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Study the pharmacokinetic of the *Staphylococcus aureus* specific bacteriophage in healthy animals

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Abstract
The objective of the study was to investigate the pharmacokinetic of the *Staphylococcus aureus* specific bacteriophage in healthy animals first time in Iraq. This research were conducted from march to July, 2017. The current study includes two experiments. The first one was isolation of bacteriophage. It was separated from sewage samples by double layer technique: the isolated phage was categorized by rounded, clear plaques extended between 2-3 mm in size. While the second main experiment was studying the pharmacokinetics properties of the phage. The main parameters measured were the mean log PFU/ml of bacteriophage in blood plasma of rabbits after intravenous administration (IV) of 3×10^8 pfu/ml was (10.40 ± 0.11) after (0.08 hr.). The phage concentrations decreased to (1.30 ± 0.23) after (7 hr.). The pharmacokinetic study showed that phage was rabidly absorbed through gut wall and highly distributed.

Keywords: Bacteriophage, *Staphylococcus aureus*, pharmacokinetics, rabbits

Introduction
Poor understanding of phage therapy kinetics remains a major problem. Some new efforts have been made to provide more data in this field. The kinetics of active phage therapy concerns the population dynamics of ecological predator-prey models and epidemiological host–parasite models. Phages, as self-replicating pharmaceuticals, substantially differ from other pharmacological agents. Very important studies of phage therapy kinetics were presented by [1]. Some very important questions still involve bacteriophage ability to penetrate higher organisms, as this determines the potential phage activity in antibacterial treatment [1]
Pharmacokinetics also is a description of a drug's ability to reach target tissues in sufficient densities to be effective while pharmacodynamics is a description of what a drug is capable of accomplishing, both positively and negatively, once those densities have been reached. It is traditional also to differentiate pharmacokinetics into what are known as absorption, distribution, metabolism, and excretion. These, respectively, represent drug uptake principally into the blood, drug movement to other body tissues (and particularly out of the blood), drug modification (usually but not exclusively towards inactivation) [2], and drug physical removal from the body.
These various pharmacological concepts require some modification to be fully applicable to phage therapy. First, movement into the blood is required only given systemic application and consequently often is not a goal with phage therapy, particularly of local infections. Second, movement for phages represents penetration to target bacteria and an important aspect of such penetration is into bacterial biofilms [3]. Third, "metabolism" for phages logically includes not just inactivation but also activation particularly of phage bactericidal activity [4]. *Staphylococcus aureus* was the most ordinarily confined pathogen for both acute and chronic osteomyelitis in all different ages of people (Jorge *et al*., 2010; Eid and Berbari, 2012). Lately there has been rise in methicillin-resistant *S. aureus* (MRSA) because of quick progression of antimicrobial resistance and development of virulent factors. Irrespective to the patient's health status (Chihara and Segreti, 2010). In a recent study, total 107 organisms were isolated from 100 samples of pus from chronic osteomyelitis cases, MRSA was detected in 14(40%) isolates of *S. aureus* (Mita *et al*., 2015). In this work we intended to present current knowledge in this area and point out the main possibilities that are the outcomes of bacteriophage treatment and study the kinetics of phage in healthy animals.

Material and Methods

Isolation and purification of *staph aureus* bacteriophage

Sample collection

The methods used adapted from USA EPA and Jaime, 2003 [5, 6]. 45 ml raw sewage was measured into graduated cylinder, the sample centrifuged at 3500 rpm for 15 min, the supernatant is filtered through 0.22 µm Millipore filter and transferred into Erlenmeyer flask and pipette 5 ml double sterile phosphate buffer saline and 5 ml *staph aureus* suspension (1.5 ×10^8 cfu/ml), then incubated at 37 °C for 24 hours.

Phage isolation

Isolation of phage and seeding by using agar overlay technique [7]. Sewage and *staph aureus* culture (isolated from patient suffering from bone infection) distributed into 8 centrifuge tubes and centrifuged the sample at 2000 RPM for 5 minute. Most of the remaining cells were pelleted. The supernatant contains bacteriophage pipetted into a Millipore filter (0.22 µm) and until all the liquid is pulled into the container, then 0.1 ml of filtrate and 0.1 ml of *S. aureus* added to 3 ml of top agar, then mixed and poured over a plate of bottom agar, the plate allowed to be harden, invert the plate and incubate at 37 °C for 24 hours and then phage plaque detected. Top and Bottom agars were prepared according to Sanders, 2012 [8].

Preparation, standardization of phage suspension stock and Storage of bacteriophage

When plaques were identified, a pure suspension is prepared by carefully removing a portion of the single phage plaque on bottom agar plate by stabbing the center of a plaque with a sterile needle. Rinse in 10 ml sterile TSB (Tryptic Soya Broth, Hi-media-India), the broth is transferred to a sterile 25 ml centrifuge tube and centrifuging at 5000 rpm for 5 min. The supernatant filter through 0.22 microfilter and then aseptically transferred to a sterile 15 ml tube and stored at 4°C till use [9, 7].

Measurement of phage titer PFU/ml by using phage plaque assay.

Ten-fold serial dilutions to 10^{-9} of phage filtrate made in PBS. 3 ml of hot, melted top agar into three 13x100 mm test tubes held in 47 ºC water bath. To each tube of 47 ºC top agar, 0.1 ml of *S. aureus* added and 0.1 ml of the 10^8 phage dilution quickly pipited into a tube of top agar containing host bacteria, mix well but gently to avoid bubbles and quickly pour the mixture onto the surface of bottom agar while turning the dish with the left hand, tap out the final drop, the plates rapidly tilted and distributed over the surface of the bottom layer, this procedure was repeated with others phage dilutions. The top agar allow to solidify and become firmly attached to the hard agar bottom, all the plates inverted and incubated for 24 hrs. The phage titer determined by counting the number of plaque forming units (p.f.u.) for each dilution. For determination (dilution factor) that used for all other experiments. Plate that had between 30 and 300 plaques were counted by applying the following formula [3]. Phage titer = number of plagues / dilution factor X plating amount

Pharmacokinetics study of bacteriophage

1. Thirty rabbits received 3 × 10^8 PFU/ml bacteriophage orally once time; blood sample was collected (parallel blood sampling) at different times 10min, 15min, 30 min, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr and 7 hrs at each time interval p.f.u. was calculated, three animals used for each withdrawal time. Modified method adapted from [10,11].
2. Thirty three rabbits received a single intravenous injection of 3 × 10^8 PFU/ml bacteriophage, blood sample was collected (parallel blood sampling) at different times 5min, 15min, 30min, 45min, 1, 2, 3, 4, 5, 6 and 7 hrs at each time interval (three animals for each time). The bacteriophage concentrations PFU/ml was determined by plaque assay [12, 13], table (1) showing the different times of blood drawing after oral and IV administration of bacteriophage.

Table 1: Shows the different times for blood collecting after oral and IV administration of bacteriophage

Intravenous administration (hr)	Oral administration (hr)
0.08 (5 minute)	0.16 (10 minute)
0.25 (15 minute)	0.25 (15 minute)
0.5 (30 minute)	0.5 (30 minute)
0.75 (45 minute)	
1	1
2	2
3	3
4	4
5	5
6	6
7	7

3. Ketamine 50 mg/kg B.W and Xylazine 4 mg/kg B.W. were given intramuscular to anesthetized animals [14]. Blood samples (4 ml) were obtained via cardiac puncture technique from each anesthetized animals using 5ml syringe from three rabbits of each group. Blood was collected in anticoagulant tubes (K2 EDTA) and mixed well then transferred to sterile tubes and centrifuged for fifteen min at 6000 rpm, and the plasma were collected in 2 ml Eppendorf tube. All tubes were labeled with time, date of blood drawing and route of administration and stored in freezer at – 80 ºC till use. Plasma from these samples was tittered for bacteriophage concentration (plaque-forming unit pfu/ml). At the same time Blood plasma was also collected from these rabbits before administration of phage to assure that animals used in these experiments were free of *staphylococcal* phage.

4. Standard curve drawing

To draw a standard curve for a bacteriophage, serial dilutions of phage stock solution was made (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}). Bacteriophage concentration (Log plaque-forming unit pfu/ml) was calculated using double layer technique. This standard curve was used to estimate the phage concentration in blood plasma. Figure (1)

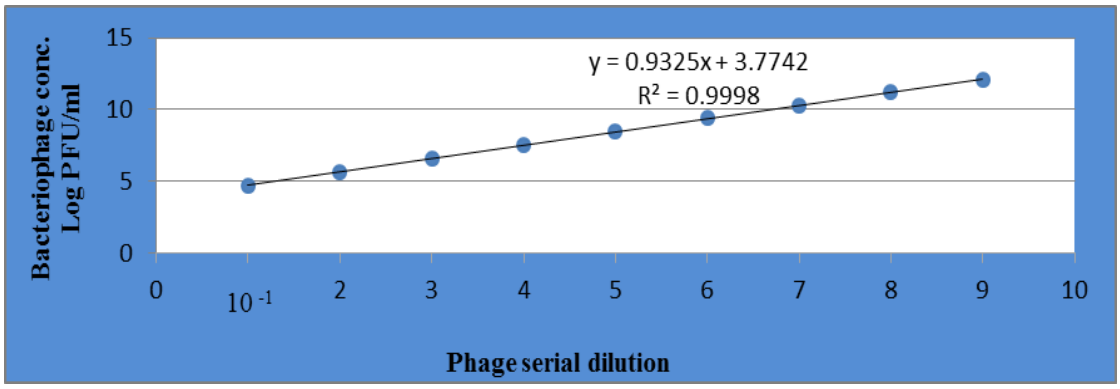

Fig 1: Standard curve for measurement of bacteriophage concentration in blood plasma

$$CP = Ae^{-\alpha t} + Be^{-\beta t}$$

$$\text{slope} = \frac{\sum x \sum y - n\sum xy}{(\sum x)^2 - n\sum x^2} =$$

$$CP^{o} = \frac{\sum x \sum xy - \sum x^2 \sum y}{(\sum x)^2 - n\sum x^2}$$

When the phage is given intravenously

a. Distribution phase slope α (hr^{-1})
   Elimination phase slope β (hr^{-1})
b. concentration of phage at zero time in α phase (A) PFU/ml.

concentration of phage at zero time in β phase (B) PFU/ml

c. biological half-life (hr)

$$t\,\frac{1}{2} = \frac{0.693}{\alpha \quad or \quad \beta}$$

d. Area Under Curve (AUC) PFU/ml.hr

Tow compartment

$$AUC = \frac{A}{\alpha} + \frac{B}{\beta}$$

e. Volume of phage distribution (Vd) L/Kg =

f. Clearance (CL) L/Kg/hr=Dose/AUC
j. Measurement of micro-constant
After determined the concentration of phage at zero time in Alpha and Beta phase, and α and β using the method of residuals, K21 and K12 were calculated from the equations:
K21=Aβ+Bα/A+B
Ke=αβ/K21
K12= α + β – K21 – Kel

2. When phage is given orally
a. Absorption phase slope Ka (hr^{-1})
Elimination phase slope Ke (hr^{-1})
b.Phage concentration at time zero in absorption phase (PFU^{a} / ml)
Phage concentration at time zero in elimination phase (PFU^{e}/ml)

$$t\,\frac{1}{2} = \frac{0.693}{Ke \quad or \quad Ka}$$

c. biological haf-life (hr)=
d. Area Under Curve (AUC) PFU/ml.hr using Trapezoidal rule.

$$\sum \frac{C_{n-1} + C_n}{2}(t_n - t_{n-1})$$

When n= blood withdrawal series, C= concentration, t= time

e. Bioavailability F % =$AUC_{oral}/AUC_{inj}\times 100$
f. Clearance (CL) L/Kg/hr = Dose×F/AUC
j. Apparent Volume of distribution = Dose×F/AUC×Ke

Statistical Analysis
Data were analyzed statistically using the Microsoft Program, SAS (Statistical Analysis System - version 9.1). Statistical analysis of data was performed on the basis of Two-Way Analysis of Variance (ANOVA) using a significant level of ($P<0.05$). post hoc test was performed to assess significant difference among means

Isolation and purification of *staphylococcus* aureus - specific bacteriophage
Phage isolation
Phage was able to lyse bacteria and form plaques after incubation 24 hr at 37 ºC. A piece of single plaque of phage on the bottom agar was picked by sterile needle. Put in broth for 3 hrs and re-infect the host to confirm obtaining phage.

Plaque morphology
Plaques were formed as clear circular with a size ranged between 2-3 mm in diameter when incubated at 37 C^{o} for 24 hrs,

Preparation of phage stock and titration

Over lay technique was used to prepared of *Staph aureus* phage stock solution and a titer between 10^1 to 10^9 PFU/ml was made.

Calculation of phage titer by using Series of dilutions was used to calculate the phage titer and the p.f.u. for each dilution summarized in the table below. Dilution factor that gave the best countable number of plaques is (10^5), this dilution factor was then used in the experiments and the pfu/ml was (3×10^8). Table (2).

Table 2: Determination of *S. aureus* phage titer.

Plate No	Plaque per plate	Titer calculation = Plaque× DF / Volume of phage plated (ml)	Titer Plaque forming unit
1	580	580×10^1 /0.1	5.8×10^4
2	540	540×10^2/0.1	5.4×10^5
3	430	430×10^3/0.1	4.3×10^6
4	400	400×10^4/0.1	4.0×10^7
5	300	300×10^5/0.1	3.0×10^8
6	250	250×10^6/0.1	2.5×10^9
7	210	210×10^7/0.1	2.1×10^{10}
8	180	180×10^8/0.1	1.8×10^{11}
9	120	120×10^9/0.1	1.2×10^{12}

Pharmacokinetics study of bacteriophage

Staphylococcus aureus – Bacteriophage kinetic appeared to be obeyed the first order kinetic, two compartment model. Results are showed in tables 3, 4, 5 and 6 and fig. 2and 3

Table 3: Bacteriophage concentration Log PFU/ml in blood plasma after intravenous administration

Time (hr)	Mean Log_{10} PFU/ml M±SE
0.08	10.40 ± 0.11
0.25	6.20 ± 0.15
0.5	3.60 ± 0.13
0.75	2.35 ± 0.12
1	2.20 ± 0.22
2	2.00 ± 0.40
3	1.85 ± 0.12
4	1.70 ± 0.11
5	1.56 ± 0.05
6	1.45 ± 0.02
7	1.30 ± 0.23

Table 4: Bacteriophage concentration Log PFU/ml in blood plasma after oral administration

Time (hr)	Mean Log_{10} PFU/ml M±SE
0.16	2.32 ± 0.17
0.25	3.40 ± 0.22
0.5	4.45 ± 0.13
1	6.10 ± 0.16
2	6.70 ± 0.41
3	6.10 ± 0.32
4	5.40 ± 0.20
5	4.85 ± 0.09
6	4.40 ± 0.12
7	3.80 ± 0.15

Table 5: Pharmacokinetic parameters for Staph- specific bacteriophage after IV. administration.

Pharmacokinetics parameters	Mean±SE
Distribution rate constant (α) hr^{-1}	6.24±0.23
Elimination rate constant (β) hr^{-1}	0.08±0.002
PFU/ml at α phase at time zero (A)	17.17±6.21
PFU/ml at β phase at time zero (B)	2.38±1.33
t 1/2 α hr	0.11±0.01
t 1/2 β hr	8.66±0.83
Volume of distribution Vd (L/kg)	3.25±0.41
Area under curve AUC (PFU/ml*hr)	32.5±3.63
Clearance CL (L/kg/hr)	0.26±0.14
K12	4.91±0.58
K21	0.82±0.37

Table 6: Pharmacokinetic parameters for Staph- specific bacteriophage after oral administration.

Pharmacokinetics parameters	Mean±SE
Absorption rate constant (Ka) hr^{-1}	1.43±0.15
Elimination rate constant (Ke) hr^{-1}	0.11±0.02
PFU/ml at absorption phase at time zero (PFU^a)	7.59±0.18
PFU/ml at elimination phase at time zero (PFU^e)	8.61±1.07
t 1/2a hr	0.48±0.12
t 1/2e hr	6.3±0.34
Volume of distribution Vd (L/kg)	2.36±0.13
Area under curve AUC (PFU/ml*hr)	17.32±4.02
Clearance CL (L/kg/hr)	0.26±0.02
Bioavailability F %	53.29±1.38

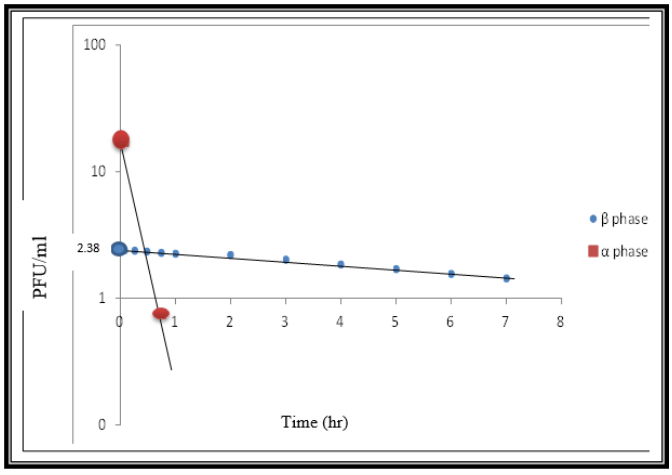

Fig 2: Semi-Log Plot of PFU/ml Versus Time (hr) Shows Residual Line and PFU/ml_{late} Line after IV administration.

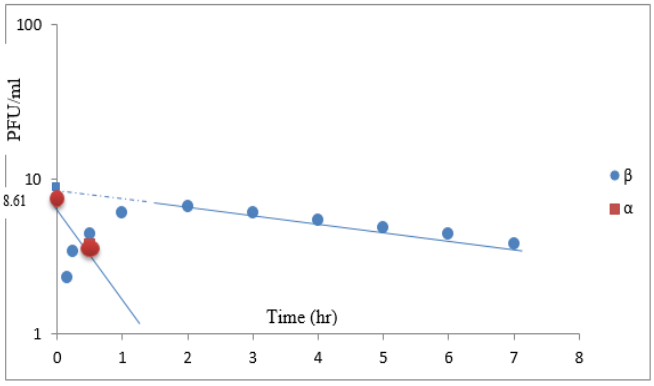

Fig 3: Semi-Log Plot of PFU/ml Versus Time (hr) Shows Residual Line and PFU/ml_{late} Line after oral administration.

Discussion

These various pharmacological concepts require some modification to be fully applicable to phage therapy. First, movement into the blood is required only given systemic application and consequently often is not a goal with phage therapy, particularly of local infections. Second, movement for phages represents penetration to target bacteria and an important aspect of such penetration is into bacterial biofilms [3]. Third, "metabolism" for phages logically includes not just inactivation but also activation particularly of phage bactericidal activity and also the often-associated *in situ* amplification of phage numbers, where the latter can be described as an "auto dosing" Auto dosing is not unique to phages but may be particularly effective for phages as antibacterial agents given that this amplification takes place in the immediate vicinity of target bacteria. Lastly and as is true for antimicrobial agents in general, the concept of "body" in pharmacology includes not only host tissues but also microorganisms, including target bacteria for antibacterial treatment [1].

Catherine *et al*., [5]. Supported the present work, they examined the pharmacokinetics of phage introduced via IM, SC or IP route in uninjured, uninfected animals. Three animals each from groups receiving (3×10^8/100 µl per inoculum phage cocktails) IP, IM, or SC were sacrificed at 0.5,12, 24, 36 and 48h. The numbers of phage detected per gm of liver and spleen and per ml of blood. Can phages also circulate in the human bloodstream? Literature addressing this fundamental question is very scarce. The novelty of the problem of phage translocation and distribution in the body can be illustrated by the number of records retrieved from PubMed discussed this issue [16]. If bacteria and viruses can translocate through the intestinal barrier, it could be expected that bacteriophages can also pass the gut wall. If phage translocation indeed takes place, this should lead to phage circulation in the peripheral blood ('phagemia') [17].

There are a number of researches have demonstrated the ability of some orally applied phages to be absorbed into the systemic circulation [18]. This phenomenon was reviewed lately by Gorski *et al*., [16]. Who concluded that some phages may not only reside within the gut lumen but also pass the intestinal wall in a process similar to bacterial translocation. Although the particular processes controlling the viral translocation remain obscure, it was suggested that phage passage is determined by a number of factors, including phage concentration, specific sequences within the phage capsid proteins interacting with enterocyte receptors, and phage interactions with gut immune cells.

In our study, in blood phage reached a high titer after the two hour following oral administration. In contrast, phage reached the highest titer after IV injection within the few minutes. This in agreement with Weber-Dabrowska, 1987 [19]. Oliveira *et al*., [18]. Supporting the present observations, phage was recovered from the GIT at least 2-3 h after the oral administration of 10^8 pfu/mL suspensions and find their way to the bloodstream.

Some researchers [20, 17, 16]. reported that orally administered phages can reach the peripheral blood and migrate to the infection sites. The phage occurrence in the blood is also supported by several authors [20, 17]. Penetration of phage to the blood circulation and urinary tract after oral administration was reported. Gastrointestinal administration for systemic phage delivery results in highly variable phage counts in the bloodstream, making the clinical importance of this administration route questionable [21]. While the physiological and immunobiological importance of phages circulating in the blood of otherwise healthy animals and humans presents an intriguing phenomenon which clearly requires further study and confirmation, they question of whether orally administered phages (e.g. for therapeutic purposes) can penetrate the intestinal wall is equally important, especially from a clinical perspective. The group of Keller & Engley [22]. have shown that Bacillus megathericum phages introduced into the gastrointestinal tracts of mice could be recovered as early as 5 min after gastric lavage or oral inoculation.

Hildebrand and Wolochow [23]. have shown that T1 coliphages instilled into the duodenum of rats can pass directly into lymph and then reach the peripheral blood. Further work of this group revealed that properties other than size alone appear to influence the capability of microorganisms (including phages) to translocate. The ratio of instilled to recovered phages became larger, with the numbers instilled subsequently reaching a plateau [24]. On the other hand, there was no correlation between recovery and the volume of instilled suspension. Hoffman [25] has shown that 15 min after oral administration in mice, T3 coliphages can be demonstrated in the majority of animals, with high fluctuations among individual mice. Phagemia is as high as after intramuscular injection, but increases more rapidly and peaks after 5 min (15 min after intramuscular administration).

Duerr *et al*., [26], used *in vivo* phage display to identify the sequences which may facilitate phage translocation through the intestine wall; however, enterocytes are able to recognize specific peptides displayed by engineered phages and transport them across the mucosal barrier, thus preserving their biological activities. In addition, a novel model of macromolecular transport has been suggested for phages bearing a specific peptide. These data indicate that at least some phages may translocate; however, their passage may also be regulated by possible phage ligands which can be recognized by intestinal cells responsible for their transport (enterocytes, M cells and dendritic cells) [27-29]. Very subtle changes in the protein structure of the phage capsid may evoke major changes in phage transport and bioavailability, a single specific substitution of glutamic acid to a lysine upgrades a phage's capacity to remain in the mouse circulatory system up to 16 000-fold [30]. This rule could also apply to phages crossing the gut barrier on their way to lymphatic and blood vessels.

Parenteral delivery of bacteriophages in experimental animal studies has proven to be one of the most popular and successful of all delivery methods for bacteriophages because of the immediate distribution of phages into the systemic circulation. and that IV administration is the most efficient method of delivering the phages throughout the body [19].

Intravenous (i.v.) injection of bacteriophages enables a fast and direct introduction of bacteriophages into the blood circulation and their spread throughout the system [31]. However, other ways of administration have proved to result in an equally effective penetration of bacteriophages into the blood. Intraperitoneal (i.p.) injections are very efficient in introducing phages into the circulatory system. In this case, bacteriophage titers usually reflect the initial dose and relative attenuation of the phage titer in the animal body. Therefore, the peritoneum-blood penetration appears to be very fast and facile. Indirect but important evidence of bacteriophage penetration to the blood is their ability to cure septicaemia in animals after i.p. injection [31, 10].

After administration, the level of bacteriophages decreased

rapidly in first 8 to 12 hours and then gradually decreased and eventually disappeared in three days, depicts a pattern just like of two-compartmental model. The initial phase of rapid decrease from 8 to 12 hours (considered as alpha-phase) is due to distribution of phages to organs and second phase (considered as betaphase) is due to elimination of phages It was determined that reticuloendothelial system is involved in elimination of bacteriophages from the human body [32]. but the potential net control of innate immunity on elimination of bacteriophages is not evident. The initially discovered bacteriophages that were studied by d'Hrelle were rapidly cleared and have shorter duration of action. Later studies made the researchers successful in making phages of longer duration of action that are long circulating mutant phages [30, 33].

Body impact on a drug's chemical structure, as a pharmacokinetic process, is described as metabolism. Metabolism for many drugs represents inactivation as mediated, for example, by liver enzymes. For a few drugs, however, these chemical changes result instead in increases in activity [34]. The metabolic impact of bacteria on drugs also can include drug inactivation, particularly as associated with bacterial resistance [35]. This bacterium-mediated inactivation is as one sees, for example, via the action of antipenicillin β-lactamase enzymes [36]. Immune responses, can have an equivalent impact on phage virions, though in the short term, antivirion immunity does not necessarily correspond to actual virion chemical modification so much as a physical blocking or sequestration of activity. Metabolism as displayed by a patient's actual body tissues, by contrast, tends to result solely in phage inactivation rather than activation [37].

Bacteriophage cleared by the spleen, liver and other filtering organs of the reticulo-endothelial system [10]. The persistence of bacteriophages in the spleen seems to be significant. It was observed for at least 12 days in rabbits [38]. It was also demonstrated in mice that bacteriophages were rapidly phagocytized by the liver Kupffer's cells and that this clearance pathway seemed to be the most important one. Bacteriophages applied intravenously to mice accumulated mainly in the liver (12 times more than in the spleen), but then the bacteriophage titer decreased much faster than in other organs [39].

Bacteriophages are also able to pass through the renal filter. The presence of bacteriophages in urine samples was observed in humans [21], mice [22] and rabbits [39]. Moreover, phage concentration in kidneys could be higher than their concentration in plasma Schultz and Neva demonstrated that phages could be observed in urine samples when phage doses passed the limit of 10^9 PFU/ mouse and phage plasma concentration was higher than 10^5 PFU ml^{-1}. The role of the kidneys in the clearance of bacteriophages was also demonstrated in fish: phages were detected in fish kidney even a month after a single administration [40]

Conclusion

Sewage water was observed to be the best environmental source to get lytic phages with aggressive infective qualities. Pharmacokinetics of active phage in healthy rabbits was studied here. The microbiological test like plaque assay method was a standard golden technique to enumeration of phage concentration (PFU) in blood plasma. And the pharmacokinetic study showed that phage was rabidly absorbed through gut wall and highly distributed.

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