

Bacteriophage Biocontrol Rescues Mice Bacteremic of Clinically Isolated Mastitis from Dairy Cows Associated with Methicillin-Resistant *Staphylococcus aureus*

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is among the most alarming pathogens affecting both humans and the global bovine industry. The current control measures in hospitals and on farms for MRSA have proven to be inadequate leaving a need for new rapid control methods to curb MRSA infections *in situ*. New control measures for bacterial infection are widely sought, with particular interest in the applications for bacteriophages (phages) as a biocontrol or therapeutic agent. The current study uses a wild highly lytic phage isolated from cow's milk taken from three farms in Baghdad, Iraq. The resulting phage was able to rescue 100% of the mice from a median lethal dose (LD₅₀) or (1 × 10⁸ CFU mL⁻¹ per mouse) for MRSA wild isolates achieved when the phage:bacteria ratio was 100:1. Even when treatment was delayed for 6 h post lethal infection, to the point where all mice were moribund, 80% of them were rescued by a single injection of this phage preparation. Based on the current results, a comprehensive study is needed to guide further research on the MRSA phage as a biocontrol for MRSA mastitis in dairy cows to replace or reduce the use of antibiotics in animal husbandry.

Keywords

Bacteriophages, Phage, Biocontrol, Methicillin-Resistant *Staphylococcus aureus*, MRSA, Mastitis, Antibiotics

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1. Introduction

Methicillin was introduced in 1959 to treat infections caused by penicillin-resistant pathogens and by 1961 in the United Kingdom and 1968 in the United States there were reports of *Staphylococcus aureus* first isolates that had acquired resistance to methicillin (methicillin-resistant *Staph. aureus*, MRSA) [1] [2]). MRSA remains a dangerous pathogen that is notorious as the prime cause of hospital-acquired (HA)-MRSA infections that are becoming increasingly difficult to combat because of emerging resistance to all current antibiotic classes [2] and represents an especially big threat to the public-health system outside of the hospital setting [3]. These community-associated (CA)-MRSA infections were first reported in the Northern USA, where they caused fatal infections in otherwise healthy children [4]. Since that time, CA-MRSA has become a global problem, with the most serious epidemic seen in the USA [5] [6] and was recently implicated in zoonotic pathogens reported worldwide [7]-[9]. It appears that the zoonotic transmission likely occurs through the same mechanisms as horizontal transfer in humans and animals. Veterinary personnel are prime candidates as a carrier of MRSA and facilitate the spread of MRSA to and within animal populations [7]. A newly described type of MRSA carried by farm animals, “Livestock Associated MRSA (LA-MRSA)”, is now causing infections in humans with and without direct livestock contact [8] and has been introduced to distinguish these strains from classical human HA-MRSA or CA-MRSA [9].

MRSA has become a prominent pathogen causing mastitis worldwide and incidence of MRSA infection is rising in dairy cows while most antibiotics are not effective against the bacterium. The rate at which *Staph. aureus* can develop or acquire resistance to new antibiotics is higher than the rate at which new antibiotics are discovered and developed. Accordingly, new control measures, rather than antibiotics, are needed to contain the increasing hazard, especially when it is known that antibiotics are also part of the problem [10]. For example, glycopeptides, cephalosporins and in particular quinolones are associated with an increased risk of colonization by MRSA. As a result, reducing the use of antibiotic classes which promote MRSA colonization, especially fluoroquinolones is recommended in current guidelines [11]. There is a need for safe, widely accepted and innovative measures for the control and therapy of MRSA since worldwide; an estimated 2 billion people carry some form of *Staph. aureus*; of these, up to 53 million (2.7% of carriers) are thought to carry MRSA [12].

One promising new proposal for the control of MRSA uses bacteriophages (phages). Phages are viruses that can attack bacteria including MRSA and can safely be applied to wounds, in a dressing or as a nose-spray, to eliminate the infection or carriage of MRSA [10]. The lytic cycle or “virulent phages” fit in the class of “natural antimicrobial controlling agents” and are arguably the most abundant biological entities on the planet while also considered safe for humans and animals [10]. Regulatory approval of the safety and efficacy of phages in human therapy, animal biocontrol (prophylaxis) and in nonclinical settings has been reported [10].

Lytic phages are being exploited in various areas of biotechnology, including rapid bacterial detection, biocontrol, food bio-processing and removal of bacterial biofilms [10] [13] [14]. This approach differs drastically from past efforts to kill the bacterial pathogens with antibiotics, which have proven counter-productive [10]. A study showed that phages can be used effectively to deliver a photosensitizer or light-activated antimicrobial agent, to a target MRSA resulting in enhanced and selective killing of the organism [15]. Such attributes are desirable in photodynamic therapy for infectious diseases.

In another study, a lytic phage (phage K) was assessed in vitro for its ability to lyse clinically isolated MRSA strains from hospitals and bovine infections with heterogeneous vancomycin resistance and teicoplanin-resistance [16]. In the same study, phage-enriched wash solution resulted in a 100-fold reduction in staphylococcal numbers on human skin in comparison with numbers remaining after washing in phage-free solution [16]. Recently, successful purification of a cloned lysin encoded by the novel *Staph. aureus* phage, revealed phi MR11, designated MV-L. It rapidly and completely lysed cells of a number of the *Staph. aureus* strains tested, including MRSA and VISA strains in growing conditions [17].

However, bacterial resistance against “lysogenic phages” remains a challenging problem [10]. Although bacteria can develop resistance to phages and their lysins, if bacteria become resistant to a phage, unlike antibiotics, a new phage matched to the new bacteria can be rapidly chosen or developed [10]. The bacterial resistance to phages can also be controlled by inhibiting the resistance development; this can be achieved by formulating a cocktail of phages or phage lysins used in MRSA control or treatment [10] [14]. In this regard, the current pace for isolating wild anti-MRSA phages can never be sufficient to cope with the resistance race. Therefore, an accelerated or divergent approach is needed to provide higher throughput of new lytic anti-MRSA phages in a rel-

atively short time. Therefore, novel approaches for reducing such antibiotic selective pressure on MRSA must be considered urgent.

2. Materials and Methods

2.1. Milk Samples

One hundred and fifteen milk samples were obtained from mastitic cows on farms of the College of Veterinary Medicine, Baghdad University at Abu-Ghraib, Al-Shulaa and Al-Dorah in Iraq during the period from November 2013 to March 2014. The udder was washed directly with tap water to remove visible dirt then dried with a clean towel, the teat was dipped in 1:1000 iodine solution and left to dry. Before samples were taken, one or two streams of milk were discarded. Samples of approximately 50 ml of cow's milk were collected aseptically in a sterile sample collection tube (100 ml) by trained technicians for quality assurance purposes then transported to the laboratory in a cooler and stored at 4°C for up to five days prior to testing.

2.2. Detection and Identification of Staphylococci

A questionnaire, designed for mastitis management was conducted on each farm. All milk samples were subjected to physical, chemical and bacteriological tests. The physical tests include the color, odor and consistency of milk. For the chemical test the California mastitis test was used for estimating the somatic cell count (SCC) according to [18]. All cows' quarters with SCC above 150,000 cells mL⁻¹ or 250,000 cells/ml, respectively, were sampled. Subsequently, milk samples from individual quarters with SCC \geq 200,000 cells mL⁻¹ were set up for culture.

The microbiological screenings were conducted as follows: All milk samples collected from dairy cows with clinical mastitis samples were centrifuged at 3000 rpm for 15 min, and the precipitate was cultured on blood agar (Oxoid, Basingstoke, UK) and NA, and then incubated at 37°C for 24 h. Diagnosis of *Staph. aureus* depends on morphological character (shape, color and size of colony) and haemolytic pattern of *Staphylococcus* were observed on blood agar, colonies with hemolysis recultured on mannitol salt agar (Oxoid, Basingstoke, UK) as a selective medium for *Staph. aureus* then incubated at 37°C for 24 h. The diagnosed *Staph. aureus* were then subjected to a series of selective and differential media to identify only MRSA bacteria which are the target bacteria of this study. Selective colonies were inoculated in 9 ml of Mueller-Hinton broth (BioRad, Hercules, USA) supplemented with 6.5% NaCl. After incubation at 37°C for 20 h, 1 ml samples were screened for methicillin-resistance using cefoxitin agar-diffusion. Cefoxitin-resistant *Staph. aureus* isolates were tested for methicillin-resistance and identification of *Staph. aureus*, respectively [19]. The suspected colonies from all types of media were then inoculated onto blood agar (Oxoid, Basingstoke, UK), cultivated at 37°C for 24 h and subsequently identified biochemically using the API Staph system (bioMérieux, France) with 99% confirmatory rate, 9 isolates of *Staph. aureus* were identify as MRSA and termed S1, S2, S3, S4, S5, S6, S7, S8 and S9.

2.3. Isolation of Wild Phages against MRSA

Phages for MRSA were attempted to be isolated from 3 different cow farms. Approximately 50 ml of cow's milk from each was collected in a sterile sample collection tube (100 ml). Then 15 ml of the milk sample was centrifuged at 3500 rpm for 15 min, the supernatant was filtered through 0.22 μ m millipore filter, the filtrate was assayed for plaque using the double agar layer technique. Ten millilitres of each supernatant was transferred into 80 ml of nutrient broth (NB; Hi-media, India) and subjected to vortex for 30 sec. Then 1ml of 8 h of each NB culture of the 9 MRSA mastitis clinical isolates were added and incubated at 37°C. After 18 h, 10 ml of the mixture was withdrawn into a sterile 15 ml test tube and centrifuged for 5 min at 5000 \times g at room temperature. Supernatant was aspirated into new sterile 15 ml test tubes. To the supernatant, 1 ml of chloroform (Sigma, USA) was added with gentle shaking of tubes for 5 min then all tubes were incubated on crushed ice for 5 min. A milky solution appears due to bacterial proteins digestion by chloroform. Next centrifugation at 5000 \times g for 5 min at room temperature was carried-out. The top aqueous supernatant was collected into 15 ml sterile tubes and stored at 4°C as a possible phage solution.

2.4. Testing for the Presence of Wild Phages (Phage Spot Lysis Test)

Thin bacterial lawns of MRSA mastitis clinical isolates were prepared by adding 500 μ l of each 9 MRSA iso-

lates NB 18 h cultures on 9 nutrient agar (NA; Hi-media, India) plates, allowing the liquid to soak into the plate. Such cells are healthy and grow rapidly; therefore to prevent bacterial growth and excessive thickening of the bacterial lawn, the plate was used within 1h at room temperature. Transferred 10 μ l of the possible phage solution onto each 9 bacterial lawns and then incubated at 37°C where plaques or lysis spot were observed after 6 h and 18 h. The detection of phage was based on visual appearance of a lysis zone at the site where a 10 μ l solution was added to the surface of the lawn. Positive results were expressed by either a clear or semi-clear (turbid) lysis zone while negative results were expressed by the absence of such lysis zones.

2.5. Increase Phage Infection Capacity

A series of optimization steps have been introduced in order to augment the efficacy and art of phage hunting/isolation techniques. These optimization manoeuvres were taken into account:

- 1) Lysis zones, if any, were cut by a sterile scalpel and plunged into 1 ml of Lambda (λ -) buffer in 1.5 ml sterile microcentrifuge tubes for 20 min with intermittent gentle shaking.
- 2) The crude phage sample collection was diversified in a way that 1 ml of a minimum of 10 different samples was used to form the crude mixture.
- 3) Samples of crude phage mixture representing 10 ml were placed in 100 ml Erlenmeyer flask with cotton-plug. Then 80 ml of NB were added and the mixture was inoculated with a total of 10 ml of 18 h cultures of 9 MRSA mastitis clinical isolates.
- 4) After 18 h, standing incubation at 37°C, a sample of 10 ml was dispensed into a sterile 15-mL plastic culture tube.
- 5) After centrifugation at 5000 \times g for 5 min at room temperature, the supernatant was transferred into 1.5 ml sterile microcentrifuge. Then 1:10 chloroform to lysate ratio was added with gentle shaking for 5 min at room temperature in order to lyse the bacterial cells, followed by a further 3 min incubation in crushed ice, the mixture was centrifuged at 5000 \times g for 15 min at room temperature and the supernatant was transferred into a 1.5 ml sterile microcentrifuge tube which became the isolated phages mixture.
- 6) Then, the produced mixture of the isolated phages was propagated on the desired target bacterial lawn, as mentioned earlier in the procedure “phage spot lysis test”.
- 7) Hence using this novel approach, an ever increasing number of crude mixture-purified and isolated multi-phages was obtained.

2.6. Production of the Transient Phage Stock

The resultant mixture of isolated phages was propagated on each target bacterial lawn as mentioned earlier in the “phage spot lysis test” where phages were propagated from their own lysis zones on the bacterial lawns. Lysis zones, if any, were cut by a sterile scalpel and plunged into 300 μ l of λ -buffer in 1.5 ml sterile microcentrifuge tubes for 20 min with intermittent gentle shaking. Chloroform to lysate ratio of 1:10 was added with gentle shaking for 5 min at room temperature in order to elute the phages from the agar and to lyse the bacterial cells. After further 3 min incubation in crushed ice, the mixture was centrifuged at 5000 \times g for 15 min at room temperature and the supernatant transferred into a 1.5 ml sterile microcentrifuge tube.

2.7. Optimization of the Phages Lytic Characteristics

The isolates of wild lytic phages from the transient stocks were propagated with the corresponding host clinical MRSA isolates using the plate method as follows: Tenfold serial dilution (10^{-1} to 10^{-6}) were made with λ -buffer for the phage stock solutions by taking 100 μ l of the phage solution into 900 μ l of λ -buffer. Next transferred 100 μ l of each dilution for each phage stock solution into 15 ml volume sterile plastic container with 100 μ l of 10^9 colony-forming units (CFU) mL^{-1} of 18 h NB culture of targeted bacteria and incubated at 37°C. After 10 min incubation, added 2.5 ml of top layer agar cooled to 45°C and poured over NA plates.

Plates were incubated overnight at 37°C and plaque morphology and growth characteristics were recorded according to the following parameters:

- 1) Diameter (mm) of the plaque
- 2) Shape of the plaque
- 3) Depth of the plaque

- 4) Margin cut
- 5) Clarity or turbidity of the plaque
- 6) Plaque visible time

By conducting a thorough examination of the plaques, it was found that very few of the tens or hundreds of plaques per single plate showed larger diameters and clearer lysis than the average. The slightly larger plaques proved to be an excellent indicator for the optimization of the phages lytic characteristics in this study. Accordingly, the best 3 - 5 well-defined, clear and largest plaques were selected at each run and used according to the above phages purification and propagation program. This was repeated for 8 - 10 times in order to magnify the outcome of the biased selection of the large and clear plaques until obtaining the ever-largest and the ever-clear 3 - 5 plaques, which reached to the limits of plaque-based optimization, reflecting the best possible natural enhancement of the lytic characteristics of the phages.

2.8. Preparation of Phage Stocks

The phage stocks were developed to high titre on an appropriate host strain by the plate lysis procedure, essentially equivalent to growing bacteriophage λ -derived vectors [20] [21] using the soft layer plaque technique as follows: An aliquot (100 μ l) of the phage sample (10-fold serially diluted with λ -buffer) was mixed with 100 μ l of an overnight NB culture of MRSA clinical isolates in a sterile Eppendorf micro-centrifuge tube (polypropylene; 1.5 ml; Sarstedt) and incubated for 10 min at 37°C to facilitate attachment of the phage to the host cells. The mixture was transferred from the Eppendorf micro-centrifuge tube to a 5 ml Bijou bottle and then 2.3 ml of "soft agar" was added (NB prepared in λ -buffer and supplemented with 0.4% w/v agar bacteriology No. 1 Oxoid which had been melted and cooled to 40°C in a water bath). The contents of each bottle were then well mixed by swirling, poured over the surface of a plate of NA and allowed to set for 15 min at room temperature. The plates were incubated for 18 h at 37°C, and a plate showing almost confluent plaques was used to prepare a concentrated phage suspension by overlaying with 5 ml of λ -buffer titre 10^{10-12} plaque-forming units (PFU) mL^{-1} . The final purification process used 1:10 chloroform to lysate ratio to separate the bacteriophage from the bacterial cells. The phage stocks were maintained in λ -buffer at 4°C.

2.9. Groups of Tested Animals

Female white BALB/c mice aged about 10-12 weeks weighing 30 - 35 grams were randomly divided into five groups of 5 animals and were housed in an animal facility and maintained throughout under standard conditions: 22°C \pm 2°C, 50% \pm 10% relative humidity and 12 hour light/12 hour dark cycle. The mice were fed a standard diet and watered.

2.10. Determination the Median Lethal Dose (LD₅₀) for MRSA

From all 9 MRSA wild clinical mastitis isolates strains S9 strain was selected for establishing LD₅₀ MRSA of BALB/c mice. Preparation of the infecting MRSA was as follows. MRSA clinical isolates mastitis was grown in NB at 37°C. Log-phase cultures of S9 was grown to an optical density at 600 nm of 1.1 (equivalent to 1×10^9 CFU mL^{-1}), followed by centrifugation and re-suspension in sterile phosphate-buffered saline (PBS) at 4°C. This log-phase preparation of bacteria was serially diluted in PBS for use in animal infections. To determine the LD₅₀, serial dilutions of the bacteria were injected intraperitoneally (i.p.) into mice in 400 μ l aliquots. The animals were observed for 100 h. and body weights were recorded pre-test and prior to termination. The animals were observed once daily for toxicity and pharmacological effects, and twice daily for morbidity and mortality. Food consumption was calculated at the end of the study.

On day 4, all animals were anesthetized with ether, sacrificed, and exsanguinated. All animals were examined for gross pathology. The kidney, liver, heart, lung and spleen were preserved in neutral buffered formalin (NBF) prepared from 10% (vol/vol) buffered formalin saline (BDH, England) embedded in paraffin wax [22]. Histopathologic preparation (cross-sections and longitudinal sections) and microscopical analysis were performed according to standardized procedures. All results were evaluated based on the relationship between the dose levels and incidence or severity of responses (if any).

While the experiments were not conducted in a double-blind manner, all animals were evaluated by two or more independent observers. These experiments established that 10^8 CFU mL^{-1} of strain S9 MRSA is the LD₅₀

for 6 weeks-old BALB/c mice, with all mice that received the LD₅₀ dying within 48 h. The inoculum size of approximately 1×10^8 CFU mL⁻¹ per mouse was chosen to produce a robust and consistent infection.

2.11. Mouse Septicemia Model

To study the comparative efficacies of phage in the mouse septicemia model, mice were infected i.p. with dairy farm acquired MRSA wild isolates (approximately 1×10^8 CFU mL⁻¹ per mouse, the LD₅₀) and then treated i.p. immediately or 6 h post-infection, and mortality was monitored for 3 days. The dosing regimen (phage titer PFU) was determined from pilot studies conducted in the mouse septicemia model (data not shown). The 50% phage effective dose (PED₅₀) and the 100% phage effective dose (PED₁₀₀), the doses at which 50% and 100% survival of animal under treatment and histologic analysis were observed, respectively.

2.12. Treatment of Mice with MRSA Infection with Wild Isolate of MRSA Phage

The efficacy of phage therapy was evaluated using the MRSA mastitis wild clinical isolates infection mouse model in order to evaluate the PED₅₀ and PED₁₀₀ on the ability of phage to rescue mice from MRSA infection. Six groups of mice (five mice in each) were used in these experiments as follows:

Group 1 were challenged by i.p. injection of the LD₅₀ (10^8 CFU mL⁻¹) of MRSA and were treated immediately “0-h” with a single injection of wild phage, administered i.p. into mice in 400 µl aliquots with titre (10^8 PFU).

Group 2 were challenged by i.p. injection of the LD₅₀ (10^8 CFU mL⁻¹) of MRSA and were treated immediately “0-h” with a single injection of wild phage, administered i.p. into mice in 400 µl aliquots with titre (10^{10} PFU).

Group 3 were challenged by i.p. injection of the LD₅₀ (10^8 CFU mL⁻¹) of MRSA and were treated after 6 h with a single injection of wild phage, administered i.p. into mice in 400 µl aliquots with titre (10^{10} PFU).

Group 4 were challenged by i.p. injection of the LD₅₀ (10^8 CFU mL⁻¹) of MRSA and immediately “0-h” after the bacterial inoculation, 400 µl of PBS was injected i.p. as a control.

Group 5 mice in this group were not challenged with bacteria, receiving only the injection of phage dose of 10^{10} PFU mL⁻¹ as a control.

Group 6 were challenged by i.p. injection of mice with 400 µl PBS alone to serve as an additional control.

On day 4, all animals were anesthetized with ether, sacrificed, and exsanguinated. All animals were examined for gross pathology. The kidney, liver, heart, lung and spleen were preserved in NBF. Histopathologic preparation (cross-sections and longitudinal sections) and microscopical analysis were performed according to standardized procedures. All results were evaluated based on the relationship between the phage dose levels and rescue mice from MRSA infection.

2.13. Histologic Analysis

All histopathologic features of a subset of the infected mice were examined to determine whether phage therapy affected microscopic damage. Tissues of subcutaneous (s.c.) lesions were collected immediately after euthanasia by inhalation of chloroform (Mommert, Germany) and postmortem examinations were completed. Skin tissue was sampled and processed according to the following procedures. All skin tissue samples were dissected to an equal size of 1×1 cm square. Each piece of tissue was immediately fixed in NBF. Subsequently, all the pieces were separately labeled and processed together, encased in a Shandon Elliot Histokinette tissue processor (Shandon Southern Products Ltd, Runcorn, UK). Sets of histological sections were mounted, and stained with Harris's hematoxylin (Thermo-Shandon, UK) and Eosin stain (Panreac Quimica, Spain) (H & E) as described previously [23]. The histological evaluation was performed using an Olympus BX 40 light microscope and a digital color video camera.

2.14. Scanning Electron Microscopy (SEM)

This was conducted from the phage stocks maintained in λ -buffer at 4°C for several months. Using previously described procedures [24]. The preparation was then spread on an electron microscope grid and negatively stained with uranyl acetate.

3. Results

3.1. Isolation and Characterization of MRSA

The API Staph system was used to identify MRSA strains from 30 isolates of *Staph. aureus* where 99% confirmatory diagnosis were obtained from 115 milk samples. Only 9 isolates termed S1 to S9 out of 30 of *Staph. aureus* were shown to be MRSA mastitis bacteria after applying consecutive selective and differential media and they were highly resistant to antibiotics ampicillin 10 mg, cefoxitin 30 mg, methicilline 5 mg and penicillin 10 mg.

All 9 MRSA clinical mastitis strains were chosen for phage isolation whilst for phage treatment, mice with clinical mastitis infection with the strain S9 were chosen to study MRSA infection.

3.2. Isolation, Characterization and Morphology of Phage

Only one phage was isolated from 115 milk samples and the SEM has revealed (data not shown; as observed by negative staining with uranyl acetate) the phage has a moderately elongated head, 97.2 nm long and 81.3 nm wide, and a contractile tail (201.1 nm long) and it was termed phage SA9. The phage SA9 was found highly lytic and forming clear plaques on host bacterial lawn of all 9 MRSA isolate of clinical mastitis on NA plates incubated overnight at 37°C where plaque morphology and growth characteristics were recorded according to the parameters described above.

3.3. Ability of the Phage Preparation to Rescue Mice from MRSA Infection

To determine whether phage SP9 could protect against MRSA infection, mice were injected i.p. with 10^8 CFU mL^{-1} of S9, and immediately, unless otherwise stated, injected i.v. with 10^8 PFU of phage where control mice were treated with phage SP9 or PBS as follow:

The Group 1, a single dose (10^8 PFU mL^{-1}) of phage 400 μl was administered i.p. immediately “0-h” after the challenged with the LD_{50} (10^8 CFU mL^{-1}) of S9 MRSA wild isolate. The ratio of phage:bacteria was 1:1. Of the 5 mice, 2 mice survived and 3 died on day 3 giving a survival rate of 40%.

The histopathological section from the kidney of two protected mice shows no clear lesions (**Figure 1**). The liver section revealed proliferation of kupffer cells with congested central veins, in the other section, the liver showed mononuclear cell aggregation around bile ducts and portal blood vessels (**Figure 2**). The phage-treated mice have a significantly protected heart, in which there are no clear lesions (**Figure 3**). However, the lung section expressed some mononuclear cell aggregation in the interstitial tissue (**Figure 4**), in addition moderate proliferation of lymphocytes in the periarteriolar sheath of the spleen (**Figure 5**).

At higher doses, treatment with phage SA9 10^{10} PFU mL^{-1} (Group 2), representing the ratio of phage:bacteria 100:1 achieved 100% survival of the animals. The histopathological change in the kidney shows mononuclear cell aggregation around blood vessels with moderate cellular degeneration of epithelial cells on renal tubules

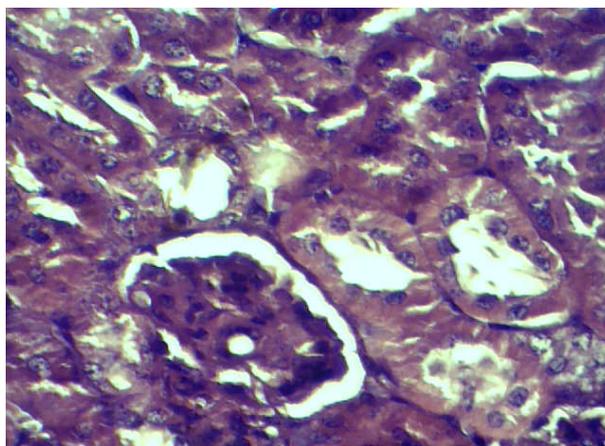


Figure 1. Section of the kidney of mice at 3 days post infection and treatment with same concentration of phage shows no clear lesions (H & E, $\times 400$).

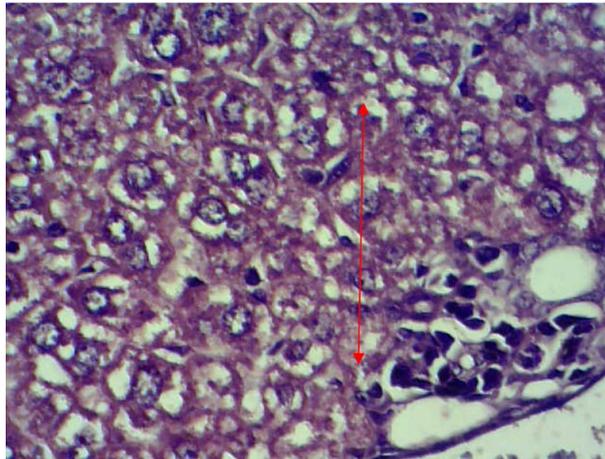


Figure 2. Section of the liver of mice at 3 days post infection shows mononuclear cell aggregation around bile ducts and portal blood vessels with vacuolar degeneration of hepatocytes (H & E, $\times 400$).

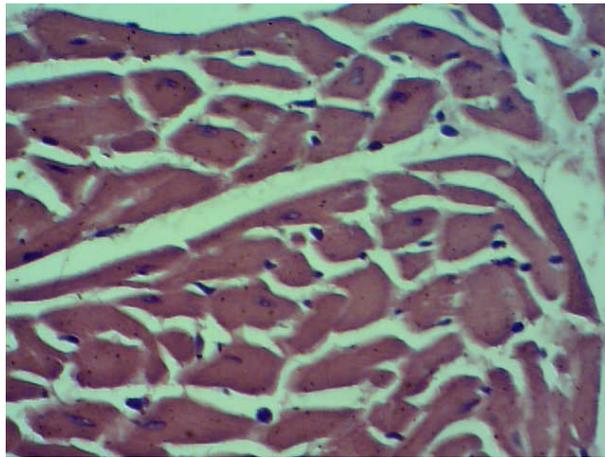


Figure 3. Section of the heart of mice at 3 days post infection shows no clear lesions (H & E, $\times 400$).

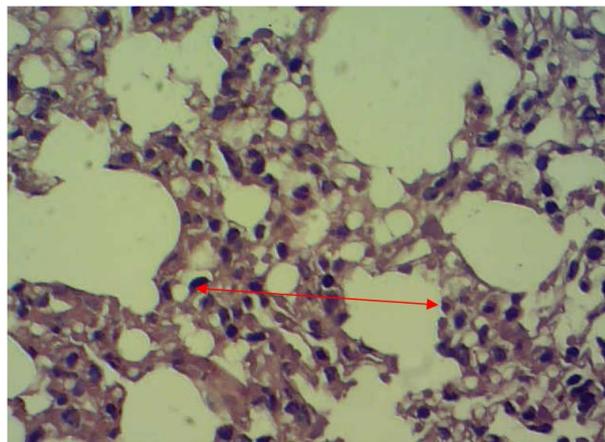


Figure 4. Section of the lung of mice at 3 days post infection and treatment with equal concentration of phage shows some mononuclear cell aggregation in the interstitial tissue (H & E, $\times 400$).

(**Figure 6**). The liver showed aggregation of mononuclear cells in the parenchyma (**Figure 7**), and in other animals, no clear lesions were seen in the liver except moderated fatty changes in the portal zone (**Figure 8**), as

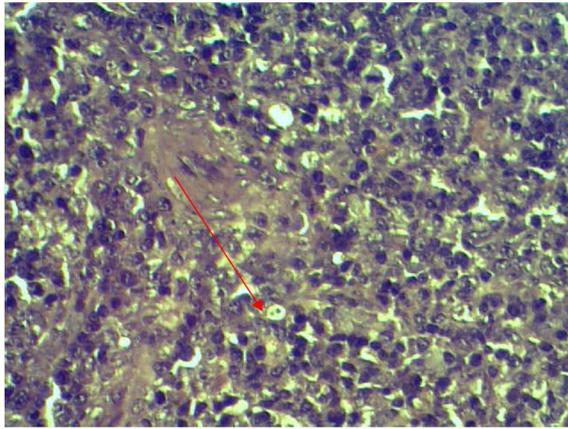


Figure 5. Section of the spleen of mice at 3 days post infection and treatment with equal concentration of phage shows moderate proliferation of lymphocytes in the periarteriolar sheath (H & E, $\times 400$).

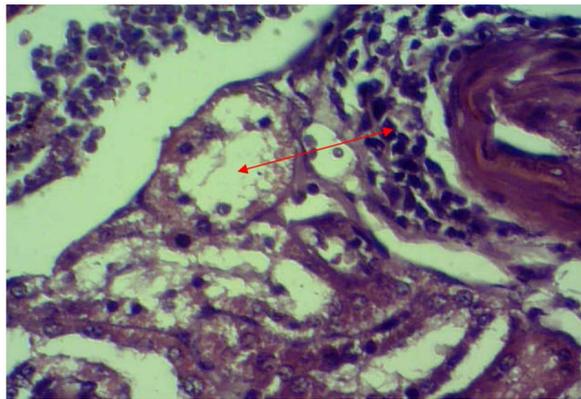


Figure 6. Section of the kidney of infected mice post treatment with phage shows mononuclear cell aggregation around blood vessels and marked vacuolar degeneration of epithelial cells of renal tubules (H & E, $\times 400$).

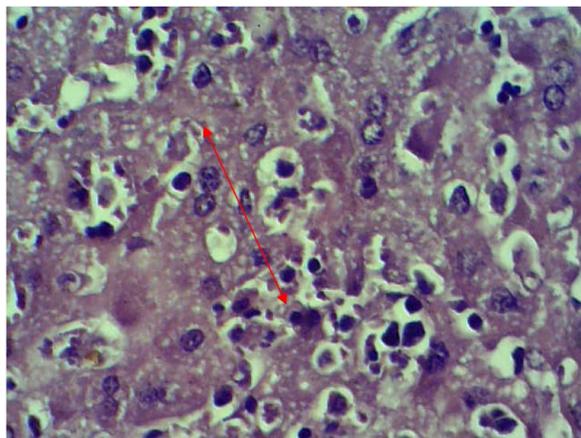


Figure 7. Section of the liver of mice shows necrosis of hepatocytes with inflammatory cells in lumen of dilated sinusoids (H & E, $\times 400$).

well as, hyperplasia of white pulp of the spleen (**Figure 9**) and no clear lesions in the heart and lung.

Using a similar ratio of phage: bacteria at 100:1, the rescue by the phage injection was delayed 6 h after the bacterial inoculation (Group 3) where the only signs of illness were seen (in the first 24 h) where the animal died on day 4. When the phage treatment was delayed for 6 hours after infection with MRSA, survival rates were 80% in comparison to the results of Group 2 where the survival rate was 100%. The histopathological section of the kidney shows increased thickness of capsular region due to fibrin networks deposition, fibrosis and inflammatory cells infiltration (**Figure 10**), in addition to thrombus in blood vessels between renal tubules. The other kidney section revealed inflammatory cells in dilated blood vessels with acute cellular degeneration of epithelial cells of renal tubules. The main lesions in the liver consisting of granulomatous lesions in liver parenchyma with inflammatory cells in dilated sinusoids (**Figure 11**), in addition to inflammatory cells particularly mononuclear cells and neutrophils in the lumen of dilated blood vessels and in their wall. In the other animal, necrosis of hepatocytes with inflammatory cells in lumen and congested blood vessels were recorded (**Figure 12**), as well as RBCs and neutrophils replacement of necrotic hepatocytes (**Figure 13**). The heart section revealed congested blood vessels between cardiac muscle with inflammatory cells particularly mononuclear cells and neutrophils in their lumen (**Figure 14**). The histopathological section of the spleen expressed depletion of white pulp and fibrin networks and inflammatory cell infiltration in the red pulp (**Figure 15**).

The MRSA infected mice untreated with the phage (Group 4) animals were visibly ill after 14 hrs where they were lethargic, associated with progressive disease states as reflected by several clinical signs such as anorexia, decrease in body weight and ruffled hair where all mice died within 48 h.

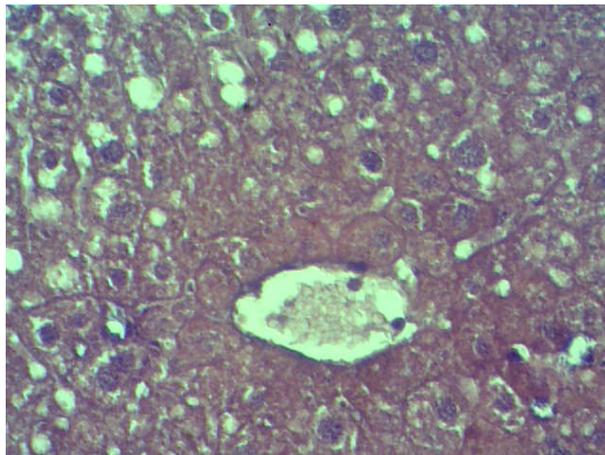


Figure 8. Section of the liver of mice shows moderate fatty changes in portal zone of the liver (H & E, $\times 400$).

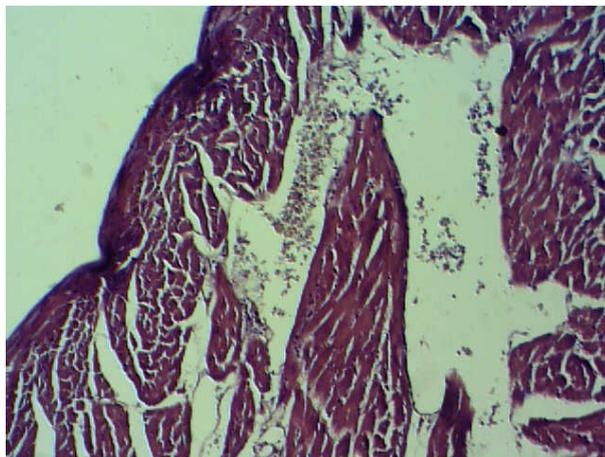


Figure 9. Section of the heart of mice shows no clear lesions (H & E, $\times 400$).

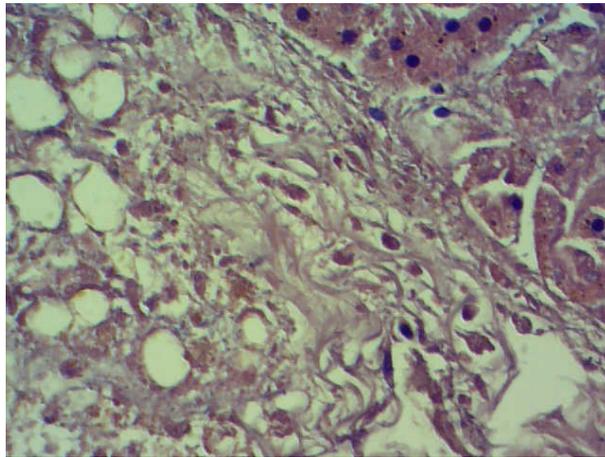


Figure 10. Section of the kidney of mice shows increased thickness of capsular region due to fibrin networks deposition, fibrosis and inflammatory cell infiltration (H & E, $\times 400$).

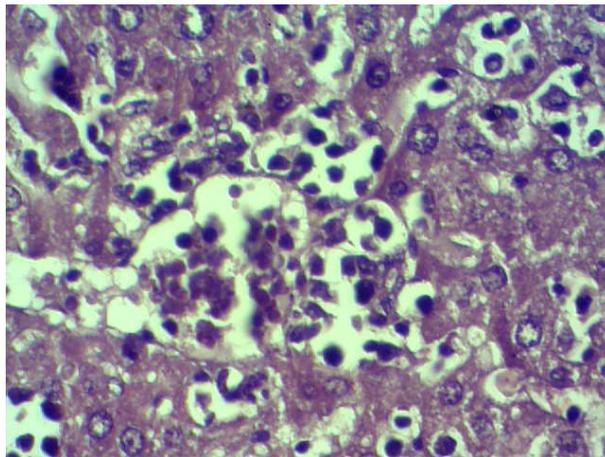


Figure 11. Section of the liver of mice shows mononuclear cell aggregation with inflammatory cells in dilated sinusoids (H & E, $\times 400$).

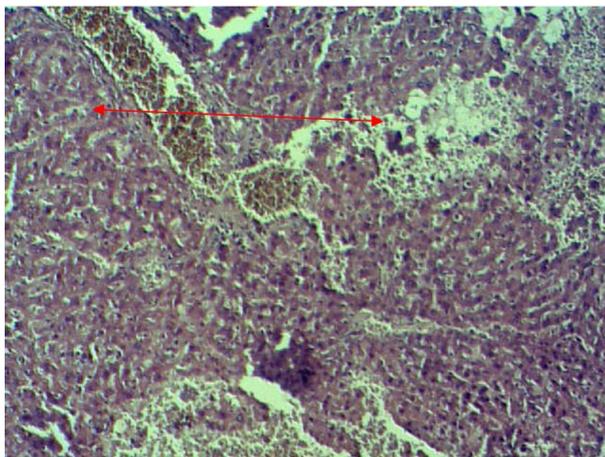


Figure 12. Section of the liver of mice shows necrosis of hepatocytes with inflammatory cells, particularly mononuclear cells and neutrophils mononuclear cell aggregation in liver parenchyma (H & E, $\times 400$).

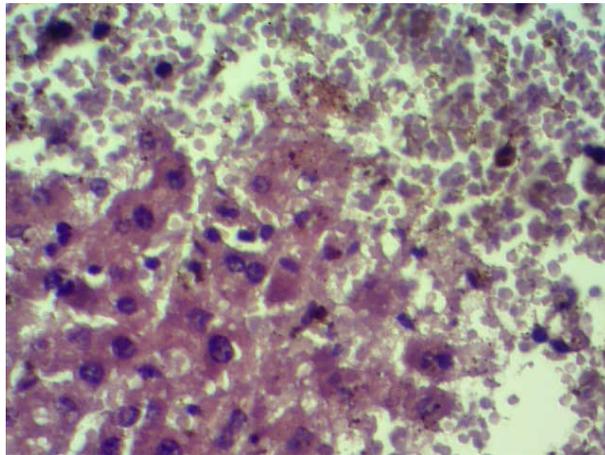


Figure 13. Section of the liver of mice shows red blood cells and neutrophils replacement of necrotic hepatocytes (H & E, $\times 400$).

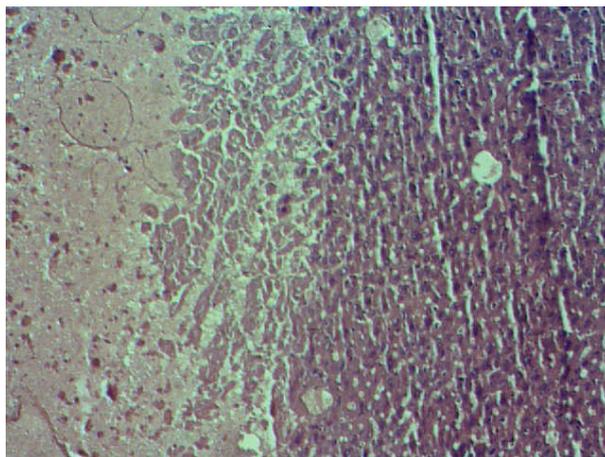


Figure 14. Section of the heart of mice shows congested blood vessels between cardiac muscle with inflammatory cells particularly mononuclear cells and neutrophils in their lumen (H & E, $\times 400$).

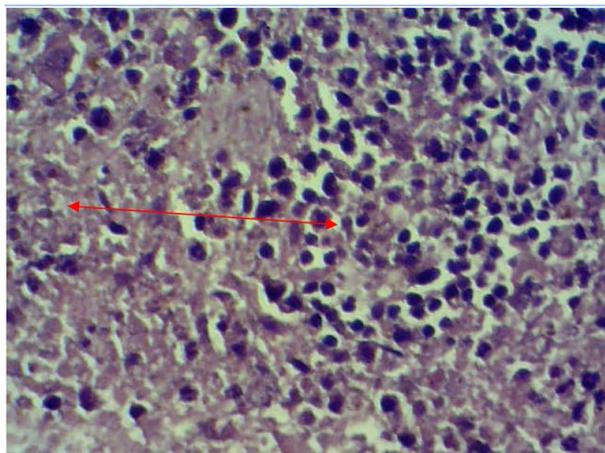


Figure 15. Section of the spleen of mice shows depletion of white pulp and fibrin networks and inflammatory cell infiltration in the red pulp (H & E, $\times 400$).

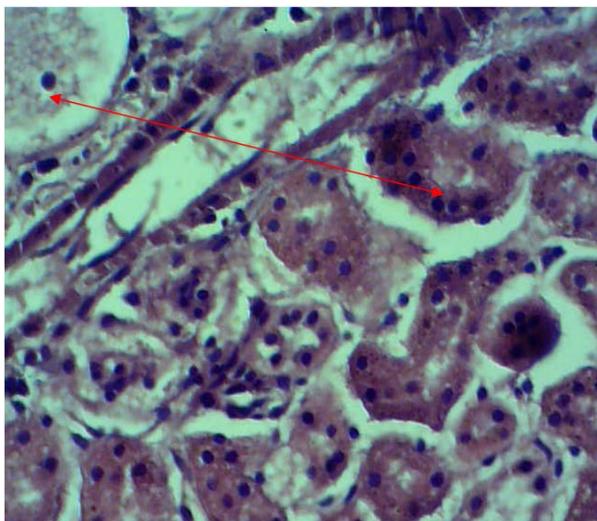


Figure 16. Section of the liver of mice at 2 days post infection shows multiple necrotic areas with atrophy of hepatic cord and neutrophils infiltration (H & E, $\times 400$).

The histopathological change in kidney tissues 48 h post infection with MRSA shows inflammatory cells in congested blood vessels between renal tubules that expressed acute cellular degeneration (**Figure 16**). The liver tissues characterized by multiple necrotic areas in liver parenchyma with atrophy of hepatic cord and neutrophils infiltration (**Figure 17**), as well as thrombus in blood vessels (**Figure 18**). In other animals, granulomatous lesions consisting of aggregation of macrophages and lymphocytes were the main lesions in the liver (**Figure 19**), in addition to mononuclear cell aggregation in one side of congested central veins. In the other section, the liver expressed necrosis of hepatocytes with inflammatory cells particularly mononuclear cells and neutrophils aggregation in liver parenchyma. The heart section showed inflammatory cell infiltration in the epicardium and characteristic haemorrhage and edema between cardiac muscle fibers (**Figure 20**).

In comparison, in Group 5 the mice were not challenged with bacteria but received only the i.p. injection $400\mu\text{l}$ of phage SA9 10^{10} PFU mL^{-1} . The state of the health of these animals was monitored for 7 days and was normal, showing no signs of illness. The histopathological sections of kidney (**Figure 21**), liver (**Figure 22**), and heart (**Figure 23**) show no clear lesions.

As an additional control, a sixth group untreated with phage and not infected with MRSA, control Group 6, were instead given PBS $400\mu\text{l}$ i.p. where all animals survived and have shown no illness. The state of the health of these animals was monitored for 7 days and they were normal and showing no signs of illness. Similar results of the histopathological changes in kidney, liver and heart were seen as in the Group 5 above.

4. Discussion

From 115 raw milk samples, 9 out of 30 staphylococci isolates were confirmed as MRSA mastitis bacteria biochemically by API Staph system with 99% confirmatory rate where they were resistant to traditional anti-staphylococcal beta-lactam antibiotics, such as cefoxitin, which is considered to be a strong beta-lactamase inducer [25], ampicillin and methicillin. This represents almost 30% of the staphylococci isolated from 115 raw milk samples. The higher resistance of most *Staph. aureus* isolates to methicillin, penicillin and ampicillin are coming from increased and frequent use of β -lactam antibiotics which lead to development of resistance due to production of penicillinase enzymes from *Staph. aureus* bacteria, found to be an induction to the penicillin binding protein 2a responsible of methicillin resistant encoded by *mecA* gene [26] [27]. Several Iraqi studies have shown an increase in MRSA prevalence in milk and cheeses and MRSA is considered endemic in most areas of Iraq [28] [29]. This may be reflected in the significant isolation of 9 isolates of MRSA from 115 raw milk samples collected from 3 dairy farms of the College of Veterinary Medicine, Baghdad University at Abu-Ghraib, Al-Shulaa and Al-Dorah, produced many exotoxins and hemolysins (data not shown). This indeed represents a high ratio with around 8% of staphylococci isolates resistant to different selected antibiotics especially methicillin,

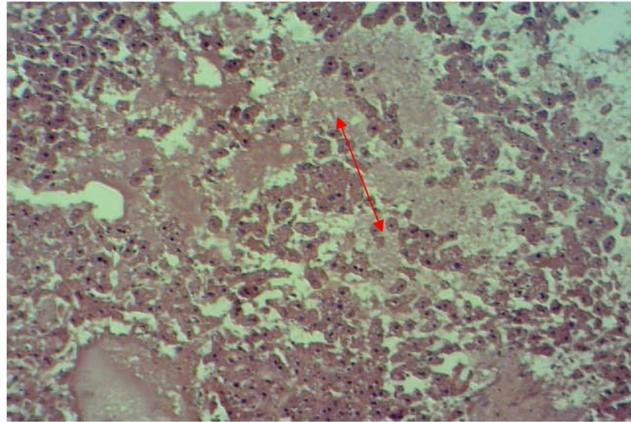


Figure 17. Section of the liver of mice at 2 days post infection shows multiple necrotic areas with atrophy of hepatic cord and neutrophils infiltration (H & E, $\times 400$).

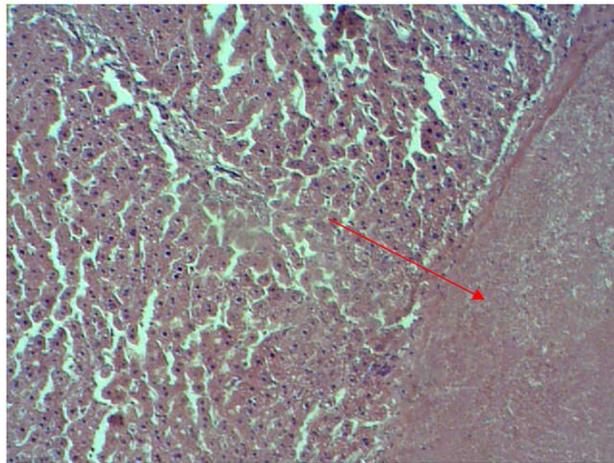


Figure 18. Section of the liver of mice at 2 days post infection shows thrombus in blood vessels (H & E, $\times 400$).

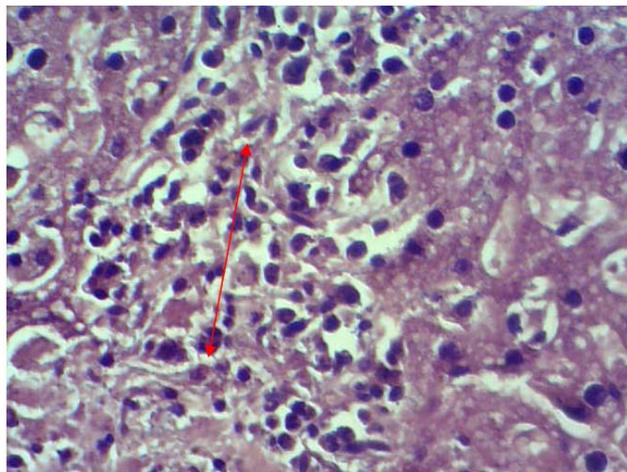


Figure 19. Section of the liver of mice at 2 days post infection shows large granulomatous lesion consisting of aggregation of macrophages and lymphocytes in liver parenchyma (H & E, $\times 400$).

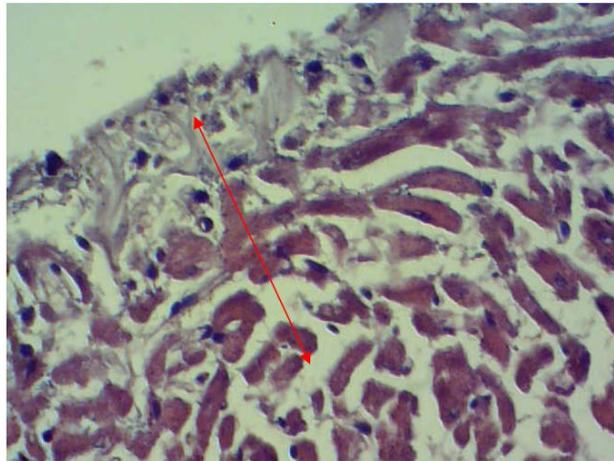


Figure 20. Section of the heart of mice at 2 days post infection shows inflammatory cells infiltration in the epicardium and edema between cardiac muscle fibers (H & E, $\times 400$).

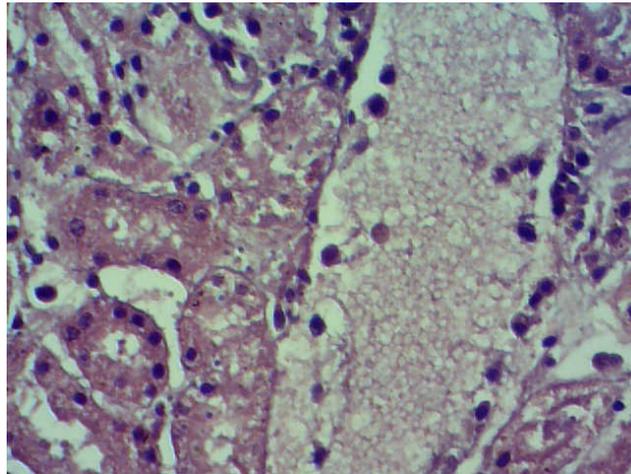


Figure 21. Section of the kidney of normal mice shows no clear lesions (H & E, $\times 400$).

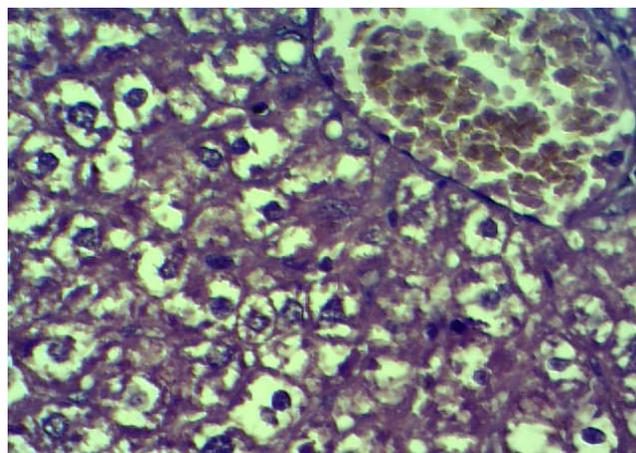


Figure 22. Section of the liver of normal mice show no clear lesions (H & E, $\times 400$).

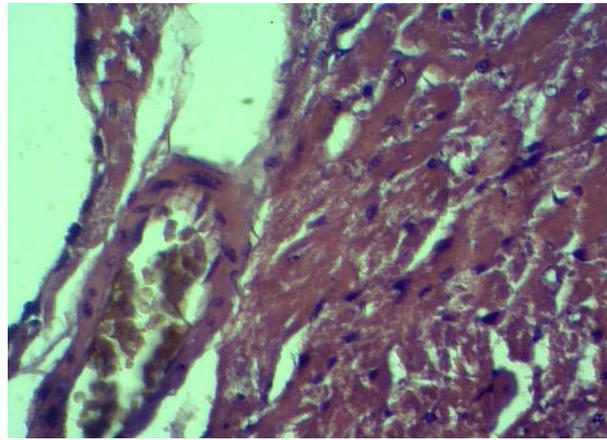


Figure 23. Section of the heart of normal mice shows no clear lesions except shows congested blood vessels between cardiac muscle (H & E, $\times 400$).

which may be a sign of unhygienic processing strategies with poor or insufficient hygienic measurements (contamination and pollution) of farm animals and post processing contamination in the milk chain [28]. From scientific and hygienic points of view if the ratio of MRSA isolates reach more than 5%, it is considered a public health redline risk forcing banning laws on products from these epidemic countries [28] [30] [31]. This may reflect a high level of contamination and development of resistance in these pathogens due partially to abuse of antibiotics in therapy or as growth promoters especially in cows on dairy farms in Iraq. This could suggest that there is a high prevalence of MRSA in Iraqi dairy herds [28] which poses a health threat to humans.

Treatment of staphylococcal infections with antibiotics is becoming increasingly difficult in view of the widespread presence of *Staph. aureus* strains resistant to multiple antibiotics. Health bodies all over the world are searching for a solution to the inevitable end of the antibiotics era, or the total resistance of MRSA against all known antibiotics. Moreover, the conventional control/therapy measures for MRSA using antibiotics were shown to be part of the problem. The emergence of infectious diseases caused by drug resistant bacteria requires alternatives to conventional antibiotics and phages are one potential solution that will help to replace, curb, or promote judicious use of antibiotics in farm animals [10].

To examine the possible usefulness of phages in treating disease caused by MRSA, we isolated phage SA9 with a similar morphology characteristic to that of A2 morphotype described by Ackermann and Dubow [32] and it was found to not inhibit bacterial growth of *Staph. aureus* non-multiresistant MRSA, thus exhibiting an antibacterial effect against all 9 MRSA mastitis strains in our collection.

The phage SA9 was isolated from milk samples collected from dairy cows with clinical mastitis and was isolated using robust isolation parameters to help increase the phage host-interaction efficiency to form clear plaques on all 9 MRSA mastitis bacteria. Given concurrently with a median lethal dose of MRSA, wild isolate S9 was used to establish the MRSA LD₅₀. Bacterial dose of 10^8 CFU mL⁻¹ of strain S9 represent the LD₅₀ for BALB/c 6 week old mice where all mice receiving i.p. died within 48 h. This LD₅₀ was challenged in the present study and highlights SP9 phage therapy as a possible solution.

The phage SP9 in the Group 1 with a ratio of phage:bacteria of 1:1 rescued 2 mice out of 5 with a survival rate of 40% around (PED₅₀). In this experiment with the ratio of 1:1 phage:MRSA we observed that there was very little, if any damage in the residual lesions in protected phage SP9-treated mice in their examined organs, this result may indicate that phage destroyed most of the pathogen at the site of inoculation or through dissemination to internal organs (Figures 1-5). The two surviving mice treated with phage showed drastically reduced inflammation likely because of the rapid bacterial lysis by the phage which leads to reduced toxins and mortality rate [33] [34].

It appears that phage can destroy the pathogen, with mononuclear cell aggregation in the liver (Figure 2) and lung (Figure 4) possibly due to immune response to the pathogen and may indicate that residual pathogen may have remained in lag phase and proliferated to log and disseminated to internal organs where host defense mechanisms attempt to destroy the pathogen through proliferation and activation mononuclear cells. This active proliferation of mononuclear cells may simply indicate a good immune response [35].

In Group 2 we achieved a 100% (PED₁₀₀) phage therapy protection rate against MRSA infection when the phage titre was raised to ratio of 100:1 phage:bacteria and completely eradicated bacteria in vivo within 4 days of phage treatment. All animals in this experiment observed a normal physical state against systemic infection of MRSA. The histopathological study (Figures 6-9) expressed more mononuclear cell aggregation around blood vessels in kidney (Figure 6) and liver (Figure 7), this result may indicate that a high concentration of phage has the potential to stimulate the production of neutrophils while concurrently eliminating susceptible microorganisms [36], though this in addition to *Staph. aureus* surface protein elicit immune responses [37]. In this regard high phage titre can lead to the destruction of most *S. aureus*, with the fragments of pathogens engulfed by phagocytic cells which processed and presented them for CD4+ T cells, and this process lead to proliferation and differentiation CD4+ T cells into Th1 cells that produced IFN- γ , and INF- γ leading to activate and attract mononuclear cells to the inflamed area [35]. The above results become more convincing when examined in the context of numerous reports documenting phage efficacy in vivo against *Staph. aureus* [34] [38] [39].

Group 3: We also attempted to test the efficacy of phage SP9 when bacterial infection was already established for 6 h. In this experiment although the phage: bacteria ratio was 100:1 the successful phage treatment rate was 4 mice out of 5, representing 80% survival rate, above PED₅₀ and less than PED₁₀₀. In comparison the 0-h treatment delay Group 2 was significantly protected in terms of all criteria compared with the 6 h treatment delay group ($P \leq 0.03$). Therefore, with delays of as little as 6 h the phage treatment is still highly effective but not perfect in this model with this particular phage-host interaction delay. The phage can be highly effective even when administered post infection [34] [40]. The histopathological section of the 4 protected mice (Figures 10-15) shows severe pathological lesions in the kidney (Figure 10) characterized by fibrino suppurative inflammatory response with thrombus formation, this result may indicate that bacteremia and septicemia occur during 6 hr post-infection, before the treatment of animals with phages, probably since toxigenic *Staph. aureus* have the ability to produce exotoxin like staphylococcal enterotoxin which lead to animal death during 6 - 48 h post-infection [41]. The pathological lesions in the heart (Figure 14) may indicate that this pathogen can spread through the blood and induce myocarditis and adhere to native valvular surface causing endocarditis [42].

Group 4: Mortality is certainly an important criterion to judge the efficacy of a treatment. Therefore, in this group the mice infected by MRSA were not treated with the phage. All animal were visibly ill 14 hr after infection, observed to be lethargic associated with progressive disease states as reflected by several clinical signs including anorexia, decrease in body weight, ruffled hair where all died within 48 hours due to MRSA virulence and associated toxins that cause the death [43] [44]. The current study demonstrated that *Staph. aureus* induced pathological lesions in the examined organs of animals in this group. *Staph. aureus* can induce virulent factors that can overcome the host defense mechanisms and disseminated from the site of inoculation to most internal organs (Figures 16-20). This can cause destruction to host tissues through inactivation of host defense mechanisms associated with the lesions induced by this pathogen, consisting of severe necrosis of hepatocytes of the animal [45] [46]. We observed in the liver multiple necrotic areas with atrophy of hepatic cord and neutrophils infiltration (Figure 17), thrombus formation in blood vessels (Figure 18), large granulomatous lesion consisting of aggregation of macrophages and lymphocytes (Figure 19). In the heart there was inflammatory cell infiltration in the epicardium and edema between cardiac muscle fibers (Figure 20) all these scenes may be due to the toxic effects of this pathogen that produced different toxins such as toxic shock syndrome toxin and induced septic shock [43] [47]. It is well known that *Staph. aureus* causes sepsis through secretion of their virulence factors such as cell wall associated and secreted protein, including protein A, hemolysin that stimulated macrophages for producing pro inflammatory cytokines such Interleukin-1, and tumor necrotic factor. The latter responsible for septic shock, inflammatory cells particularly neutrophils infiltration in the liver, kidney and heart indicate the pathogen induced pyogenic lesions [35] [48].

Group 5: Healthy animals displayed no apparent reaction to the phage, there are no anaphylactic reactions or alterations in body temperature and no other adverse effects in the control group inoculated with a high dose (10^{10} PFU mL⁻¹) of the SP9 phage preparation. In general the phages are considered safe and have no adverse effects on mice used as controls for phage therapy experiments [36] [49].

Group 6 was made as an additional control by injecting i.p. healthy mice with the PBS to serve as control group. All the mice in this group 5/5 survive with normal physical activity.

In general, efficacy in murine models of MRSA infection were used in this study since it was taken as a key to develop new antibacterial agents targeted for the treatment of staphylococcal infections, including MRSA infections in the community setting [50]. This MRSA mice treatment with phage is time and ratio-dependent be-

tween the phage titre to bacterial titre. Best results were achieved when the bacterial phage to bacteria is 100:1 (PFU:CFU).

The use of sub-therapeutic antibiotics to promote both health and growth in animals rose by about 50% between 1985 and 2001 and this has increased gradually until today where approximately 80% of all antibiotics in the US are used on farm animals [10]. In the dairy cow industries, antibiotic treatment was also used to treat mastitis [10]. This has led to the evolution of bacteria that are resistant to the drug's effects and a new type of MRSA, the livestock associated MRSA (LA-MRSA) [51]. It was noticed that after 2000, the majority of MRSA infections in most countries are acquired in the community outside of healthcare settings. People who live near livestock or in livestock farming communities are at a greater risk of acquiring of LA-MRSA and today this new type of MRSA is a vital public health concern that urgently needs prevention [51].

Newly approved antibiotics have steadily declined since the 1980s [10]. This fact, associated with the emergence of infectious disease caused by drug resistant bacteria, has resulted in about 70% of bacteria developing resistance to antibiotics [52]. It appears there is a great need to investigate alternative anti-MRSA agents. The present study demonstrated that i) a single injection of phage can rescue 100% of the animals and improve survival in a model of bacteraemia caused by MRSA in mice, even when treatment is delayed until 6 h after lethal bacterial challenge; and ii) if treatment is delayed beyond that point, morbidity increases and mortality begins to appear. However, these findings suggest that MRSA phage SP9 may have utility for treatment of MRSA bacteraemia infections within a specific time after lethal infection, not exceed more than 6 hr to obtain around 80% survival.

5. Conclusion

Antibiotics are currently being phased out of animal production in many countries. Our finding, further reinforces the view that phages could be used in situations where there is no substitutes available for treating multi-resistant strains. Phages are naturally occurring predators of MRSA which can be found in dairy farm-acquired MRSA. These phages can be further developed to produce effective natural living drugs targeting specifically MRSA with PED₁₀₀, using dairy farm-acquired MRSA strains for which the selection of the phages can be determined most efficiently. In general, our successful phage treatment to experimentally prevent MRSA infection suggests that phage could be used to control this disease. Moreover, the finding that phage can inhibit and cure post infectious animals suggests that the phage could be used i.p. to prevent horizontal and vertical transmission of MRSA in agriculture animals. Further experiments to determine the effectiveness of phage against natural infections should be performed in order to develop a phage biocontrol and treatment for the disease.

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