then incubated at 37°C. Plaque assay technique was performed in triplicate and phage counts determined. The logarithmic reduction in phage titer was determined.

Temperature stability: A wide range temperature degrees (-20°C, 4°C, room temperature, 37°C) were chosen to test phage stability for 1 day, 1 week, or 2 weeks. Temperature treatments were repeated and the average of phage counts was determined.

About salinity, Phage was tested against several salinity conditions (5%, 10%, and 20%) for 1hrs and one day. The lytic activity of phages was tested using spot test.

Determination of the Bacteriophage Insensitive Mutant Frequency.

The frequency of bacteriophage insensitive mutant (BIM) was tested through overlay method as mentioned previously (O'Flynn et al., 2006) at a multiplicity of infection MOI of 10. The BIM of phage cocktail, containing same volume and titers of four phages, was tested. The emerged colonies were counted. The BIM frequency was determined as:

Number of surviving colonies divided/ The original bacterial titer.

Bio-control application using the cocktail of Salmonella Phages

In vitro: The bio-control efficacy of a phage cocktail composed of 4 lytic *S. Typhi* phages was assessed in broth medium. MOI of 5 was used by mixing 10³ CFU/ml of overnight *S. Typhi* with phage cocktail suspension. Samples were incubated for 1, 3, 5, 7 and 24hrs at 37°C. After incubation, the surviving bacterial cells were counted in sterile saline to determine the bacterial log₁₀ reduction.

In vivo: Treatment of tap water, full fat and skim milk.

Tap water, full and skim milk were initially screened for contamination with *Salmonella* spp. on specific SS agar medium. An equal volume of the 4 lytic *S. Typhi* phages were applied to tap water artificially contaminated by 10³ CFU/ml *S. Typhi* culture suspended in saline. The artificially contaminated tap water was treated with phage cocktail with MOI 5. Positive controls (untreated) were made with 10³ CFU/ml bacterial culture and CM buffer. Treated, untreated and negative

controls of tap water were stored for 0, 1, 3, 5 and 24hrs at room temperature (25°C). On the same approach, full fat and skim milk were artificially treated with *S. Typhi* and phages. Treated, untreated and negative control of full fat milk samples were stored 0, 1, 3, 5, and 24hrs at 25°C and 4°C. For skim milk sample, treated, untreated, negative control were stored at 25°C and 4°C for 1hrs, 1 day, 2 days and 3 days. The developed Colonies were counted and calculated in (CFU/ml). The viability of phages was determined using plaque assay.

Statistical analysis

All experiments were performed in triplicates; the averages and standard errors were then calculated. The data represent the mean± standard deviation (SD). A *t*-test was performed and considered significant at $P < 0.05$, for three independent trials.

Results

Serotyping of *Salmonella* spp.

The Widal test in diagnosing typhoid fever was assessed in Specimens with suspected enteric fever in Benha, Egypt. The test was done on sera specimens from 233 samples, 133 specimens with non-typhoidal fever and 100 bacteriologically proven specimens of typhoid fever. This study suggests that a positive Widel test is of considerable importance in diagnosing typhoid fever and identified the clinical isolates of *Salmonella* as *Salmonella* Typhi.

Identification of *Salmonella* isolates

Twenty out of 100 of *S. Typhi* isolates which gave positive blood culture and found sensitive to isolated phages were selected and identified. The colonies were detected as white, smooth and non-hemolytic on blood agar media; and as smooth colonies non-lactose fermenting, on Macconkey agar media; while colonies with circular shape, moderate to large in size with black centers were detected on SS agar media.

A variety of biochemical tests conventionally used to identify *S. isolates* was performed on *S. Typhi* isolates and showed that all the bacterial isolates were identified as gram negative bacilli and catalase positive. Whereas they gave negative result with indole, citrate and urease test.

Identification of the *S. Typhi* isolates using VITEK® 2 GN analyzer, modified version 2018,

was carried out in Benha university hospital and reported that the tested isolates were identified as *Salmonella* spp. with 94% similarity.

Antibiotic sensitivity test

Using antibiotic susceptibility testing by disk diffusion, the twenty *S. Typhi* isolates, sensitive to phages, were tested against different antibiotic groups. They showed resistance to conventional antibiotics such as Ampicillin (100%), Amoxicillin (100%), Tetracycline (80%), Chloramphenicol (75%), Ceftriaxone (55%), Cefadroxil (65%) and Co-trimoxazole (75%). On the other hand, most of *S. Typhi* isolates showed reduced resistance to Fluoroquinolone including Ciprofloxacin (40%), Levofloxacin (50%) and Nalidixic acid (45%). The sensitivity of the four *S. Typhi* isolates selected for phage isolation was reported in Table 1.

Characterization of *S. Typhi* phages.

Four phages specific to *S. Typhi* were isolated from sewage water and selected based on their lytic activity by spot test. They were showed different plaque morphologies and named as vb_StyM EN 1, vb_StyM EN 2, vb_StyS EN 3 and vb_StyP EN 4. The titration of phages was determined using overlay method and counted approximately 10^9 PFU/ml. The size, morphology and turbidity of plaques were determined as showed in Table 2.

Morphology of the phage isolates.

The selected *S. Typhi* phages were examined using TEM and represent four different morphotypes (Fig. 1). *Salmonella* phage EN-1 and EN-2 have contractile tails and related to the family *Myoviridae* (Fig. 1 A, B). EN-3 is member of the *Siphoviridae* family as determined by the presence of a flexible, long tail and the absence of a contractile sheath (Fig. 1 C), whereas *Salmonella* phage EN-4 is characterized by icosahedral head without tail that related to *Podoviridae* (Fig. 1 D). The diameter of head and tail lengths of phages were calculated (Table 3).

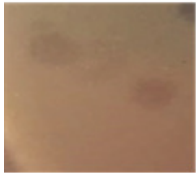



Phage stability

Wide range of stability criteria of *S. Typhi* phages were characterized due to their possible application in bio-control of food borne pathogens in food matrix usually exposed to different processing conditions such as pH, temperature and salinity.

TABLE 1. The antibiotic susceptibility test on pure cultures of four *S. Typhi* isolates.

t group	Antimicrobial agents	Dose	<i>S. Typhi</i> isolates			
			<i>S. Typhi</i> 1	<i>S. Typhi</i> 2	<i>S. Typhi</i> 3	<i>S. Typhi</i> 4
β-lactam	Ampicillin-AM	10μg	R	R	R	R
	Amoxicillin-AX	25μg	R	R	R	R
Tetracyclines	Tertracycline-TE	30μg	R	R	I	R
Chloramphenicol	Chloramphenicol-CMP	15μg	S	R	S	R
Cephalosporin	Ceftriaxone-CRO	30μg	R	S	I	R
	Cefadroxil-CFR	30μg	S	R	R	R
Co-trimoxazole	Trimthoprim-TMP/ Sulfamethoxazole-SMZ	1.25/ 23.75	R	R	I	I
Fluoroquinolone	Ciprofloxacin- CIP	5μg	S	I	I	R
	Levofloxacin- LEV	5μg	R	S	R	S
	Nalidixic acid-NA	30μg	R	S	S	I

TABLE 2. Plaques morphology of *S. Typhi* phages.

Phage isolates	Plaque morphology	
vb_StyM EN-1	Turbid, regular, mediate, circular plaques with 1.5 to 2mm	
vb_StyM EN-2	Turbid, irregular, Large, circular plaques with 2.5 to 3.5mm	
vb_StyS EN-3	Clear, regular, mediate, circular plaques with 2 to 3mm	
vb_StyP EN-4	Clear, regular, small, circular plaques with 1 to 2mm	

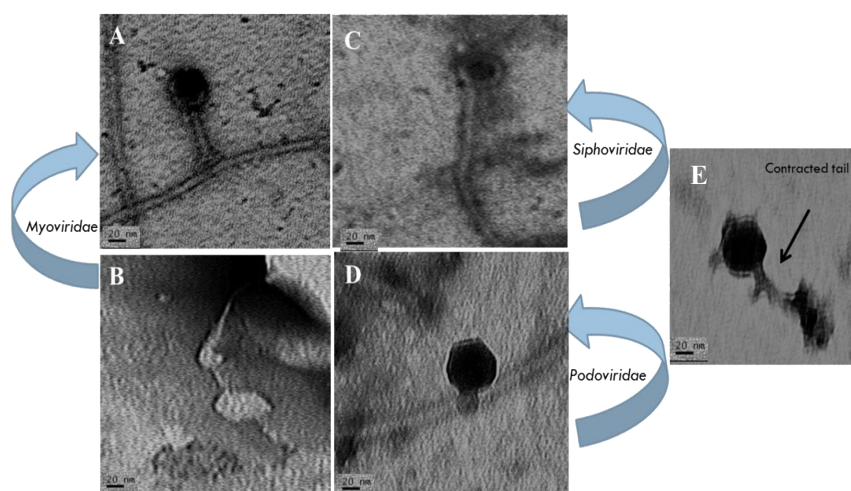


Fig. 1. Electro-micrograph shows the four different phages morphology specific to *S. Typhi* using TEM showed that: (A) EN-1 phage and (B) EN-2 phage are related to Myoviridae, (C) EN-3 phage is related to Siphoviridae, and (D) EN-4 phage is related to Podoviridae, (E) Contracted tail of Myoviridae phage.

TABLE 3. Head diameters and tail lengths of phages.

Size Phage	Head diameter (nm)	Tail length (nm)	Family
vb_StyM EN-1	76.03±1	103.21±3	Myoviridae
vb_StyM EN-2	79.47±1	98.44±2	Myoviridae
vb_StyS EN-3	69.98±0.5	211.54±2	Siphoviridae
vb_StyP EN-4	88.02±2	—	Podovirida

The stability of phage cocktail was tested in buffers of various pH values (5.0, 7.0 and 9.0) and temperatures (-20°C, 4°C, 25°C and 37°C). Then the count and log reductions (PFU/ml) of phage cocktail were determined through overlay technique. The result showed that the storage of phage cocktail under tested pH values for up to 24hrs did not affect- ($P > 0.05$) their stability (Fig. 2 A). In addition, there was no reduction of phage titre under different temperatures ($P > 0.05$) up to 2 weeks (Fig. 2 B.).

The *Salmonella* phages cocktail were subjected to different salinity conditions (5%, 10% and 13%). Phages did not encounter any significant reduction in their titer ($P > 0.05$) when subjected to different salinity conditions (5%, 10% and 13%) after 1hrs and 24hrs.

Bacteriophage insensitive mutants (BIM)

BIM against the isolated phages was tested using phage cocktail. Resistant mutants of *S. Typhi* were developed against phage cocktail. Using phage cocktail resulted in a frequency of

BIM about 1.62×10^{-5} . Phage cocktail caused significantly low BIM frequency when compared to the effect of the other *Salmonella* phages against *S. Typhi*.

S. Typhi challenge using Salmonella phage cocktail.

In broth media

The efficacy of phages cocktail to control *S. Typhi* was tested in blood culture broth medium. Phage cocktail was used to achieve significant reduction in *S. Typhi* growth and to reduce the chance of resistance development. A significantly high number of phages are required to ensure sufficiently rapid contact and infection of the few targeted bacterial cells present. In blood culture broth, the addition of phage cocktail with MOI 5 resulting in 2 log reduction of bacterial growth after 1hrs ($P < 0.05$). While significant reduction and complete inhibition (below detection limit 1 log CFU/ml) of bacterial growth was achieved after 3, 5, 7 and 24hrs at 4°C and 25°C compared to control counts (Fig. 3 A, B).

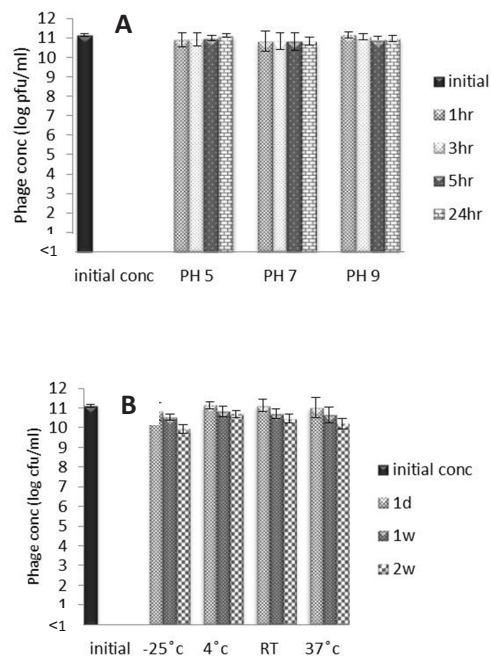


Fig. 2. Stability of *Salmonella* phage cocktail under different pH values (A) and temperatures (B).

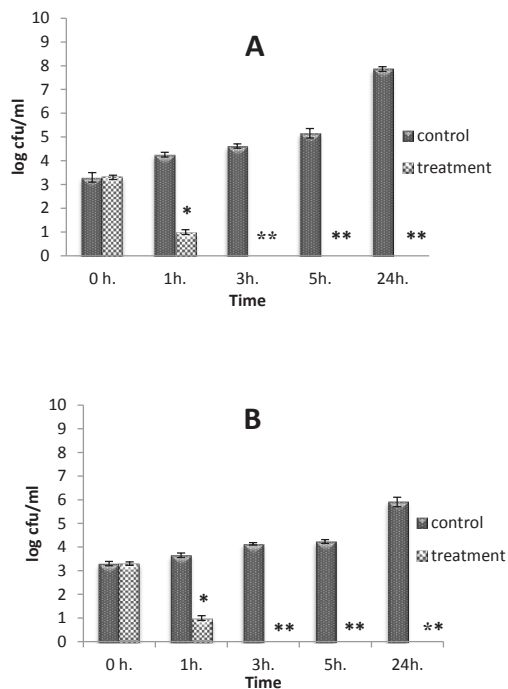


Fig. 3. Bio-control of *S. Typhi* in broth medium using MOI 5. (A) After addition of phage cocktail with MOI 5 at 25°C and (B) After addition of phage cocktail with MOI 5 at 4°C [Statistically significant treatments compared to the control were indicated by “**” where $P < 0.05$. The population count below detection limit (less than 1 log CFU/ml) is indicated by “***”].

In Tap water

The efficacy of the phage cocktails to control *S. Typhi* in a drinking water was examined. The tap water was artificially contaminated with *S. Typhi*, and then the corresponding phage cocktails were added and incubated at room temperature. The count of the surviving *S. Typhi* was determined at regular intervals and compared to the control. Figure 4 showed the effect of phage cocktail with MOI 5 to reduce *S. Typhi* count in tap water incubated at room temperature. Treatment using with MOI 5 resulted in inhibition of *S. Typhi* after 1hrs ($P < 0.05$) and complete reduction after 3 h compared to the control (below 1 log CFU/ml) (Fig. 4).

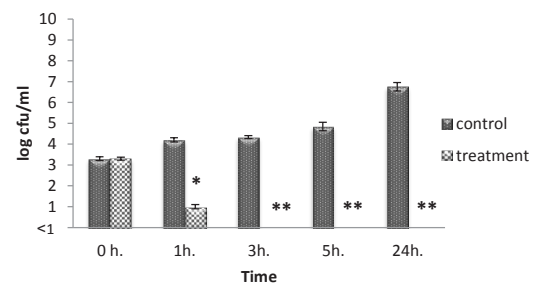


Fig. 4. bio-control of *S. Typhi* in tap after addition of phage cocktail with MOI 5 [Statistically significant treatments compared to the control were indicated by “**” where $P < 0.05$. The population count below detection limit (less than 1 log CFU/ml) is indicated by “***”].

In full fat and skim milk

Bio-control of *S. Typhi* using phage cocktail resulted in decrease of viable *Salmonella* counts of at least 1 log CFU/ml after 1h. (Fig. 5 A, B) in treated full fat milk stored at 4 °C and 25 °C, followed by regrowth during the remaining incubation period at 25 °C (Fig. 5 A). On the other hand, a complete elimination of viable bacteria in pasteurized full fat milk was observed at 4°C after 3hrs (Fig. 5 B) compared to the control (less than 1 log CFU/ml).

Further, the efficacy of the phage cocktail in skim milk was better than for full fat milk. At 25°C, the growth of *S. Typhi* was reduced by about 2 log CFU/ml in treated skim milk after 1hrs. followed by a small amount of regrowth during the remaining incubation period with about 5 log reduction after 3 days (Fig. 5 C) compared to the control ($P < 0.05$). The efficacious phages showed complete eradication in treated skim milk at 4 °C over the incubation period (below detection limit) (Fig. 5 D).

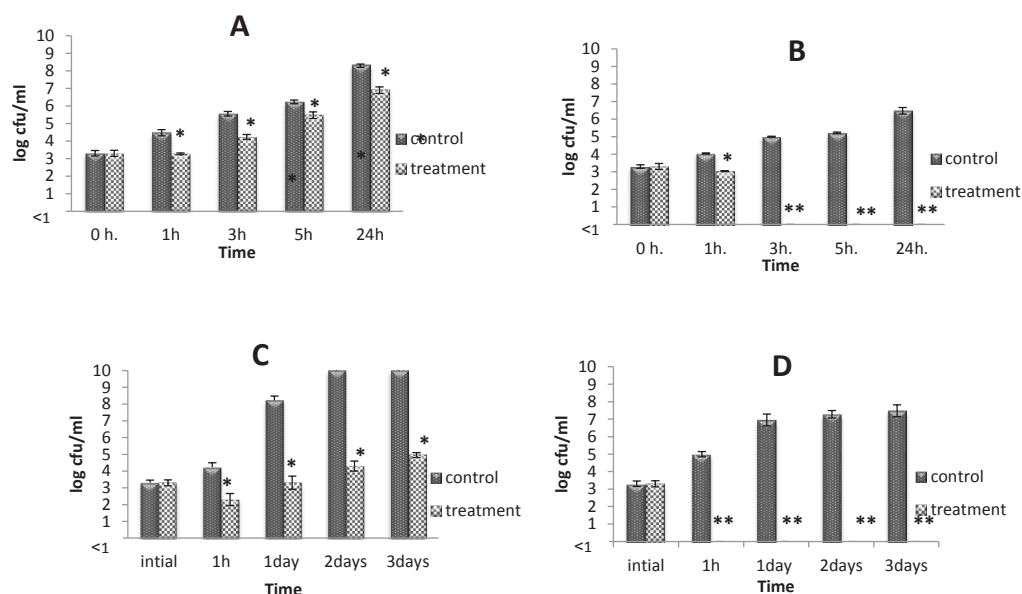


Fig. 5. Histogram showing bio-control of *S. Typhi* in treated full fat and skim milk after addition of phage cocktail with MOI 5 (A) Treatment of *S. Typhi* in full fat milk using phage cocktail at 25°C, (B) Treatment of *S. Typhi* in full fat milk using phage cocktail at 4°C, (C) Treatment of *S. Typhi* in skim milk using phage cocktail at 25°C and (D) Treatment of *S. Typhi* in skim milk using phage cocktail at 4°C [Statistically significant treatments compared to the control were indicated by “*” where $P < 0.05$. The population count below detection limit (less than 1 log CFU/ml) is indicated by “*”].**

Stability of Phage cocktail on samples

The concentration of phage cocktail added to the samples was monitored over different incubation periods. In tap water, we observed a slight rising (approximately 2 log) in the phage titer at 25°C. In milk samples, no significant loss in titers of phage cocktail inoculated into full fat milk at 4°C ($P > 0.05$), in contrast the titer of phage cocktail was reduced up to 6 log after 24hrs incubation at 25°C ($P < 0.05$) (Fig. 6 A). On the other hand, phage titers remained stable at 4°C and decreased by 1 log at 25°C in skim milk after 3 days (Fig. 6 B).

Discussion

S. Typhi is globally a major clinical problem

causing typhoid fever. In an effort to develop effective strategies to control these pathogens, a mixture of lytic bacteriophage specific for *S. Typhi* with antibacterial activity is ready to use for bio-control of bacterial pathogen was used. In our study, twenty isolates of *S. Typhi* were obtained from blood cultures (Tack, 2019) and identified phenotypically by biochemical tests as described previously (Karmakar et al., 2016). Additionally, *S. Typhi* isolates were characterized morphologically by observing colonies on the plate and using light microscope to investigate cell morphology and all the bacterial isolates were identified as being gram negative rods (Asmelash et al., 2016).

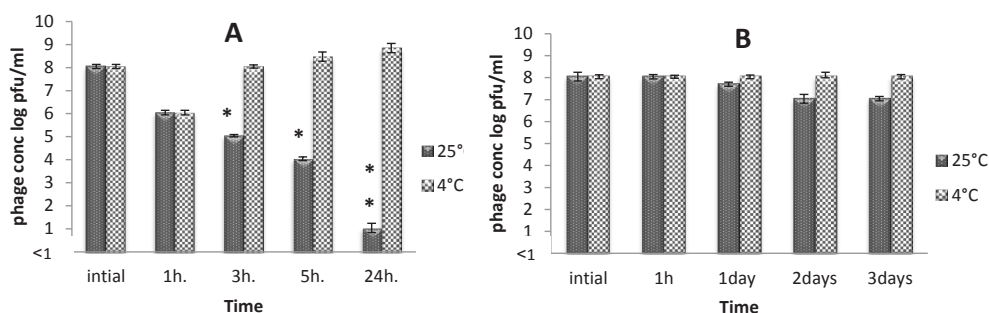


Fig. 6. Stability of *S. Typhi* phages in (A) Full fat milk and in (B) Skim milk at 4°C and 25°C [Statistically significant treatments compared to the control (initial conc.) were indicated by “*” where $P < 0.05$].

The clinical isolates were identified using Vitek analyzer as *Salmonella* spp. (Andrews et al., 2014; Pincus, 2014; Barman et al., 2018). Furthermore, they were identified serologically as *Salmonella enterica* serotype Typhi using Widal test (Somily et al., 2011; Ameya et al., 2017). Widal test is used to diagnose typhoid fever in the developing countries. The test measures agglutination antibody levels against O and H antigens (Tankeshwar, 2015).

Emergence of resistance in *S. Typhi* is a serious public health concern (Karkey et al., 2017). The antibiotic sensitivity test for *S. Typhi* was performed and showed resistance to fluoroquinolones and other families. The resistance to the fluoroquinolones has reduced therapeutic options in this completely treatable disease (Raveendran et al., 2010). Resistance Mechanism of *S. Typhi* against antibiotics was reported (Ugboko & De, 2014) and was attributed to drug inactivation, alteration of active efflux and target site in *Salmonella*. These resistance mechanisms could either be plasmid or chromosomal mediated.

Bacteriophages have been considered as a promising trend to detect and control foodborne pathogenic bacteria (Anany et al., 2017; Moye et al., 2018). The main step for successful application and to boost the food safety is cautiously selection of lytic phages. In this study, *S. Typhi* phages, targeting the causal of typhoid fever; *S. Typhi*, were isolated and identified with various plaque morphologies and sizes. These phages are belonging to *Myoviridae*, *Siphoviridae* and *Podoviridae*. The behavior and susceptibility of isolated phages depends on several phenomenons such as restriction endonuclease modification and blocking the adsorption step (Pires et al., 2016). Host range is one of main criteria affecting the selection of lytic phages to be used for bio-control applications (Holmfeldt, 2007). Most of *Salmonella* phages has a broad host range against different *Salmonella* serovars (Whichard et al., 2003). Using a cocktail of phages could overcome the limitations of phages with a narrow host range (Goodridge & Bisha, 2011). Consequently, in this work, a cocktail of *S. Typhi* phages was used.

The applications of bacteriophages for bio-control were based on stability of phages at various environmental conditions. The phages showed high tolerance under alkaline and acidic

conditions for up to 24hrs without a significant decrease in their titers which was in agreement with previous work (Jończyk et al., 2011).

The low frequency of emergence of resistant mutants against *S. Typhi* phages was investigated, as shown in a previous study (O'Flynn et al., 2006). The BIM result of *S. Typhi* phages in the current work suggests the phages are able to overcome resistance mechanisms (Labrie et al., 2010). The development of resistant strains of phage is considered as one of the problems of using phages for bio-control against pathogenic bacteria (Maura & Debarbieux, 2011).

Interestingly, bacteriophages with lytic activity specific to *S. enterica* have been considered as a new and alternative strategy to manage and control pathogenic bacteria in food matrices (Goodridge & Bisha, 2011; Moye et al., 2018). Currently, phages are considered as new antimicrobials to fight multi-drug resistant bacteria. Bacteriophages possess a number of properties helping them to combat pathogenic bacteria such as specificity which is a very valuable property, as it would prevent interference with the microflora or inhibition of starter culture in dairy production in addition to their auto-replication which gives lifetime controlling (Molina et al., 2018). Outbreaks of *S. Typhi* thought to be related to the consumption of tap water contaminated with sewage, raw and pastoralized milk represents a threat of the basic public health (Kaur et al., 2018; Liu et al., 2018). Therapeutic application of bacteriophage has many pros over chemical conventional preservatives including natural activity, specificity, lack toxicity, and a high degree of safety (Huang et al., 2018). Treating contaminated tap water with a cocktail of *S. Typhi* phages using MOI 5 resulted in complete eradication in the count of bacterial pathogen comparing with the untreated samples after 24hrs.

EFSA and ECDC, (2017) reported that *Salmonella* being the most frequently causative agent in outbreaks associated with milk and dairy products (37.7%). Furthermore, pathogenic bacteria can be emerged during food processing and storage, that is mainly linked to cross-contamination, changes in temperature and poor handling practices.

In milk, the phage cocktail showed high efficacy on the bacterial growth as described

previously (Huang et al., 2018; Phontang et al., 2018).

Bacteriophages can also be an effective control in pasteurized whole milk contaminated with *Salmonella* spp. The inhibitory effect of this phage cocktail was higher at 4°C than at 25°C (Bao et al., 2015). Thus, phages were able to successfully inhibit the growth of targeted bacteria in milk even at refrigeration temperatures (Lee et al., 2017; Molina et al., 2018. Gutiérrez et al., 2019).

In spite of this, the effectiveness of phages decreases in full fat milk than in skimmed milk and this may attributed to the organic substances such as proteins, fat content, inhibitory substances and the conditions of medium. The adsorption of phages can be affected by changes in temperature, pH or ionic strength of the medium which is particularly troublesome for biocontrol application (Hosseinidoust et al., 2014). The composition and pH of milk (Slightly acidic) have a lower impact on the activity of phages. Where the phages are prone to aggregates at low pH (Langlet et al., 2008). In addition to that, the tail fiber of phage, responsible for attachment to bacterial cell, has a positive charges (Serwer, 1987) could be attracted to the negative charges on Fatty acids in milk rather than those on bacterial cells thus render the interaction between phage and its host. Consequently, this study showed no significant reduction in bacterial growth in full fat milk at 25°C comparing to skimmed milk.

Nevertheless, other studies showed the lytic activity of bacteriophages for bio-control *Salmonella* spp. and other pathogenic bacteria with relevance in different food matrices and dairy industry (Heyse et al., 2015; Nannapaneni & Soni, 2015; Gutiérrez et al., 2019).

Conclusion

S. Typhi showed resistance against traditional antibiotics such as ampicillin, chloramphenicol, and trimethoprim-sulfonamide combinations. Although fluoroquinolones such as ciprofloxacin are used to treat severe *Salmonella* infections, some of *S. Typhi* showed resistance to fluoroquinolones. Consequently, many trends are looking for new safe, effective and potential antimicrobials alternative to antibiotic. Therefore, this study led to isolation of lytic phages specific to *S. Typhi*. Stability of the isolated phages in

different environmental conditions suggests their applicability and use as bio-control tools throughout the food supply chain. This study proves the efficacy of these lytic phages for controlling the growth of *S. Typhi* in milk and drinking water, and so potentially increases the shelf life of milk and enhances the safety of tap water.

References

- Ackermann, H.-W. (2012) Bacteriophage electron microscopy. *Adv. Virus Res.* **82**, 1-32.
- Ameya, G., Atalel, E., Kebede, B., Yohannes, B. (2017) Comparative study of Widal test against stool culture for typhoid fever suspected cases in southern Ethiopia. *Pathol. Lab. Med. Internat.* **9**, 1-7.
- Anany, H., Brovko, L., El-Arabi, T., Griffiths, M. (2014) Bacteriophages as antimicrobials in food products: History, biology and application. In: "*Handbook of Nat. Antimicrobials for Food Saf. and Qual.*". 69.
- Anany, H., Chou, Y., Cucic, S., Derda, R., Evoy, S., Griffiths, M.W. (2017) From bits and pieces to whole phage to nanomachines: pathogen detection using bacteriophages. *Annu. Rev. Food Sci. Technol.* **8**(1), 305-29.
- Anany, H., Brovko, L., El Dougdoug, N.K., Sohar, J., Fenn, H., Alasiri, N., Jabrane, T., Mangin, P., Ali, M.M., Kannan, B., Filipe, C.D.M., Griffiths, M. W. (2018) Print to detect: A rapid and ultrasensitive phage-based dipstick assay for foodborne pathogens. *Anal. Bioanal. Chem.* **410**, 1217-1230.
- Andrews, W.H., Wang, H., Jacobson, A., Hammack, T. (2014) "*Bacteriological Analytical*" The VITEK 2 method of Presumptive generic identification of *Salmonella* was updated. *Bacteriol. Analytical Man.* Chapter 5.
- Asmelash, T., Mesfin, N., Addisu, D., Aklilu, F., Biruk, T., Tesfaye, S. (2016) Isolation, identification and drug resistance patterns of methicillin resistant *Staphylococcus aureus* from mastitic cows milk from selected dairy farms in and around Kombolcha, Ethiopia. *J. Vet. Med. Anim. Heal.* **8**, 1-10.
- Bao, H., Zhang, P., Zhang, H., Zhou, Y., Zhang, L., Wang, R. (2015) Bio- control of *Salmonella*

- enteritidis* in foods using bacteriophages. *Viruses*, **7**, 4836-4853.
- Barman, P., Chopra, S., Thukral, T. (2018) Direct testing by VITEK® 2: A dependable method to reduce turnaround time in Gram-negative blood stream infections. *J. Lab. Physicians*. **10**(3), 260-264.
- Beke, G., Stano, M., Klucar, L. (2016) Modelling the interaction between bacteriophages and their bacterial hosts. *Math.Biosci.* **279**, 27-32.
- Bergey, D.H., Whitman, W.B., De, V.P., Garrity, G.M., Jones, D. (2009) "*Bergey's Manual of Systematic Bacteriology*", 3.
- CDC (2008) "*Outbreak-associated Salmonella enterica Serotypes and Food Commodities*", United States.
- CDC (2010) Multistate Outbreak of Human Typhoid Fever Infections Associated with Frozen Mamey Fruit Pulp. **United States**.
- Clarke, T. (2003) Drug companies snub antibiotics as pipeline threatens to run dry. *Nat.* **425**, 225.
- CLSI (2019) M100, Performance Standards for Antimicrobial Susceptibility Testing, 29th ed. *Clinical and Laboratory Standards Institute*, **4**(1), 2-20.
- Cooke, F.J., Wain, J. (2004) The emergence of antibiotic resistance in Typhoid fever. *Travel Medicine and Infect Dis.* **2**(2), 67-74.
- EFSA, ECDC. (2017) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *Sci. Rep. EFSA J.* **15**, 5077.
- El-Dougdoug, N.K., Cucic, S., Abdelhamid, A.G., Brovkob, L., Kropinskib, A.M., Griffith, M.W., Anany, H. (2019) Control of *Salmonella* Newport on cherry tomato using a cocktail of lytic bacteriophages. *Intern. J. Food Microbiol.* **239**, 60-7.
- Goodridge, L.D., Bisha, B. (2011) Phage-based biocontrol strategies to reduce foodborne pathogens in foods. *Bacteriophage*, **1**, 130-137.
- Gutiérrez, D., Fernández, L., Rodríguez, A., García, P. (2019) Role of bacteriophages in the implementation of a sustainable dairy chain. *Front. Microbiol.* **10**(12), 1-14.
- Heyse, S., Hanna, L.F., Woolston, J., Sulakvelidze, A., Charbonneau, D. (2015) Bacteriophage cocktail for biocontrol of *Salmonella* in dried pet food. *J. Food Prot.* **78**, 97-103.
- Holmfeldt, K., Middelboe, M., Nybroe, O., Riemann, L. (2007) Large variabilities in host strain susceptibility and phage host range govern interactions between lytic marine phages and their *Flavobacterium* hosts. *Appl. Environ. Microbiol.* **73**, 6730-6739.
- Hosseini Doust, Z., Olsson, A.L.J., Tufenkji, (2014) Going viral: Designing bioactive surfaces with bacteriophages. *Colloids and Sur. B: Bioinfect.* **124**, 2-16.
- Huang, C., Virk, S.M., Shi, J., Zhou, Y., Willias, S.P., Morsy, M.K., Abdelnabby, H.E., Liu, J., Wang, X., Li, J. (2018) Isolation, characterization, and application of bacteriophage LPSE1 against *Salmonella enterica* in ready to eat (RTE) foods. **Front. Microbiol.**
- Jams, M. (2003) Typhoid fever. *JAMC*, **169**(2), 132.
- Jończyk, E., Kłak, M., Międzybrodzki, R., Górski, A. (2011) The influence of external factors on bacteriophages--review. *Folia Microbiol. (Praha)*. **56**, 191-200.
- Karkey, A., Thwaites, G.E., Barker, S. (2017) The evolution of antimicrobial resistance in *Salmonella* Typhi. *Cur. Opinion in Gastroenterol.* **34**(1), 1.
- Karmakar, A., Dua, P., Ghosh, C., (2016) Biochemical and molecular analysis of *Staphylococcus aureus* clinical isolates **from hospitalized patients**.
- Kaur, A., Das, R., Nigam, M.R., Elangovan, R., Pandya, D., Jha, S., Kalyanasundaram, D. (2018) Rapid detection device for *Salmonella typhi* in Milk, Juice, Water and Calf Serum. *Ind. J. Microbiol.* **58**(3), 381-392.
- Labrie, S.J., Samson, J.E., Moineau, S. (2010) Bacteriophage resistance mechanisms. *Nat. Rev. Microbiol.* **8**, 317-327.
- Langlet, J., Gaboriaud, F., Duval, J., Gantzer, C. (2008) Aggregation and surface properties of F-specific RNA phages: Implication for membrane filtration processes. *Water Res.* **42**, 2769-2777.

- Lee, S., Kim, M.G., Lee, H.S., Heo, S., Kwon, M., Kim, G. (2017) Isolation and characterization of *Listeria* phages for control of growth of *Listeria monocytogenes* in milk. *Korean J. Food Sci. Anim. Resour.* **37**, 320-328.
- Liu, H., Chris, A., Whitehouse, C.A., Baoguang Li, B. (2018) Presence and Persistence of *Salmonella* in Water: The impact on microbial quality of water and food safety. *Front. Pub. Heal.* **6**, 159.
- Marus, J.R., Magee, M.J., Manikonda, K., Nichols, M.C. (2019) Outbreaks of *Salmonella enterica* infections linked to animal contact: Demographic and outbreak characteristics and comparison to foodborne outbreaks. *Zoon. Pub. Heal.* **00**, 1-7.
- Maura, D., Debarbieux, L., (2011) Bacteriophages as twenty-first century antibacterial tools for food and medicine. *Appl. Microbiol. Biotechnol.* **90**, 851-859.79, **1956-1968**.
- Molina, F., Simancas, A., Díaz, M., Nuñez, R., Rebollo, J.E., Tabla, R., et al. (2018) Isolation and host-specificity characterization of coliphages suitable for biocontrol in dairy industries. *Explor. Microorg. Recent Adv. Appl. Microbiol.* **91**, 91-95.
- Moye, Z., Woolston, J., Sulakvelidze, A. (2018) Bacteriophage applications for food production and processing. *Vir.* **10**, 205.
- Mukesh, S., Sandeep, R., Shobha, B. (2018) Comparison of Slide Widal with Tube Widal Agglutination Test for the Diagnosis of Enteric Fever in Febrile Patients. *Ind. J. Heal. Sci. Care*, **5**(1),
- Nannapaneni, R., Soni, K.A. (2015) Use of bacteriophages to remove biofilms of *Listeria monocytogenes* and other foodborne bacterial pathogens in the food environment. *Food Environ.* **131-144**.
- O'Flynn, G., Ross, R.P., Fitzgerald, G.F., Coffey, A. (2006) Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* **70**, 3417-3424.
- Phontang, W., Choi, G., Chuksaitote, E., Ahn, J. (2018) Bacteriophage control of *Salmonella* Typhimurium in milk. *Food Sci. Biotechnol.* **28**(1), 297-301.
- Pincus, D.H. (2014) Microbial Identification using the BIOMÉRIEUX Vitek ® 2 System, bioMérieux, Inc. Hazelwood, MO, USA, pp. 1-32.
- Pires, D.P., Oliveira, H., Melo, L.D.R., Sillankorva, S., Azeredo, J., (2016) Bacteriophage-encoded depolymerases: Their diversity and biotechnological applications. *Appl. Microbiol. Biotechnol.* **1-11**.
- Rasul, F., Sughra, K., Mushtaq, A., Zeeshan, N., Mehmood, S., Rashid, U. (2017) Surveillance report on typhoid fever epidemiology and risk factor assessment in district Gujrat, Punjab, Pakistan. *Biomedical. Res.* **16**, 0976-1683.
- Raveendran, R., Datta, S., Wattal, C. (2010) Drug resistance in *Salmonella enterica* serotype Typhi and Paratyphi. *JIMSA*, **23**, 1-21.
- Sambrook, J., Fritsch, E.F., Maniatis, T., (1989) "*Molecular Cloning: A Laboratory Manual*". Cold Spring Harbor Laboratory Press.
- Scallan, E., Hoekstra, R.M., Mahon, B.E., Jones, T.F., Griffin, P.M. (2015) An assessment of the human health impact of seven leading foodborne pathogens in the United States using disability adjusted life years. *Epidemiol. Infect.* **143**(13), 2795-804.
- Serwer, P. (1987) Agarose gel electrophoresis of bacteriophages and related particles. *J. Chromatogr. B: Biomed. Sci. Appl.* **245-357**.
- Somily, A.M., Adam, M.H., Gad El Rab, M.O., Morshed, M.G., Shakoor, Z. (2011) Detection of *Salmonella typhi* agglutinins in sera of patients with other febrile illnesses and healthy individuals. *Ann. Afr. Med.* **10**(1), 41-4.
- Steele, A.D., Burgess, D.C.H., Diaz, Z., Carey, M.E., Zaidi, A.K.M. (2016) Challenges and opportunities for typhoid fever control: A call for coordinated action. *Clin. Infect. Dis.* **62**, S4-S8.
- Tack, B., Phoba, M.F., Puyvelde, S.V., Kalonji, L.M., Hardy, L., Barbe, B., Sande, M.V., Monsieurs, E., Deborggraeve, S., Lunguya, O. Jacobs, O.J. (2019) *Salmonella* Typhi from blood cultures in the Democratic Republic of the Congo: A 10-Year Surveillance. *Clin. Infect. Dis.* **68**, 2-15.
- Tankeshwar, A. (2015) "*Widal Test: Principle, Procedure, Results, Interpretation, Significance and Limitation*". Medical Microbiology guide.
- Egypt. J. Bot.* **60**, No. 1 (2020)

- Ugboko, H., De, N. (2014) Mechanisms of Antibiotic resistance in *Salmonella typhi*. *Int. J. Curr. Microbiol. App. Sci.* **3**(12), 461-476.
- 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. *J. Food Prot.* **66**, 220-225.
- WHO (2018) Typhoid: Global Report on Surveillance. World Health Organization.
- Widal, F.M. (1896) Serodiagnostic de la fièvre typhoïde a-propos d'une modification par MMC. *Bull. Soc. Med. Hop.* **13**, 561-6.
- Zhan, A., Buchan, A., Chen, F. (2015) Novel N4 bacteriophages prevail in the cold biosphere. *Appl. Environ. Microbiol.* **81**, 5196-5202.



يرجع التأثير المرضي لبكتريا السالمونيلا المعوية (سالمونيلا تيفي) المقاومة للمضادات الحيوية والمسببة لحمى التيفويد بشكل رئيسي إلى تلوث الطعام ومياه الشرب الملوثة بمياه الصرف. ولذلك الهدف من هذه الدراسة هو تقييم حساسية السالمونيلا تيفي للمضادات المختلفة وتطبيق البكتريوفاجات المحللة والمتخصصة لسالمونيلا تيفي لتحسين سلامة مياه الشرب والحليب. وشملت الدراسة على عزلات السالمونيلا المعوية المستخرجة من عينات دم سريرية. ولقد تم تعريف العزلات البكتيرية عن طريق الإختبارات الكيميائية الحيوية التقليدية، و system Vitek بالإضافة إلى اختبار سيرولوجي للكشف عن الأنماط المصلية لعزلات السالمونيلا. وعندما تم عمل اختبار الحساسية للمضادات الحيوية اظهرت عزلات السالمونيلا تيفي مقاومة للعديد من المضادات الحيوية. ومن ثم تم عزل وتعريف أربع أنواع من البكتريوفاجات ذات نشاط محلل للبكتريا وسميت بـ EN-1 و vb_StyM و EN-2 و b_StyM و EN-3 و vb_StyS و EN-4 lytic و vb_StyP وتطبيقها للتحكم في نمو بكتريا السالمونيلا تيفي. وعند استخدام كوكنتيل من الفاجات لمقاومة البكتريا المستهدفة، اظهرت النتائج القضاء التام للنمو البكتيري في الوسط السائل عند 4 درجة مئوية ودرجة حرارة الغرفة لمدة 24 ساعة. وبالإضافة إلى ذلك، لوحظ تثبيط كامل في عدد البكتريا عند تطبيق كوكنتيل الفاجات في مياه الشرب المعالجة عند 25 درجة مئوية لمدة 24 ساعة. وعند تطبيق الفاجات لمقاومة السالمونيلا تيفي في الحليب، اظهرت إنخفاض بمقدار 5 درجات لوغارتية في نمو البكتريا في الحليب الخالي من الدسم مقارنة بالحليب كامل الدسم بمقدار درجة لوغارتية. وتشير هذه النتائج إلى أن البكتريوفاجات تعد بدائل واعدة مضادة للميكروبات وبكتريا السالمونيلا تيفي المقاومة للمضادات الحيوية.

