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Potentiality of lytic bacteriophages and their virolysins in lysing multi-drug resistant *Salmonella typhi*

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Bacteriophage virolysins or lytic enzymes are bacterial peptidoglycan hydrolases responsible for lysing bacterial cells. Consequently, they are used as enzybiotics alongside with bacteriophage therapy to remedy multi-drug resistant *Salmonella typhi*. The objective of this study was to evaluate the potentiality of lytic bacteriophages and their virolysins in curing multi-drug resistant *S. typhi*. *S. typhi* was isolated and identified according to WHO and ISO guidelines. Antibiotics susceptibilities were tested using CLSI recommendations. Correspondingly, bacteriophage-lysing efficiency was assayed by plaques formation using the double-layer agar technique. Virolysins were extracted using ultracentrifugation and purified by dialysis after buffering in ammonium sulfate. Virolysins activity was determined by measuring the reaction mixtures spectrophotometrically (bacteria incubated as substrate in 37°C for 4 h). The phages and virolysins kinetics exponential rates were calculated using specific differential equations. Susceptibility data plotted based on antibiogram criteria confirmed that 33% of *S. typhi* isolates were multi-drug resistant. For bacteriophage replication and multiplicity of infection, phages were amplified to produce the maximum particles of titers. The phage titration data fit on sonogram revealed exponential decay of *S. typhi* incubated for 12 h. Meanwhile, the enzyme kinetics exponential decay on double reciprocal plot showed irretrievable relationship of host decay in 4 h. Since phages depend on their lytic cycle in lysing bacterial host, their enzymes have more capability in decaying the host; besides they are safe and time-saving when used in the treatment of antibiotics resistant *S. typhi*.

Key words: Antibiotics, bacteriophage, enzybiotics, *Salmonella typhi,* virolysins.

INTRODUCTION

With the emergence of multi-drug resistant (MDR) strains of *Salmonella typhi* worldwide, the increasing incidence of enteric fever leads to death, especially in poor communities (Gupta et al., 2009; Weill, 2010; Feasey et al., 2015). *S. typhi* rapidly gained resistance to antibiotics like ampicillin, chloramphenicol and cotrimoxazole; also the earlier efficacious drugs of fluoroquinolones like ciprofloxacin acquired a reduced sensitivity against MDR

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S. typhi as many cases reported (Madhulika et al., 2004; Jones et al., 2014). Therefore, bacteriophage therapy is an alternative drug to cure bacterial infections because they are highly specific in lysing bacteria, they do not contain integrated genes in their genomes and cannot coexist with the host and carry virulent genes from one host after decay to infect another (Borris et al., 2001). Phage therapy is the application of bacteriophages to bacterial infections of humans (or other animals) with the goal of reducing bacterial load. Phage therapy is complicated by the self-replicating nature of phage (Patel et al., 2011).

Bacteriophage virolysins including lysins, endolysins and lysozymes seem to be the most promising alternative antibiotics as a result of a few unique characteristics of this group of lytic enzymes (so-called enzybiotics). This word is coined to describe a treatment, which uses purified phage-encoded enzymes as antibacterial agents (Yang et al., 2014; Meaney et al., 2015). Virolysins are bacteriolytic agents that cause bacterial lysis by cleaving to bonds in the cell wall. They are presumed to be a phage-induced enzyme participating in lysis through its destructive action on the host cell wall (Courchesne et al., 2009). The peptidoglycan network is responsible for cell rigidity and containment of the cytoplasmic membrane. They share the specificity found in the bacteriophage in which it was produced during its lytic cycle (Skariyachan et al., 2016). Using phage-encoded bacterial cell wall virolysins to eliminate pathogenic bacteria has led to the designation of muralytic enzymes as enzybiotics. There are tremendous reviews that have dealt with the characteristics of virolysins and their applications as alternative enzybiotics in medical-oriented *in vitro* and *in vivo* tests (Maszewska, 2015). Although, lytic enzymes are more efficient for the lysis of Gram-positive bacteria, they also assist the lysis of Gram-negative bacteria such as *Salmonella* and *Shigella* (Ahluwalia et al., 2012; Bryan et al., 2016). Phage virolysins involved in the lytic cycle of phage replication have two classes of peptidoglycan hydrolase enzymes, namely, endolysins and virion associated hydrolase. They are characterized as phage-encoded proteins (Salazar and Asenjo, 2007). Bacteriophages encode holins and lysins to achieve their exit from the host bacteria. They form pores in the cytoplasmic membrane, following lysins accumulated in the cytoplasm and degrade the peptidoglycan layer. Damage to this layer results in cell rupture and concomitant virus released through loss of osmotic integrity (Buyuktimkin and Saier, 2015; Buyuktimkin and Saier, 2016).

Virolysin catalyzes the hydrolysis of 1, 4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycans and between the N-acetyl-D-glucosamine residues in chitodextrins (Narasimhan et al., 1988). However, virolysins are promising enzybiotics for bacterial cell wall hydrolases even if these bacteria are resistant to lysozymes. They have narrow spectra of sensitive bacteria, minimizing the disturbance to normal microflora and a tremendous diversity of lytic bacteriophages in the biosphere, which guarantees their ability of targeting almost any bacteria (Filatova et al., 2015). To determine the kinetics exponential decay of virolysins mathematically, the resulting data points of equations were fit to curves solved on double reciprocal plot (Courchesne et al., 2009). Meanwhile, the bacteriophage values of kinetic lysing rate are usually obtained from the exponential growth data sets plotted on sensorgrams (Groman and Suzuki, 1963; Ploss and Kuhn, 2010). Clinical trials conducted to assess the safety and pharmacokinetic properties of virolysins confirmed that they are safe for humans and can enhance the lysing *S. typhi;* so they are highly recommended to be used for antibiotics resistant bacterial infections (Lopez-Cuevas et al., 2011). In the recent study, the potentiality of bacteriophages' virolysins lysing efficiency was evaluated and used *in vitro* as antimicrobial agents against multi-drug resistant *S. typhi.*

MATERIALS AND METHODS

Microorganisms' isolation and identification

S. typhi was recovered from Khartoum Wastewater Station. Cultural characteristics, biochemical and serological tests were performed according to the recommendations of ISO 6579 standard optimized by Mainar-Jaime et al. (2013). Each bacterium was tested for its susceptibility using National Committee for Clinical and Laboratory Standards Institute guidelines (CLSI, 2012). *S. typhi* allied lytic phages were isolated using the double-layer agar technique recommended by Mooijman et al. (2001).

Bacteriophages multiplicity of infection [MOI]

For lysing, *S. typhi* isolates lytic phages (with MOI = 50 phage/cell) were added to 5 ml of *S. typhi* culture in exponential growth phase until turbidity decreased (OD_{600nm}>0.3). The MDR-*S. typhi* allied phages' concentrations in the sample were amplified and maximized using plaque assay on double-layer agar technique. In it, bacteria were inoculated for the second time using serial dilutions of 10^{-8}, 10^{-9} and 10^{-10}; to release progeny phages, multidrug resistant *S. typhi* (I = 16 and R ≥32 µg/ml) was used as a host bacterial strain and stored at 4°C. When the OD_{600nm} was below 0.3, the inocula were filtered through 0.45 µm Whatman filter paper. The filtrates were collected in screw cap bottles containing 50 ml SM buffer which was prepared by dissolving 2.0% w/v gelatin, 2.0% w/v $MgSO_4.7H_2O$, 5.8% w/v NaCl in 200 ml tris HCl (1M, pH 7.5); the demineralized water volume was completed to 1.0 L.

Extraction of virolysins (lytic enzymes)

The bacteria-free filtrates were kept at 30°C without stirring for 2 days to sustain the phages' infection and replication before extracting the enzymes from the virion particles. Virolysins were extracted by centrifugation at 6,010 g for 30 min; ammonium sulfate (up to 40%) was added as a buffer to the supernatant and gently

mixed for 4 h at 4°C. The filtrates were sealed in dialysis bags (molecular weight cutoff, 12,000 to 14,000), and dialyzed at 4°C for 2 days against deionized water to purify the enzyme (Savary et al., 2001).

Detection of enzyme activity

Bacterial cultures were prepared and used to detect the lytic bacteriophages' virolysins activity. The enzymes were added to the culture (5 µg/ml) with a final proportion of 1:20 and incubated at 37°C for 4 h at OD_{600nm}. The enzyme activity was determined by measuring the reducing sugars released from lysed bacterial cells used as substrate in the reaction solution using Benedict's reagent.

Enzyme kinetics exponential decay

Salmonella typhi was added as a substrate (initial amount 1.0×10^6 cell/ml) to the extracted enzyme and incubated at 37°C for 4 h. The change in the mixture turbidity was recorded. The lysis rate was expressed as a reduction in the turbidity; it resembled the enzyme kinetics exponential decay rate $V = (\frac{dy}{dt} = -\lambda x)$.

RESULTS

Patterns of multi-drug resistant *S. typhi*

From the recovered bacteria species, 128 were identified as *S. typhi.* The isolates were tested for their susceptibility against antibiotics. The means of n=42 (33.0%) of the isolates were found to be multi-drug resistance and categorized in 8 main groups, namely, *S. typhi* 16, *S. typhi* 32, S.7 16, S.7 32, Dr11 16, Dr11 32, Sal C 16, and Sal C 32, based on the collection. The Minimum Inhibitory Concentrations (MICs) were determined at 16 and 32 µg/ml concentrations and 67.0% isolates that resist ciprofloxacin as Minimum Bactericidal Concentration (MBCs).

Antibiogram or *in vitro*-sensitivity showed patterns of *S. typhi* isolates that have developed resistance against most antibiotics (MDR), including ciprofloxacin and was interpreted as MBC ≥ 32 µg/ml (Figure 1).

Bacteriophage multiplicity of infection and amplification

The specific bacteriophage for each of the *S. typhi* was determined using the plaque assay. Phages were harvested at various intervals time and assayed by double-layer plaque assay on *S. typhi* lawn. This contributes significantly to the phage amplification by providing substrates for phage growth (Table 1). The multiplicity of infection of 50 phage/cell was assessed for the lysing of initial amount ($n\times10^6$ cell/ml) of the host, and the progeny phages released during the phage lytic cycle in 12 h incubation were amplified from $n\times10^{+8}$ up to $n\times10^{+11}$ phage/ml, where n = numerical digit.

Since the highest number of the plaques was obtained at high titers that correlated to the initial number of bacterial cells, then initial amounts of *S. typhi* isolates ($n\times10^6$) were infected by their lytic phages (MOI = 50 phage/cell) and replicated for titer (10^8). To obtain the maximum numbers of phage's cells per sample amplified for 12 h at 37°C, the numbers of phages' plaques (pfu/ml) were recorded for titers 10^{-8} up to 10^{-9} and 10^{-10}.

Bacteriophage exponential lysing rate

The capacity of phage to lyse bacterial lawns producing high numbers of pure plaques occurred when higher titers were selected after 12 h of incubation. To calculate the phages potentiallity in lysing *S. typhi* per serial dilution, the bacteria were tittered at 10^{-8}, 10^{-9} and 10^{-10} using the formula: [$y = a .10^n e^{(bx)}$], where a = exponential constant, 10^n = titer, e = exponential number and (x) = plaque formation/isolate at certain point (Figure 2).

The exponential portion of the curve was used to determine the slope of lysing rate and the data were used to calculate the decrease in OD_{600} per unit of time. The bacteriophage amplification rate given by [$y = (2\times10^{+9}) e^{0.2908x}$, $y = (2\times10^{+10}) e^{0.02085x}$ and $y = (7\times10^{+11}) e^{0.0373x}$] and their Spearman's rank correlation coefficient (R^2) = 0.5, 0.4 and 0.1 are in low values.

Bacteriophage kinetics titrations

The phage lysing rates of their host were determined by the time needed for bacteria to produce phages after infection and decreasing of optical density (OD_{600nm}) per minute. To confirm the phage specificity toward the specific bacteria, the numbers of plaques/phages increased for each isolate by increasing their titration rates (Equations 1 to 5).

The kinetic titration given by:

$$A+B \overset{ki}{\rightleftharpoons}_{kd} AB \quad (1)$$

$$\frac{dA}{dt} + \frac{dB}{dt} \overset{ki}{\rightleftharpoons}_{kd} \frac{d(AB)}{dt} \quad (2)$$

[A] = Initial amount of Salmonella (10^6 cell/ml), [B] = Phages ([MOI = 50 phage/cell], [LA]=concentration of reaction mixture, k_i= Initial kinetics absorbance (OD_{600nm} = 1), k_d= decreasing kinetics absorbance rate (OD_{600}<0.3). The k called rate constants are constants of proportionality in the application of the Law of Mass Action. Response was calculated for bacteriophage exponential kinetics titration: where R = replicated titer, t= total time of incubation and t_0 = initial time of incubation.

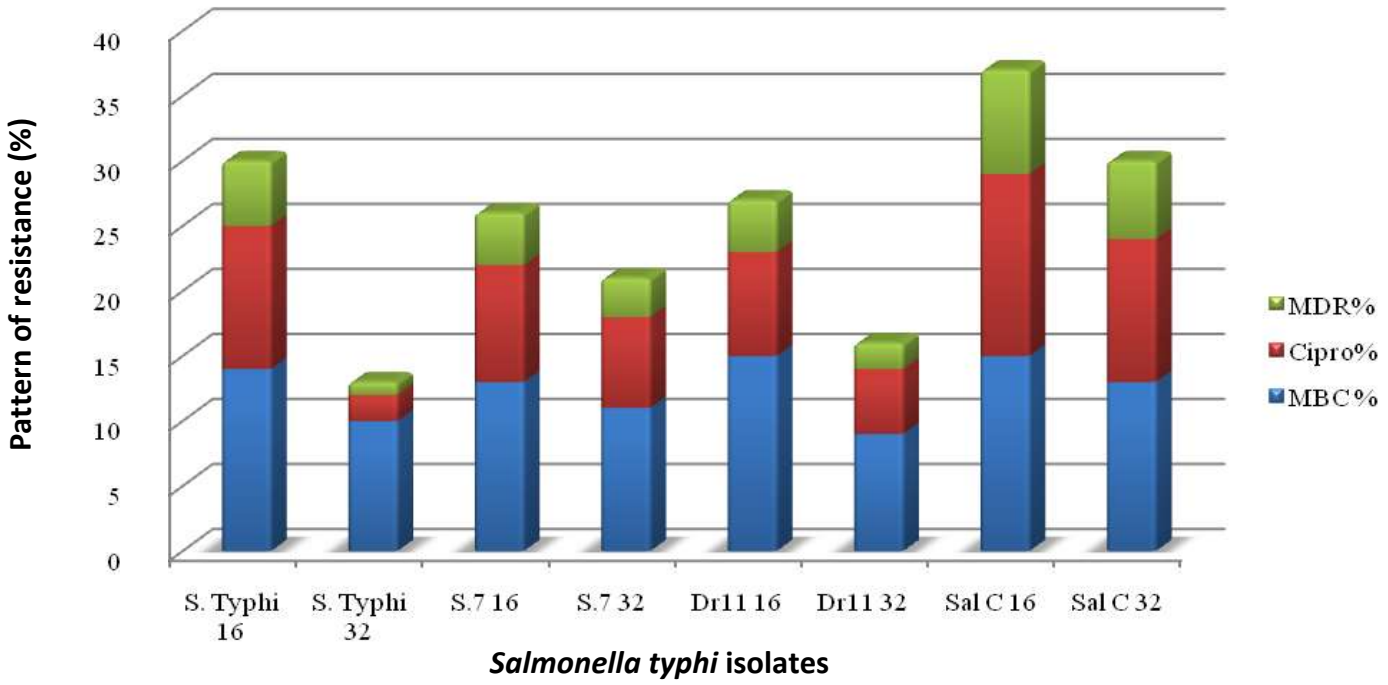

Figure 1. Antibiogram criteria for multi-drug resistant strains. *MDR: Multi-drugs, Cipro: ciprofloxacin, MBC: minimum bactericidal concentration.

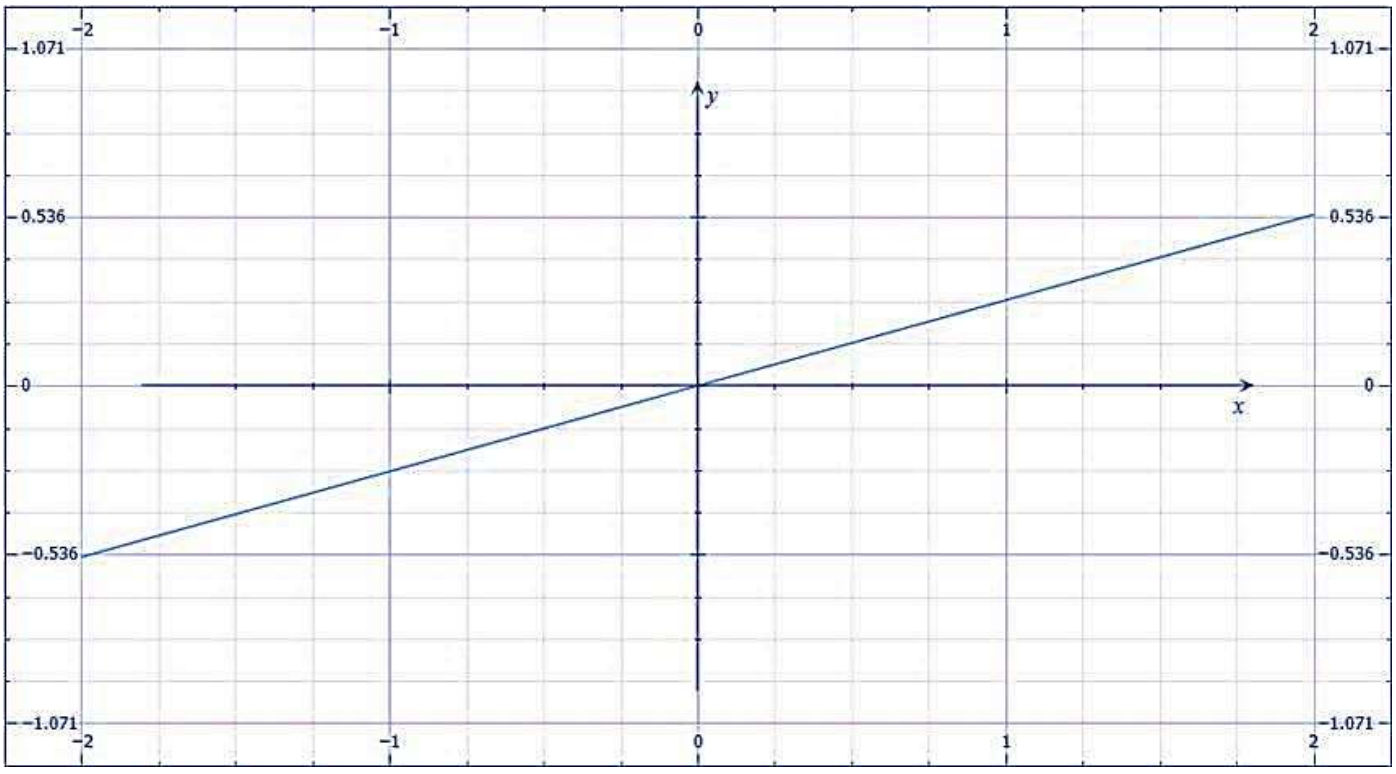

Figure 2. Plotting the slope of bacteriophages' amplification rates.

Table 1. Bacteriophage lytic replication and amplification to get the highest numbers of phage cells/sample.

Salmonella **(Initial amount OD $_{600nm}$ = 1)**	**cell/ml**	**Bacteriophage**								
		Dilution	**Plaques**	**PFU/ml**	**Dilution**	**Plaques**	**PFU/ml**	**Dilution**	**Plaques**	**PFU/ml**
S. typhi 16	1.6×10^{6}	10^{-8}	62.0	6.2×10^{9}	10^{-9}	42.0	4.2×10^{10}	10^{-10}	52.0	5.2×10^{11}
S. typhi 32	2.8×10^{6}	10^{-8}	78.0	7.8×10^{9}	10^{-9}	80.0	9.0×10^{9}	10^{-10}	84.0	8.4×10^{11}
S.7 16	1.7×10^{6}	10^{-8}	46.0	4.6×10^{9}	10^{-9}	66.0	6.6×10^{10}	10^{-10}	94.0	9.4×10^{11}
S.7 32	1.1×10^{6}	10^{-8}	58.0	5.8×10^{9}	10^{-9}	64.0	6.4×10^{10}	10^{-10}	78.0	7.8×10^{11}
Dr11 16	9.0×10^{5}	10^{-8}	90.0	9.0×10^{9}	10^{-9}	88.0	8.8×10^{10}	10^{-10}	96.0	9.6×10^{11}
Dr11 32	1.3×10^{6}	10^{-8}	74.0	7.4×10^{10}	10^{-9}	76.0	7.6×10^{10}	10^{-10}	58.0	5.8×10^{11}
Sal C 16	1.5×10^{6}	10^{-8}	24.0	2.4×10^{10}	10^{-9}	86.0	8.6×10^{10}	10^{-10}	88.0	8.8×10^{11}
Sal C 32	1.6×10^{6}	10^{-8}	26.0	2.6×10^{10}	10^{-9}	92.0	9.2×10^{10}	10^{10}	94.0	9.4×10^{11}

Table 2. Solving equations of bacteriophage kinetic titration in lysing bacterial host.

Salmonella isolates OD $_{600nm}$ = 1.0	**Initial amount (cell/ml)**	**Bacteriophage 1st infection**			**Bacteriophage 2nd infection**		
		12 h OD$_{600\ nm}$	**Phage ml^{-1}**	***Salmonella*** **(cell/ml)**	**12 h OD$_{600\ nm}$**	**Phage ml^{-1}**	***Salmonella*** **(cell/ml)**
S. typhi 16	$1.6 \times 10^{+6}$	0.40	$1.2 \times 10^{+8}$	$6.4\times^{+5}$	0.20	$3.31 \times 10^{+10}$	$1.3\times^{+5}$
S. typhi 32	$2.8 \times 10^{+6}$	0.36	$1.4 \times 10^{+8}$	$1.0\times^{+6}$	0.15	$3.72 \times 10^{+10}$	$1.5\times^{+5}$
S.7 16	$1.7 \times 10^{+6}$	0.38	$9.5 \times 10^{+8}$	$6.4\times^{+5}$	0.33	$4.14 \times 10^{+10}$	$2.1\times^{+5}$
S.7 32	$1.1 \times 10^{+6}$	0.42	$1.23 \times 10^{+9}$	$4.7\times^{+5}$	0.36	$4.55 \times 10^{+10}$	$1.7\times^{+5}$
Dr11 16	$0.9 \times 10^{+6}$	0.48	$1.65 \times 10^{+9}$	$4.3\times^{+5}$	0.42	$4.97 \times 10^{+10}$	$1.8\times^{+5}$
Dr11 32	$1.3 \times 10^{+6}$	0.54	$2.06 \times 10^{+9}$	$7.0\times^{+5}$	0.49	$5.38 \times 10^{+10}$	$3.4\times^{+5}$
Sal C 16	$1.5 \times 10^{+6}$	0.36	$2.48 \times 10^{+9}$	$5.4\times^{+5}$	0.55	$5.80 \times 10^{+10}$	$3.0\times^{+5}$
Sal C 32	$1.6 \times 10^{+6}$	0.44	$4.43 \times 10^{+9}$	$7.0\times^{+5}$	0.60	$6.32 \times 10^{+10}$	$4.2\times^{+5}$

$$\frac{dA}{dt} = -(ki.A.B - kd.AB) \quad (3)$$

$$\frac{d[AB]}{dt} = ki.[A].[B] - kd.[AB] \quad (4)$$

$$\boldsymbol{R} = R^{1a} + R^{0} + R^{1} + [drift(t - t_0)] \quad (5)$$

In the numerical kinetics equation, each replicated titer individually started from the *Salmonella* initial amount, and the total response calculated (Equations 1 to 5). The equations also contain terms for bacteria mass transport from initial amount of bacterial host to the first and second infection by phage, drift and time refractive index intervals.

To determine the kinetics lysis of bacteriophage efficancy and bacterial host cells decay rates, the lysis rates of *Salmonella* initial amounts were plotted exponentially against the phage potentiality in lysing bacteria. Furthermore, the decay profiles varied for each sample as shown in Table 2. While the viability of the phage decreased in a trend consistent with first infection decay kinetics, the bacteria displayed degradation patterns depending on incubation period before entering a second phase of infection that increased rates of lysing.

Table 3. Reducing absorbency of extracted virolysins OD/h.

Tested sample OD $_{600nm}$=1.0	Virolysins decay/h OD $_{600nm}$ <1.0
S. typhi 16	0.75
S. typhi 32	0.98
S.7 16	0.80
S.7 32	0.79
Dr11 16	0.92
Dr11 32	0.81
Sal C 16	0.85
Sal C 32	0.69

Solving bacteriophage exponential lysing rate; 1st infection [$y = 702302e^{-0.027x}$, 2nd infection $y = 107473e^{0.1582x}$] and their Spearman's rank correlation coefficient (R^2) = 0.06 and 0.8. The exponent positive integer corresponds to the repeat multiplication of the base (bacteria) and (e^{+bx}) with positive integer indicates the bacteria exponential decay and multiplication of phage in 12 h of incubation period.

Virolysins potentiality in lysing bacteria

Virolysin acts on *S. typhi* peptidoglycan, which has been used as a substrate to form the framework of cell wall hydrolysis. The enzyme activity and concentration were performed by turbidimetric measurement of the lysis under standardized conditions. The ability of the extracted phages' enzymes to lyse the *S. typhi* isolates was detected using Benedict's reagent; each at 16 and 32 µg/ml of ciprofloxacin was determined by measuring its optical densities at 600_{nm} as shown in Table 3.

The *S. typhi* lysis rate was determined as the reduction of OD_{600nm} by the active enzyme in the absence of bacteriophage particles and incubation time of 12 to 4 h.

The lytic potentialities of the virolysins and their release in the phage lytic cycle suggested that they are responsible for bacteria cell wall degradation and peptidoglycan hydrolysis. The estimated mechanisms of enzyme lysing rates of bacteria were based on determinations of the reaction velocity constants V/min calculated from the equation (Equations 6 to 11):

$$V = \frac{\Delta E}{t} ln \frac{C_0}{C_0 - C_t} \quad (6)$$

where $C_{0>0}\ and\ C_0 - c_t \neq 0$; in which V = the velocity of the reaction, ΔE = distance displacement, C_0= the initial concentration of *S. typhi* ml^{-1} (10^6 cell/ml at OD_{600nm}), C_t=final concentration of *S. typhi* ml^{-1} lysed at any given time interval = t.

Virolysins exponential kinetics

Enzyme kinetics are usually described by Michaelis-Menten equation (Bezerra et al., 2016), where the time-dependent decrease of substrate ($-\frac{dx}{dt}$) is a hyperbolic function of maximal velocity (V_{max}). If x = t is time and y = f (t) is the displacement (function) of a moving object, then:

$$\frac{dy}{dx} = f\ (V_{max}) \quad (7)$$

where f_0 (t) is the velocity (function). Thus f_0 (t_0) = 0 means that the velocity at time t_0 is 0, that is, the object is stationary at that moment.

$$\frac{dy}{dx} = f\ [\frac{\Delta E}{t}\ ln \frac{C_0}{C_0 - C_t}] \quad (8)$$

The substrate is catalyzed and the time-dependent decrease ($-\frac{dx}{dt}$) is a function of the quantity of the complex (C) at time (t):

$$\frac{dy}{dx} = \frac{d[\frac{\Delta E}{t}\ ln \frac{C_0}{C_0 - C_t}]}{dx} = -\frac{dx}{dt} = \lambda x \quad (9)$$

Kinetics exponential decay rate

The rate of the exponential decay can be measured by the ''recession of reaction velocity constants'' V, which has dimensions of V/time.

$$V = (\frac{dy}{dt} = -\lambda x) \quad (10)$$

where $x = -\frac{ty}{\lambda}, d \neq 0\ and\ \lambda \neq 0$ and solved by

$$[Y_t = y_0 e^{-bx}] \quad (11)$$

Graphically, V is the slope of the recession hydrograph on exponential axes tending to (0) and t is its reciprocal that solves the exponential decay in 4 h (Figure 3). Lysing of *S. typhi* using lytic enzymes showed irreversible interaction in 4 h, the initial optical density of tested samples was ($OD_{600\ nm}$< 1.0) when λ > 0 and the b is between 0 and 1. Solving the data on the double reciprocal plot showed that bacteria are decaying when each time x is increased, y decreased exponentially as shown in Figure 4.

The virolysins kinetics data that fit the exponential decay rate of *S. typhi* was given by negative exponential decay equation ($Y = ye^{-bx}$), with negative integer.

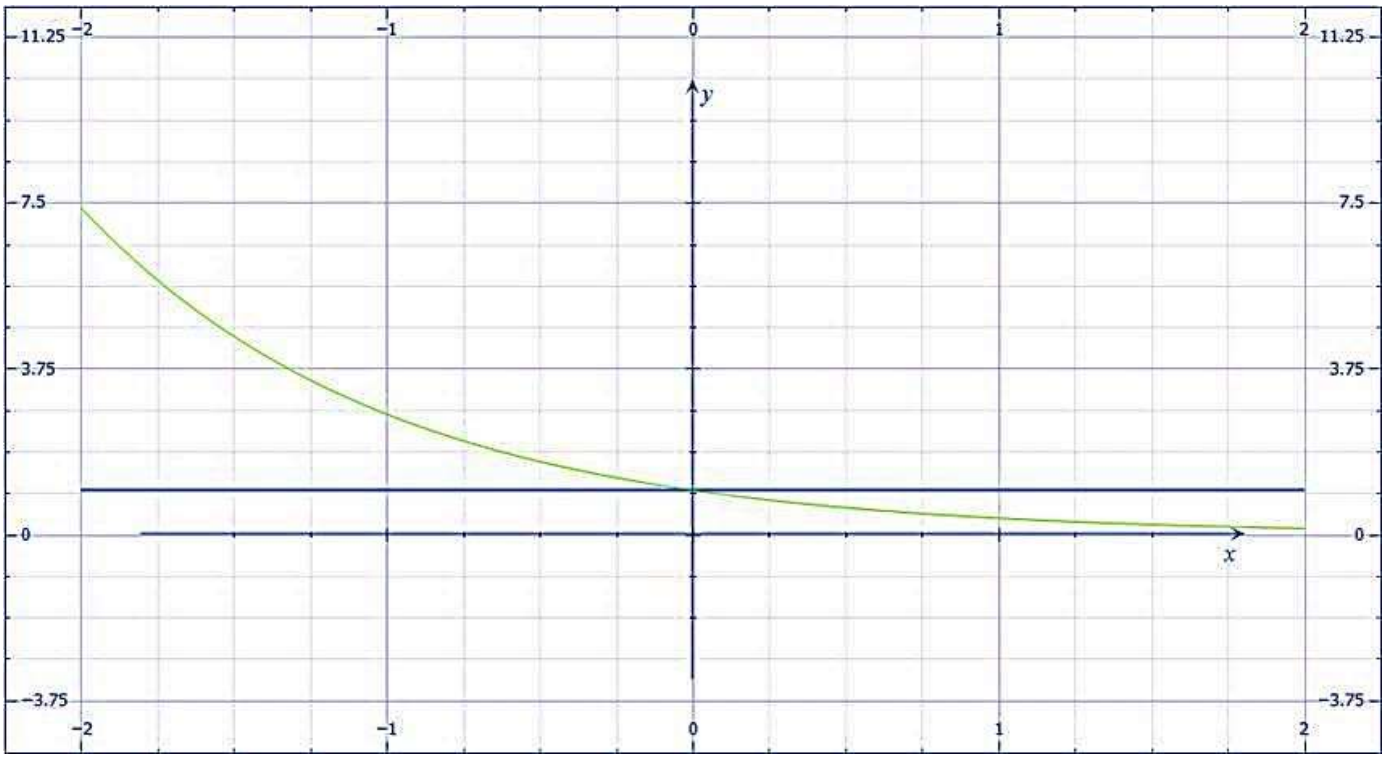

Figure 3. Virolysins kinetics exponential decay. Where V is the kinetics velocity, λ (lambda) is a positive rate termed the exponential decay constant, (Y_t) is the quantity at time t, and (y_0) is the initial absorbance = 1.0 at OD $_{600nm}$.

Table 4. The exponential decay rate of virolysins.

Sample	Exponential decay rate	coefficient
S. typhi 16	$y = 1.0262e^{-0.018x}$	$R^2 = 0.988$
S. typhi 32	$y = 1.0017e^{-0.001x}$	$R^2 = 0.954$
S.7 16	$y = 1.0192e^{-0.014x}$	$R^2 = 0.995$
S.7 32	$y = 1.0207e^{-0.014x}$	$R^2 = 0.988$
Dr11 16	$y = 1.0064e^{-0.005x}$	$R^2 = 0.997$
Dr11 32	$y = 1.0173e^{-0.014x}$	$R^2 = 0.999$
Sal C 16	$y = 1.0135e^{-0.01x}$	$R^2 = 0.991$
Sal C 32	$y = 1.0352e^{-0.025x}$	$R^2 = 0.997$

Coefficient $R^2 = 0.9$ is a very high value, and enzyme activity of the samples has a significant value ($P < 0.01$) which also indicates strong correlation of the reactions. If $b > 0$, we have exponential growth; if $b < 0$ exponential decay, b as actual rate can also be interpreted as the difference between an underlying growth rate and an underlying decay rate as shown in Table 4.

DISCUSSION

Enteric or typhoid fever is still a significant public health issue all over the world. It is a dangerous disease because of its long course and associated complications unless it is diagnosed early and treated (Effa et al., 2011). There are reports of changing clinical features in typhoid fever caused by multi-drug resistant *S. typhi.* This agrees with the susceptibility patterns of isolates' resistance that the present study revealed. However, drug resistance in typhoid fever is considered as one of the most important factors in the morbidity and mortality of the disease (Jain and Das Chugh, 2013). Therefore, bacteriophage therapy is one of the best antimicrobial alternative against resistant *S. typhi* due to its different mechanisms of action in lysing bacteria (Albino et al., 2014). The bacteriophages were specific, generally lysing

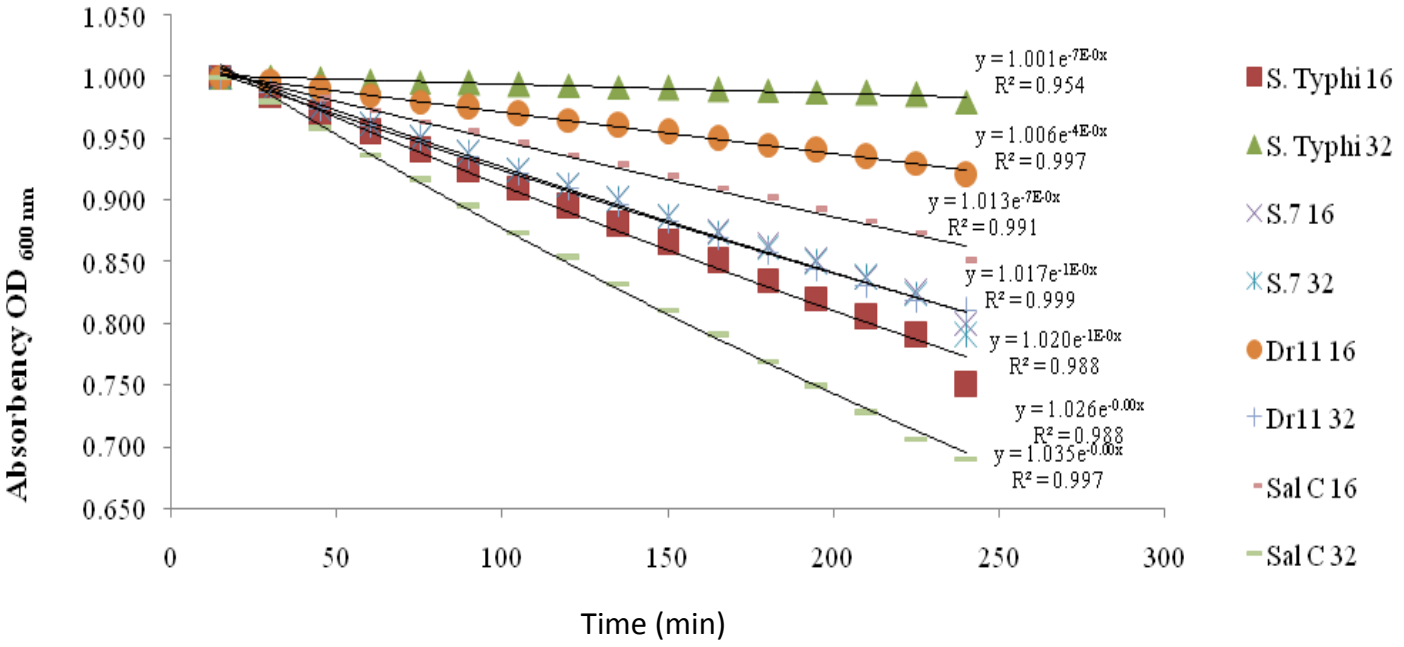

Figure 4. Virolysins kinetics exponential decay.

only the hosts on which they were isolated; the lysing rate and/or titeration kinetics are essential in bacteria decay measurements (Aviram and Rabinovitch, 2014). Consequently, it is required to isolate and characterize the infectious bacterium. Then, it can be matched with an effective lytic phage before treating using the multiplicity of infection [MOI] for bacteriophage amplification. This is in line with Chan and Abedon (2012) and Sillankorva et al. (2012), who stated that, the infection cycle utilized by a phage is an important phonemonon when choosing a phage for antibacterial application.

Another innovative approach of phage therapy is the utlization of phages' lytic enzymes namely virolysins instead of the whole virus, which can be generated via a targeted bacterium. Screening of phages-host interaction to increase lysis rates precisely (the kinetics exponential decay rate) with a functional lytic enzyme during phage infecting bacterial cells causes bacteriolysis and results in dead but intact bacterial cells (Filatova et al., 2015; Sun et al., 2015). Virolysins lysed cell wall peptidoglycan that yielded to primary amino sugars released and free intracellular components from hydrolyzed bacteria in the reaction qualitatively and quantitatively (Kadurugamuwa et al., 1998; Li et al., 2000). The capability of virolysins in lysing *S. typhi* can be induced in the absence of bacteriophage due to expression of functions from cell wall degraded by the enzyme (Clement et al., 1990; Sanz-Gaitero et al., 2013). Better results were obtained when phages and virolysins were mixed with bacterial cells in the solution because they adsorbed cell surfaces and cleaved to bonds, eventually causing lysis (Nelson et al., 2001; Busk and Lange, 2015).

Solving the kinetics equations by plotting data on sensorgram for bacteriophage decay and the double reciprocal for the enzyme activity support the *in vitro* reactions where lysis rate is constant meaning for exponentially distributed latent period of phage and bacterial cultures over incubation period (Precious and Barrett, 1993; Shinozaki-Kuwahara et al., 2001; Fong et al., 2002; Karthik et al., 2014). Lysing activity corresponding to an exponential descent in optical density (OD_{600}) was calculated to identify the area of each curve. The maximal determination coefficient (R^2) was calculated for an increasing sample size (n= number of measurements in time). This maximized R^2-value ensures the most reliable fit and hence the most reliable exponential regression. The slope of the curves was used to calculate the decrease in OD_{600} per unit of titer dilution at a certain time. The activity was calculated based on the amount of lytic enzymes added to a volume of 1 ml of cell suspension (Marova and Kovar, 1993; Cheng et al., 1994). The difference of exponential decay rate between bacteriophage and their extracted enzyme was mathematically approved by the occurrence of positive (e^{+bx}) and negative (e^{-bx}) exponent integers in the solved equations. This indicates multiplication and mortality of living organisms. The negative exponent integer described the population decay. It makes sense to define the exponential number raised to a negative integer exponent as decreasing of living bacteria and absence of

phages. Therefore, no resistant bacteria occurred after enzyme lysing and the susceptibility was 100% after 4 h of incubation period. As for antibiotics, bacteria have been reported to develop resistance against phage infection. A significant point with phage resistance is that it is associated with reduced bacterial virulence using enzybiotics; it is a great value to prevent phage resistance and have safe manipulation (Laanto et al., 2012).

Conclusion

Bacteriophage therapy and phage enzybiotics have been proven as effective agents for the elimination of a wide range of infectious bacteria including antibiotic resistant strains. Bacteriophage therapy showed lytic multiplication in their bacterial host. The lytic enzymes showed irreversible relationship of host decay in the absence of phage. Lytic enzymes are safe and time saving in the *in vitro* treatment of antibiotics resistant strains of *S. typhi.*

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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