Potentiality of Bacteriophage’s Virolysins in lysing Multi-Drug Resistant Salmonella Typhi

Enzybiotics against multidrug-resistant bacteria

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Phages are enteric viruses that can replicate only inside susceptible bacteria. They use the DNA, ribosomes, protein-synthesising factors, amino acids, and energy-generating systems of the host cell to replicate, and multiply only in metabolising host bacteria.

Phages are excellent sources of many enzymes and biochemical transactions that are broadly represented in all divisions of life.

They have evolved several structural and chemical adaptations, which allow them to survive and grow in different environments.

They are very important therapeutic resources against bacterial infections because they have adapted variables mechanisms.

The emergence of antibiotic-resistant bacteria have spread globally and has highlighted the need to explore the potential therapeutic applications of alternative therapies, such as the utilization of bacteriophages, or the use of phage-coded lytic enzymes.

A better understanding of the phage enzymology, is the availability of highly purified phage Virolysins which facilitate the development of these new therapies.
The overall objective of this study: is to isolate and identify bacteriophages’ Virolysins for the treatment of the Multi Drug resistant Salmonella enterica subsp. enterica serovar Typhi.
Objectives

Isolate and purify phages enzymes for

Isolation of Multi Drug resistant *Salmonella* Typhi from wastewater

Application of bacteriophages’ Virolysins against Multi Drug resistant *Salmonella* Typhi

Analysis and characterization of the bacteriophages Enzymes (Virolysins).

Developing Bacteriophage Therapy
Samples were collected from Soba stabilization station which located in Khartoum - Sudan.

A volume of 250 ml/sample from the crude sewage was taken to survey the phage and its bacterial host existence.

Laboratorial investigations for microorganisms were done according to (WHO) & (ISO standard 6579, 2002).

Antigens: Possess polysaccharide capsule (K antigen) on most *Salmonella* spp. or (Vi antigen) of *Salmonella* Typhi and *Salmonella* paratyphi.

Note: *Salmonella* Typhi is a non-gas producer and produces minimal H$_2$S

*Salmonella* spp. are relatively resistant to bile acids and this trait is utilized for selective isolation media.
Antimicrobial susceptibility: was evaluated according to the Kirby-Bauer disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI).

- The minimum inhibitory concentration (MIC)
- The minimum Bactericidal Concentration (MBC)
- MIC & MBC of resistant isolates to antibiotics were performed by broth dilution method that have been established by the Clinical and Laboratory Standards Institute (CLSI).
➢ **Phage head:** composed of coat protein and genome in the core

➢ **Genome:** DNA codes for enzymes and proteins necessary to replicate more viruses

➢ **Tail Sheath:** DNA travels from head to bacteria through sheath

➢ **Tail fiber:** helps anchor the phage on the cell membrane
➢ The mechanical action of the bacteriophage depends on their receptors that adsorb them to their bacterial hosts.

➢ Therefore, it is preferably to treat bacterial infections by Bacteriophage Therapy in case of antibiotic resistant bacteria occurrence.
Enzymes: are proteins that catalyze various chemical reactions which occur in living organisms.

Phages Enzymes include: lytic enzymes, lysozyme, endolysin, lysin, enzybiotics, muralysin, muramidase, virolysin and designations such as Ply, PAE and others.
➢ **Dialysis:** The enzymes were partially purified using dialysis tubing that possesses various molecular weights.

➢ **After dialysis:** ammonium sulfate was used to remove other proteins’ fractionations followed by chromatography.
Enzyme activity: influenced by pH, temperature, enzyme amount, substrate specificity, inhibitors, modulators, and product amounts.

Enzyme activity: determined by measuring Optical density of culture turbidity (OD$_{600nm}$)
Detection of enzyme activity

*Salmonella* Typhi were prepared and used to detect the activity of bacteriophages’ enzymes.

Enzymes were added to bacterial culture (5µg/ml) with a final proportion of 1:20 and incubated at 37°C for 4 hours.

The enzyme activity was determined by measuring the reduction of sugars released from lysed bacterial cells (substrate) using Benedict's reagent.
Changes in the turbidity of mixture were recorded and the lysis rate was expressed as a reduction in the turbidity.

The rate of the exponential decay was measured by the “recession of reaction velocity constants” \( V \), which has dimensions of \((\text{Velocity v/s time})\) and given by:

\[
V = \frac{dy}{dt} = -\lambda x
\]
From the isolated bacteria species, 128 were identified as *Salmonella Typhi*,

(33.0%) of the isolates were found to be multi-drug resistance.

MICs concentrations were interpreted at 16 and 32 µg/ml.

(67.0%) isolates that resist at least one antibiotic were subjected as (MBCs) and selected for phage treatment.

Figure 1: Antibiogram criteria for multi-drug resistant strains
The phage Multiplicity Of Infection (MOI = 50 phage/cell) was assessed for lysing initial amount of bacteria = (n x10^6 cell/ml).

The capacity of phage to lyse bacterial lawns and produce high numbers of pure plaques occurred when higher titers were selected.

Phages were harvested at various intervals time on S. Typhi lawn that significantly contributed to the phage amplification.

During phage lytic cycle (12 hours of incubation with bacteria), the releasing progeny phages were amplified from (nx10^8) up to (nx10^11) phage/ml; where n = numerical digit.
Clear plaques were observed on the *Salmonella* Typhi lawn.

Plaques were counted to confirm the phage’s specificity towards bacteria.

Figure 2: Phage plaque formation (Pfu/ml 10^n)
Results and Discussion

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

<table>
<thead>
<tr>
<th>Salmonella</th>
<th>Initial amount OD&lt;sub&gt;600nm = 1&lt;/sub&gt; cell/ml</th>
<th>Dilution</th>
<th>Plaques</th>
<th>PFU/ml</th>
<th>Dilution</th>
<th>Plaques</th>
<th>PFU/ml</th>
<th>Dilution</th>
<th>Plaques</th>
<th>PFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. Typhi 16</strong></td>
<td>1.6 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>62.0</td>
<td>6.2 x10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>42.0</td>
<td>4.2 x10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-10&lt;/sup&gt;</td>
<td>52.0</td>
<td>5.2 x10&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>S. Typhi 32</strong></td>
<td>2.8 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>78.0</td>
<td>7.8 x10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>80.0</td>
<td>9.0 x10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-10&lt;/sup&gt;</td>
<td>84.0</td>
<td>8.4 x10&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>S.7 16</strong></td>
<td>1.7 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>46.0</td>
<td>4.6 x10&lt;sup&gt;9&lt;/sup&gt;</td>
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<td>66.0</td>
<td>6.6 x10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-10&lt;/sup&gt;</td>
<td>94.0</td>
<td>9.4 x10&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>S.7 32</strong></td>
<td>1.1 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>58.0</td>
<td>5.8 x10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>64.0</td>
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<td>78.0</td>
<td>7.8 x10&lt;sup&gt;11&lt;/sup&gt;</td>
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<td><strong>Dr11 16</strong></td>
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<td>10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>90.0</td>
<td>9.0 x10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>88.0</td>
<td>8.8 x10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-10&lt;/sup&gt;</td>
<td>96.0</td>
<td>9.6 x10&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
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<td><strong>Dr11 32</strong></td>
<td>1.3 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>74.0</td>
<td>7.4 x10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>76.0</td>
<td>7.6 x10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-10&lt;/sup&gt;</td>
<td>58.0</td>
<td>5.8 x10&lt;sup&gt;11&lt;/sup&gt;</td>
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<td><strong>Sal C 16</strong></td>
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<td>10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>24.0</td>
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<td>10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>86.0</td>
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<td>10&lt;sup&gt;-10&lt;/sup&gt;</td>
<td>88.0</td>
<td>8.8 x10&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Sal C 32</strong></td>
<td>1.6 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>26.0</td>
<td>2.6 x10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>92.0</td>
<td>9.2 x10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>94.0</td>
<td>9.4 x10&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
To calculate the phages’ potentiality in lysing *Salmonella* Typhi per serial dilution; samples were titered and plotted.

Plotted titres were solved by exponential formula: \[ y = a \cdot 10^n e^{(bx)} \]
where; \( a \) = exponential constant, \( 10^n = \) titer, \( e \) = exponential number and \( (x) = \) plaque formation and/or isolate at certain point.

**Figure 3:** Phage plaque formation (Pfu/ml \( 10^n \))
The phage lysing rates of their host was determined by the time needed for bacteria to produce phages after infection and decreasing of optical density (OD$$_{600_{nm}}$$) per minute. For confirming the phage specificity toward the specific bacteria, the numbers of plaques / phages increased for each isolate by increasing their titration rates (Equations 1 – 5).

### The kinetic titration given by:

$$A + B \xrightleftharpoons{\text{ki}}^{\text{kd}} AB \quad (1)$$

$$\frac{dA}{dt} + \frac{dB}{dt} \xrightleftharpoons{\text{ki}}^{\text{kd}} \frac{d(AB)}{dt} \quad (2)$$

- [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$$_{600_{nm}}$$ = 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 calculation for bacteriophage exponential kinetics titration: Where R = replicated titer, t= total time of incubation and t$$^o$$ = initial time of incubation

$$\frac{dA}{dt} = -(k_i.A.B - k_d.AB) \quad (3) = \frac{d[AB]}{dt} = k_i.[A].[B] - k_d.[AB] \quad (4)$$

$$R = R^{la} + R^0 + R^1 + [drift(t-t^o)] \quad (5)$$
## Results and Discussion

### Table 2: Solving Equations of Bacteriophage kinetic titration in lysing bacterial host:

<table>
<thead>
<tr>
<th>Salmonella isolates OD&lt;sub&gt;600nm&lt;/sub&gt; = 1.0</th>
<th>Initial amount cell/ml</th>
<th>Bacteriophage 1&lt;sup&gt;st&lt;/sup&gt; infection</th>
<th>Bacteriophage 2&lt;sup&gt;nd&lt;/sup&gt; infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 hrs OD&lt;sub&gt;600 nm&lt;/sub&gt;</td>
<td>phage/ml</td>
<td>Salmonella cell/ml</td>
<td>12 hrs OD&lt;sub&gt;600 nm&lt;/sub&gt;</td>
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<td>E. coli ATCC25922</td>
<td>1.0 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.30</td>
<td>1.9 x10&lt;sup&gt;8&lt;/sup&gt;</td>
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<tr>
<td>S. Typhi 16</td>
<td>1.6 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.40</td>
<td>1.2 x10&lt;sup&gt;8&lt;/sup&gt;</td>
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<td>0.36</td>
<td>1.4 x10&lt;sup&gt;8&lt;/sup&gt;</td>
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<td>0.54</td>
<td>2.06 x10&lt;sup&gt;9&lt;/sup&gt;</td>
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<tr>
<td>Sal C 16</td>
<td>1.5 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.36</td>
<td>2.48 x10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sal C 32</td>
<td>1.6 x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.44</td>
<td>4.43 x10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Potentiality of released virolysins acts on bacterial peptidoglycan (substrate) forming the framework of cell-wall hydrolysis.

The enzyme activity and concentration performed by turbidimetric measurement under standardized conditions using Benedict's reagent at OD$_{600nm}$. 

Salmonella Typhi lysis rate was determined as the reduction of OD$_{600nm}$ by the active enzyme in absent of bacteriophages’ particles and incubation time reduction from 12 to 4 hours.
Virolysins were mixed with bacterial cells in broth cultures at OD$_{600\text{nm}}$ they were adsorbed to cell surfaces and cleave bonds, eventually causing lysis.

The precise quantitative lysing of *Salmonella* Typhi associated with the reaction of the enzyme and substrate was detected by Benedict's reagent.

It is possible to compute enzyme velocity/time

Figure 4: Detection of Virolysin activity by using Benedict's reagent
## Results and Discussion

### Table 3: Reducing absorbency of extracted virolysins OD/hour

<table>
<thead>
<tr>
<th>Tested Sample OD&lt;sub&gt;600nm&lt;/sub&gt; = 1.0</th>
<th>Virolysins decay/ hour</th>
<th>OD&lt;sub&gt;600nm&lt;/sub&gt; &lt;1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli ATCC25922</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. Typhi 16</td>
<td></td>
<td>0.95</td>
</tr>
<tr>
<td>S. Typhi 32</td>
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<td>0.75</td>
</tr>
<tr>
<td><strong>S.7 16</strong></td>
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<tr>
<td>S.7 32</td>
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<td>0.98</td>
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<td>Dr11 16</td>
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<td><strong>Sal C 16</strong></td>
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<tr>
<td>Sal C 32</td>
<td></td>
<td>0.81</td>
</tr>
<tr>
<td><strong>Sal C 32</strong></td>
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<td>0.69</td>
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The estimated mechanisms of enzyme lysing rates of bacteria based upon determinations of the reaction velocity constants $V/$min that calculated from the equation (Equations 6-11):

$$V = \frac{\Delta E}{t} \ln \frac{C_0}{C_0 - C_t}$$  \hspace{1cm} (6)

Where $C_0 > 0$ and $C_0 - c_t \neq 0$

In which; $V =$ the velocity of the reaction, $\Delta E =$ distance displacement, $C_0 =$ the initial concentration of *Salmonella Typhi* /ml ($10^6$ cell/ml at OD$_{600}$ nm), $C_t =$ final concentration of *Salmonella Typhi* /ml that lysed in any given time interval = $t$. 
Enzyme kinetics are usually described by the Michaelis-Menten equation (Bezerra et al. 2016), where the time-dependent decrease of substrate ($\frac{dx}{dt}$) is a hyperbolic function of maximal velocity ($V_{\text{max}}$). If $x = t$ is time and $y = f(t)$ is the displacement (function) of a moving object, then

\[ \frac{dy}{dx} = f(V_{\text{max}}) \]  \hspace{1cm} (7)

\[ = f_0(t) \] 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.

\[ \frac{dy}{dx} = f \left[ \frac{\Delta E}{t} \ln \frac{c_0}{c_0 - c_t} \right] \]  \hspace{1cm} (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)

\[ \frac{dy}{dx} = \frac{d[\frac{\Delta E}{t} \ln \frac{c_0}{c_0 - c_t}]}{dx} = - \frac{dx}{dt} = \lambda x \]  \hspace{1cm} (9)
The rate of the exponential decay can be measured by the “recession of reaction velocity constants” V, which has dimensions of V/time.

\[ V = \left( \frac{dy}{dt} = -\lambda x \right) \] (10)

Where \( x = -\frac{ty}{\lambda} \), \( d \neq 0 \) and \( \lambda \neq 0 \), and solved by;

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

Figure 5: Virolysins Kinetics exponential decay
Lysing of *Salmonella Typhi* using Virolysins showed irreversible interaction in 4 hours.

The initial optical density of tested samples was (OD$_{600nm}$ < 1.0). When $\lambda > 0$ 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.

*Figure 6: Virolysins Kinetics exponential decay*
The kinetics data that fit the exponential decay rate of phages’ enzyme was given by negative exponential decay equation \( Y = ye^{-bx} \), with negative integer.

If \( b > 0 \), it means 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.

Coefficient \( R^2 = 0.9 \) resembled very high value, and enzyme activity among samples has a significant value \( (P < 0.01) \) which also indicates strong correlation of the reactions.
### Results and Discussion

**Table 4: The exponential decay rate of virolysins**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Exponential decay rate</th>
<th>coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>( y = 1.063e^{-0.003x} )</td>
<td>( R^2 = 0.988 )</td>
</tr>
<tr>
<td>ATCC25922</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. Typhi 16</td>
<td>( y = 1.0262e^{-0.018x} )</td>
<td>( R^2 = 0.988 )</td>
</tr>
<tr>
<td>S. Typhi 32</td>
<td>( y = 1.0017e^{-0.001x} )</td>
<td>( R^2 = 0.954 )</td>
</tr>
<tr>
<td>S.7 16</td>
<td>( y = 1.0192e^{-0.014x} )</td>
<td>( R^2 = 0.995 )</td>
</tr>
<tr>
<td>S.7 32</td>
<td>( y = 1.0207e^{-0.014x} )</td>
<td>( R^2 = 0.988 )</td>
</tr>
<tr>
<td>Dr11 16</td>
<td>( y = 1.0064e^{-0.005x} )</td>
<td>( R^2 = 0.997 )</td>
</tr>
<tr>
<td>Dr11 32</td>
<td>( y = 1.0173e^{-0.014x} )</td>
<td>( R^2 = 0.999 )</td>
</tr>
<tr>
<td>Sal C 16</td>
<td>( y = 1.0135e^{-0.01x} )</td>
<td>( R^2 = 0.991 )</td>
</tr>
<tr>
<td>Sal C 32</td>
<td>( y = 1.0352e^{-0.025x} )</td>
<td>( R^2 = 0.997 )</td>
</tr>
</tbody>
</table>
Bacteriophage therapy and phage enzymo-therapy (enzybiotics) have been proven as effective agents for the elimination of a wide-ranging of infectious bacteria including antibiotic resistant strains.

- **Bacteriophage therapy:** showed lytic multiplication in their bacterial host.

- **Enzymo- therapy:** showed irreversible relationship of host decay in the absent of phage.

- **Virolysins:** are safe and time saving in the *in vitro* treatment for antibiotics resistant *Salmonella* Typhi.