Research Article

CrossMark
click for updates

Activity of Isolated *Staphylococcal* Bacteriophage in Treatment of Experimentally Induced Chronic Osteomyelitis in Rabbits

Orooba Mohammed Saeed Ibrahim[1], Sarhan Rashid Sarhan[1*], Serwa Ibrahim Salih[2]

[1]Department of Physiology and Pharmacology; [2]Department of Surgery, College of Veterinary Medicine, Baghdad University, Iraq.

Abstract | The central goal of this research was to explore the use of *Staphylococcus aureus* specific-bacteriophage as an alternative to antibiotics in treatment of chronic osteomyelitis in rabbits by inoculation of methicillin resistant *Staphylococcus aureus* (MRSA) in tibia. The current study included isolation of bacteriophage from swage water by using agar overlay method. While the second experiment was evaluation of phage activity in treatment of chronic osteomyelitis in rabbits in comparison with ceftaroline. Results of our research showed that all animals of infected groups with MRSA before treatment exhibited clinical signs of osteomyelitis after 21 days of inducing infection. Group C animals that dosed with 3×10^8 pfu/ml of Staph-specific bacteriophage for two weeks intramuscularly showed faster recovery than group D that treated with 40 mg/ kg B.W of ceftaroline for two weeks intramuscularly. We came in concluded that a bacteriophage was a good choice for treatment of a multiple drug resistant pathogen such as *Staphylococcus aureus* in shorter time with minimal side effects.

INTRODUCTION

Osteomyelitis is illustrated as the infection of medullary tissue, periosteum, and cortical bone (Rabillard et al., 2011). It is an inflammatory process combined by bone damage and brought on by a infecting microorganism, the infection can be constrained to a single portion of the bone or can include many areas, for example, marrow, cortex, periosteum, and the encompassing soft tissue. Inflammatory responses to the attacking pathogen make an exudate that gathers locally and packs veins, prompting osteonecrosis (Zuluaga et al., 2006). Osteomyelitis is for the most part categorized as 'acute or chronic' and 'haematogenous or contigouos' based on site, source and duration of infection and patient health status and more specifically to histopathological and radiographical finding. With an incident range of 6 % of people suffering from bone diseases are associated with a death rate of 2-10%, and a danger of losing of joint capability of 40% (Mathews et al., 2010; Garcia-Arias et al., 2011).

Staphylococcus aureus is the most commonly isolated pathogen for both acute and chronic osteomyelitis in all age groups (Jorge et al., 2010; Eid and Berbari, 2012). Lately there has been an increase in methicillin-resistant *S. aureus* (MRSA) due to rapid development of antimicrobial resistance and expression of virulence factors, regardless of the patient´s immune status. Coagulase-negative *Staphylococci* are often seen in association with foreign bodies, such as prosthetic joints (Chihara and Segreti, 2010).

Animals such as dogs, cats and horses have become an important part of most families particularly in developed countries like USA and UK (Chomel and Sun, 2011). In the UK, 1.5% of MRSA were recovered from samples of infected companion animals (Rich and Roberts, 2004)

and dogs are more infected / colonized with MRSA in comparison to cats (Morgan, 2008). Reports of MRSA colonization in horses with a percentage rate of 0 to 11% has been published (Loeffler et al., 2011). Most cases and outbreak of MRSA infections were reported in large stables and post-operative complications (Weese et al., 2005; Morgan, 2008).

Most MRSA strains are frequently resistant to most common antibiotics including tetracycline, aminoglycosides, macrolides, chloramphenicol and fluoroquinolones (Lee, 2003; Middleton et al., 2005; CDC, 2006). Antibiotics used to treat serious, multiple drug resistant MRSA infections include vancomycin, as well as newer drugs such as linezolid, tigecycline, quinupristin/dalfopristin and daptomycin (Lee, 2003; Gorwitz et al., 2006; Boucher et al., 2010; Catry et al., 2010). The indiscriminate exposure of humans and animals to antibiotics created problem through acquisition and dissemination of MRSA which limit the choice of treatment. Most antibiotics used for treatment of MRSA infection has been reported to have developed resistance (Ayliffe, 1997).

During the last 30 years, no new classes of antibiotics have been found, even with the help of modern biotechnology such as genetic engineering. Pharmaceutical companies have mainly focused on the development of new products derived from the known classes of antibiotics (Sulakvelidze et al., 2001) which is a cause of major concern. Thus, exploring alternative approaches to develop antibacterial products is also a worthwhile task, and re-examining the potential of promising older methods might be of value. One of the possible replacements for antibiotics is the use of bacteriophages or simply phages as antimicrobial agents (Shasha et al., 2004; Vinodkumar et al., 2008).

Phage therapy involves the use of lytic phages for the treatment of bacterial infections, especially those caused by antibiotic resistant bacteria. In general, there are two major types of phages, lytic and lysogenic. Only the lytic phages (also known as virulent phages) are a good choice for developing therapeutic phage preparations (Sandeep, 2006; Borysowski and Gorski, 2008). The bactericidal ability of phages has been used to treat human infections for years as a complement or alternative to antibiotic therapy (Matsuzaki et al., 2005; Kysela and Turner, 2007). Phages are bactericidal, their number increased in responding to the incidence of pathogens over the course of treatment, they are in the same manner effective against antibiotic-resistant bacteria and are capable of damaging bacterial biofilms, and have low inherent toxicities. The investment of phages as a pragmatic approach in the control of pathogens has attracted considerable interest in recent years (Jassim, et al., 2012).

MATERIALS AND METHODS

ISOLATION AND IDENTIFICATION OF *S. aureus*

A total of 50 samples of pus were taken from patient suffering from post-traumatic bone infection. All patient who were suspected to had *Staphylococcal* infections. Culture specimens were obtained at the time of admission, after the surface of the wound had been washed vigorously by saline, and followed by debridement of superficial exudates. Pus was collected by sterile syringe. Specimens were promptly took to the laboratory and processed. Standard methods for isolation and identification of *S. aureus* were used (Sowmya et al., 2014).

DETECTION OF METHICILLIN RESISTANT *Staphylococcus aureus* BY USING CEFOXITIN, METHICILLIN AND PENICILLIN G DISC DIFFUSION (D.D.) METHOD

The isolates of *S. aureus* were subjected to cefoxitin, methicillin and penicillin G Discs (Bioanalyse, Turkey), using a 30 μg, 10 μg and 10 U disc respectively. A 0.5 McFarland standard suspension of the isolate (1.5×10^8 PFU/ml) was made and lawn culture was done on Muller-Hinton agar, (Oxoid-England) plate. Plates were incubated at 37ºC for 18 hr and zone diameters were measured (Table 1) (EUCAST, 2009; CLSI, 2012).

Table 1: Antimicrobial susceptibility testing standards (EUCAST, 2009; CLSI, 2012)

Antibiotic Discs	Disc concentration	Diameter of inhibition zone (mm) Interpretive criteria		
		Resistant	Intermediate	Sensitive
Cefoxitin	30 μg	≤ 21	-	≥ 22
Methicillin	10 μg	≤ 9	10-13	≥ 14
Penicillin G	10 U	≤ 28	-	≥ 29

ISOLATION AND PURIFICATION OF *S. aureus* BACTERIOPHAGE

Sample Collection: The methods used adapted from (USEPA, 2001; Jaime, 2003). 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 decanted into Erlenmeyer flask and pipette 5 ml double sterile phosphate buffer saline and 5 ml *Staphylococcus 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 (Dias et al., 2013). Sewage and *S. aureus* culture distributed into 8 centrifuge tubes and centrifuged the sample at 2000 RPM for 5 minute. Most of the remaining cells was 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).

EXPERIMENTAL DESIGN

Twenty rabbits divided into 4 groups, five rabbits for each group (treatment started immediately when the infection appeared). Group A: Negative control group (not infected nor treated). Group B: Positive control group (infected with MRSA and not treated). Group C: This group infected with MRSA and treated with Staph-specific Bacteriophage intramuscularly with 3×10^8 PFU/ml once daily for 14 days. Group D: This group infected with MRSA and treated with 40 mg/KG Ceftaroline intramuscularly once daily for 14 days (Cedric et al., 2010).

EXPERIMENTAL ANIMALS

Twenty healthy male rabbits (local breed), were divided equally into 4 groups, five rabbits of each. Their age extended between 10-13 weeks, animals weighing between 1.8-2 kg. Rabbits were put in standard rabbit's cage. Experimental animals fed with a commercial lab animals pellets and tap water was available freely. Room temperature was maintained at 20-25 °C. Room air was changed constantly by utilizing ventilation vacuum. Litter of cages was changed daily.

METHICILLIN RESISTANT *Staphylococcus aureus*

The pathogenic isolates of Methicillin resistant *Staphylococcus aureus* were obtained from orthopedic department in Alkarama hospital and it was used as a challenge strain.

PREPARATION OF RABBITS

After two weeks adaptation period, Ketamine 50 mg/kg B.W and Xylazine 4 mg/kg B.W. were given intramuscular to anesthetized rabbits (Nuh, 2004). Right hind limb are prepared surgically (clipping the hair with electrical clipper, then shaved to remove the hair completely) from the metatarsus to the stifle joint.

INDUCTION OF CHRONIC OSTEOMYELITIS

About (1 - 1.5 cm) surgical skin incision was made at the Cranio-medial site of the tibia bone of rabbit, subcutaneous structures were pushed to aside to uncover tibia bone. Dental drill was used to make a hole (one mm), then 0.04 ml of MRSA suspension was inoculated intramedully. The challenge dose that induced infection was 1.5×10^8 cfu/ml of bacterial suspension after pilot study, then the hole was closed with sterile bone-wax, subcutaneous fascia closed by simple continuous using chromic catgut 2/0 USP. Simple interrupted stitches used to close skin using silk suture (2/0). The animals were followed up clinically for signs of inflammation and radiographically for any pathological changes in the bone (Alt et al., 2006; El-Kamel and Baddour, 2007; Li et al., 2009) (Figure 1). All animals were given analgesic agent daily after surgery for 5 days.
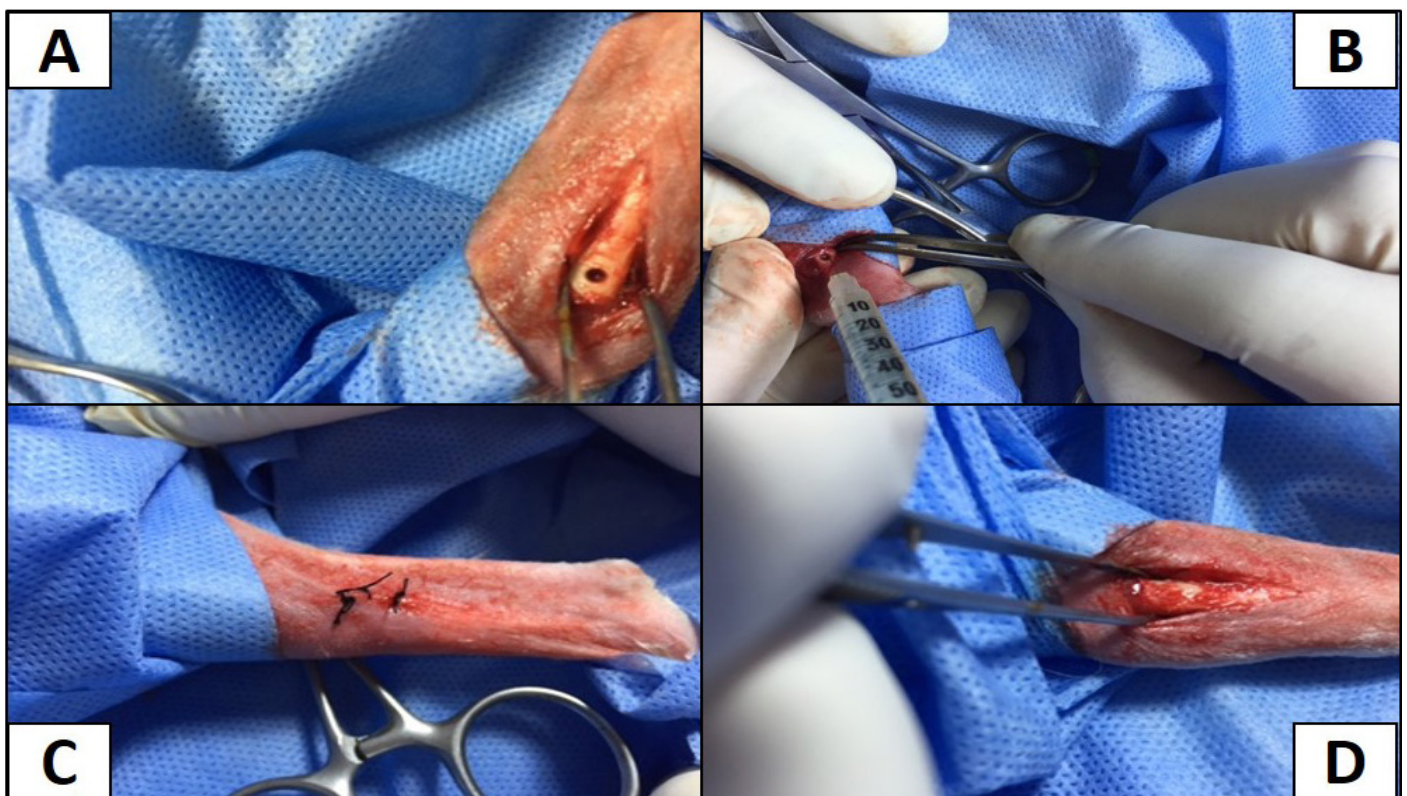

Figure 1: A) The site of operation shows the hole in right tibia; **B)** Inoculation of MRSA intramedullary; **C)** The hole filled with bone wax; **D)** The site of operation sutured with silk.

STATISTICAL ANALYSIS

Data was 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).

BONE RADIOGRAPHICAL FINDING

These findings was accomplished for all groups weekly, after infection and after treatment.

RESULTS

ISOLATION AND IDENTIFICATION OF *S. aureus*

The results of isolation and culturing of bacteria including biochemical tests showed that a total of 50 pus samples which were collected from patients suffering from post-traumatic bone infection, 42 samples, showed positive results for the presence of *Staphylococcus aureus* (Table 2).

Table 2: Prevalence of *Staphylococcus* aureus from the collected samples

Bacteria	No. of samples	Percentage %
Staphylococcus aureus	42	84 %
Other	8	16 %
Total	50	100 %

DETECTION OF METHICILLIN RESISTANT *S. aureus* BY USING DISC DIFFUSION METHOD

After the identification of *S. aureus,* susceptibility test was done for all *S. aureus* isolates by disk diffusion method to detect the presence of MRSA according to National Committee for Clinical Laboratory Standards guidelines recommendation (NCCLS), as shown in Table 3. Results of antibacterial sensitivity test of *Staphylococcus aureus* shown that all isolates were resistance to penicillin 100%, this may explain why *S. aureus* is totally resistant to pencillin G. Also it was found that 92.85 % were resistant to cefoxitin and 95.23 % of *S. aureus* were resistant to methicillin.

Table 3: Antibiotic sensitivity test of *S. aureus*

Bacteria	Staphylococcus aureus (n = 42)					
	R		I		S	
Antibiotic discs	No	Ration	No	Ration	No	Ration
Cefoxitin 30 µg	39	92.85 %	-	-	3	7.15 %
Methicillin 10 µg	40	%95.23	-	-	2	4.77%
Penicillin G 10 U	42	100%	-	-	-	-

ISOLATION AND PURIFICATION OF *S. aureus* BACTERIOPHAGE

Sample Collection: *Staph aureus* specific- Bacteriophage associated with osteomyelitis which had been separated from sewage water near to the hospital Lab and Surgery Theatre through agar method. Phage were able to lyse bacteria and form plaques after incubation 24 hr at 37 ℃. A piece of single plaque of phage on the bottom agar were picked by sterile needle. Put in broth for 3 hrs and re-infect the host to confirm obtaining phage (Figure 2).

Figure 2: Plaques of bacteria lysis by Phage after incubation 24 hr at 37°C.

Figure 3: Site of operation, shows mediolateral view of the right tibia in non-infected group 21 days post-operatively, the hole had disappeared completely (healed, ML view)

BONE RADIOGRAPHICAL FINDING

Radiographic findings of tibia in group A exhibited normal bone anatomy throughout the experimental (no radiographic changes) and the hole had disappeared completely (healed) (Figure 3), while infected groups showed radiographic changes after 14 days post infection with MRSA such as area of bone lysis (Radiolucent spot) surrounded by bone production (Radiopaque area) and thickened cortex and severe periosteal reaction (Figure 4), while after 21 days post infection there was significant chronic osteomyelitis characterized by present Soft tissue suppuration, severe bone lysis, abscesses and Seqeustrum appeared (A segment of bone devascularized with osteonecrosis and resorption of adjacent bone tissue leaving a 'separated' piece. (Reservoir for infection) which is a typical sign of chronic

osteomyelitis (Figure 5A and B).

Figure 4: 14 days post infection with MRSA, Area of bone lysis (Radiolucent spot) (→) surrounded by bone production (Radiopaque area) (→).Thickened cortex and severe periosteal reaction (→) (ML view)
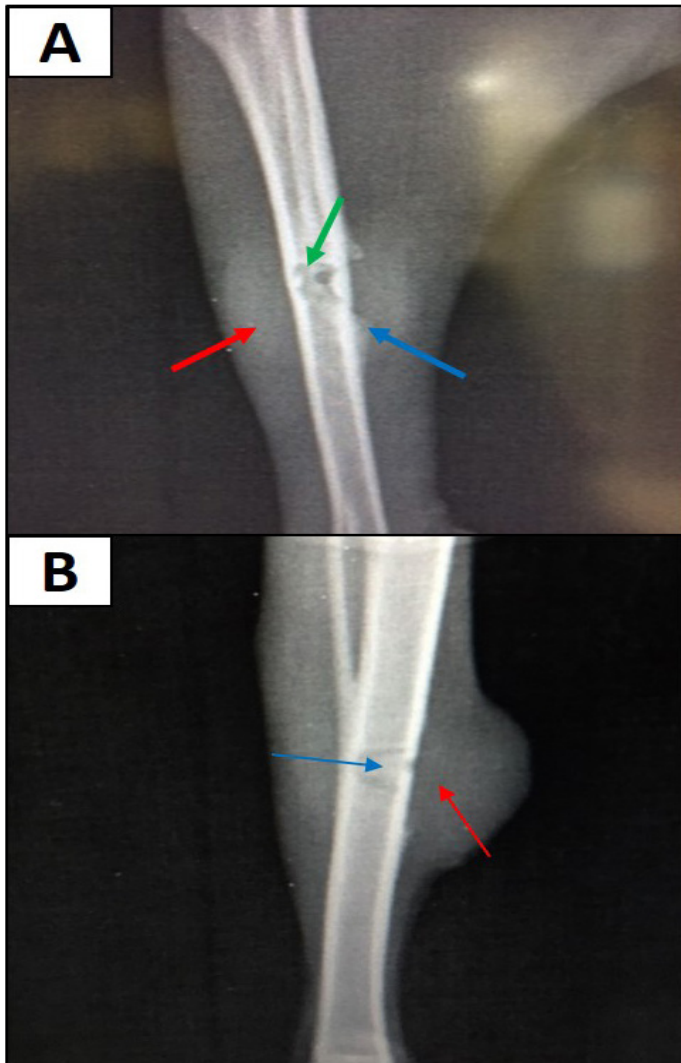

Figure 5: A) 21 days post infection with MRSA shows Sever bone lysis (→), Thickened of cortex and sever periosteal reaction (→), Soft tissue suppuration (→) (ML view); **B)** Seqeustrum

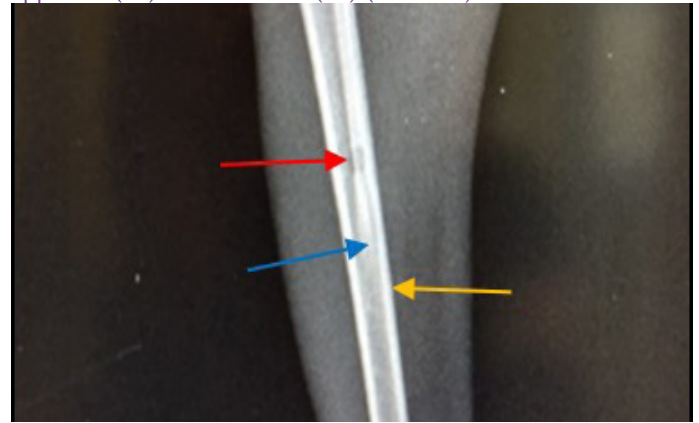appeared (→) and abscesses (→) (AP view)

Figure 6: Site of operation, 7 days post treatment with Staph specific – bacteriophage given intramuscularly (Group C), all features of osteomyelitis disappeared, almost clear medullary canal (→), uniformed bone density and cortex returned to normal thickness (→), but the hole still exists (→) (ML View)

Figure 7: Site of operation, 7 days post treatment with Ceftaroline given intramuscularly (group D): **A)** Chronic Osteomyelitis (Seqeustrum still exists) (→) (ML View); **B)** Radiopaque area in soft tissue present represent accumulated pus (→) (AP View)

In group C (treated with 3×10^8 pfu/ml bacteriophage intramuscularly) showed a fast recovery at the first week of treatment and the signs of osteomyelitis had disappeared (Medullary canal is clean), bone cortex uniformed, normal bone density but the hole still present (Figure 6 and 8). Two week post treatment with Ceftaroline 40 mg/kg given intramuscularly (group D), still there is signs of osteomyelitis (Figure 7 and 9).

Susceptibility test of *S. aureus* isolates revealed that all isolate were resistance to penicillin 100%, this may explain why *S. aureus* is totally resistant to pencillin G which agreed with Al-Jundiy (2005), Zeidan (2005) and Al-Geobory (2011). The percentage of penicillin G resistant in these studies was 100%, respectively. The present study results were compatible with results of Aghazadeh et al. (2009); likewise, this results were compatible with those obtained by Brady et al. (2007) as they observed that all isolates were

resistant to penicillin and other β-lactam antibiotics.

Figure 8: Site of operation, 14 days post treatment with Staph specific –bacteriophage given intramuscularly (Group C). Signs of osteomyelitis had disappeared. (Medullary canal is clear), bone cortex uniformed, normal bone density, the hole still present (ML View)

Figure 9: Site of operation, 14 days post treatment with Ceftaroline given intramuscularly (Group D). Still the medullary canal is not clear. Thickened cortex (→) and Endosteal reaction appeared clearly (→) (ML View)

It was found that 92.85 % were resistant to cefoxitin and also 95.23 % of *S. aureus* were resistant to methicillin. This result is close to work of Kalmus et al. (2011) and Deresse et al. (2012).

Several groups of investigators have reported that the results of cefoxitin disk diffusion (DD) tests correlate better with the presence of mecA than do the results of disk diffusion tests using oxacillin (Swenson et al., 2005). Using cefoxitin and CLSI criteria for disc diffusion, the sensitivity and specificity for recognizing methicillin resistance were both 100 % (Fernande et al., 2005).

Cefoxitin and moxalactam have been reported as surrogate markers for the detection of methicillin resistance (Felten et al., 2002; Skov et al., 2003). The NCCLS has recently reported cefoxitin zone diameter interpretive criteria for the

prediction of mecA-mediated resistance (NCCLS, 2000). The common gold standard method for enumerating and differentiating phages is the double agar overlay assay. Therefore this current study are in agreement with Mansura et al. (2015) in possibility of isolation of phage from sewage by using double agar overlay assay. Oot et al. (2007) emphasized the ubiquitous nature of phage populations, although bacteriophage counts can only rise after an increase in host bacteria population. In studies involving dairy livestock, milk and udders were primary sources for samples from which phages were isolated (Brüssow and Desiere 2001; Garcia et al., 2007, 2009; Shi et al., 2010; Kwiatek et al., 2012). Alternate sources from which phages could be isolated include sewage or wastewater (Yoon et al., 2010), and feces (Oot et al., 2007; Niu et al., 2009; Santos et al., 2011).

Plaques were formed as clear circular with a size ranged between 2-3 mm in diameter when incubated at 37 Co for 24 hrs. This result agreed with Aidan et al. (2009) and Longping et al. (2014) due to lyses of the bacterial host (MRSA). Sewage water was observed to be the best environmental source to get lytic phages with aggressive infective qualities; this might be attributed to the fact that phages from sewage tolerate hard conditions in the sewage; thus, these phages show high degree of lysis with high tolerance to harsh physical environment. Therefore, lytic phages from sewage showed larger plaques, higher titers, and clearer plaques than others.

This is in agreement with Marwa and Ahmed (2014), they reported that the different sources of phages' isolation and the finding that each phage showed unique profile of size, shape, clarity, and margin cut of plaques provided preliminary evidence that isolated phages are unique and no phages are identical to each other.

The radiograph is an image made up of shadows of different opacities, increased opacity (radiopacity) denoted more white shadow on the radiograph, while decreased opacity (radiolucency) denoted a darker shadow on the radiograph (Elliot and Skerritt, 2010). It can be concluded that the diagnosis of osteomyelitis is more often based on radiographic evidence of bone lysis, production and necrosis. Our results were in an agreement with Chandan et al. (2016). There are numerous reports in the literature using animal models of osteomyelitis for investigating pathogenesis, diagnosis, and treatment of bone infections. When designing an animal model of osteomyelitis, it is important to learn the currently available models as well as their advantages and disadvantages. For use as an animal model, rabbit tibia is a suitable size and has only a limited amount of surrounding soft tissues allowing easier manipulation and accessibility. The rabbit tibial osteomyelitis model was the first attempted animal model of osteomyelitis (El-kamel

and Baddour, 2007; Sener et al., 2010) by injecting into the tibial medullary cavity, using an 18 gauge needle, 0.1 mL 5% sodium morrhuate, followed by 0.1 mL *S. aureus* suspension inoculated bacteria into the medullary canal. Detailed radiographic study of the infection demonstrated this to be a progressive chronic osteomyelitis with typical bone destruction, periosteal reaction, sequestra formation, and new bone formation.

The osteomyelitis radiologic signs are loss of trabecular pattern, which may be the first sign, lysis or destruction of bone appeared as an area of radiolucency within the bone, there was periosteal reaction and this may appear in 14 to 21 day after the infection and the periosteum often becomes elevated and there was sub-periosteal new bone formation and a sequestrum may be seen as fragment of cortical bone of normal opacity or it may appear more opaque than normal because it contrasted with the surrounding lucent zone. An involucrum was seen as an area of decreased bone opacity surrounding the sequestrum and also there was cortical destruction (The cortex became thin) (Kealy et al., 2011).

Staphylococcus aureus is the commonest aetiological agent implicated in acute and chronic osteomyelitis (Krogstad, 2009). *S. aureus* has an extraordinary capacity to adapt the hostile environment with a proven ability to develop resistance against antibiotics. Non-antimicrobial approaches to prevent and treat the infection have also been tried. Use of lysostaphin (Dajcs et al., 2001), antimicrobial peptides (Lawton et al., 2007), some natural products (tea, tree, oil) (Stapleton et al., 2007), anti-staphylococcal vaccines (Bubeck and Schneewind, 2008), and bacteriophages (Sulakvelidze and Kutter, 2005; Górski et al., 2007) are some of the alternative modalities to deal with *S. aureus* including methicillin resistant (MRSA) and vancomycin resistant (VRSA).

Previous *in vitro* studies have investigated the interaction between *S. aureus* and the cells responsible for bone remodeling, osteoblasts and osteoclasts. *S. aureus* is capable of invading and persisting within osteoblasts (Shi and Zhang, 2012). The interaction of *S. aureus* and osteoblasts leads to physiologic alterations that favor bone resorption by two primary mechanisms. First, *S. aureus*-infected osteoblasts incite osteoclastogenesis through increased expression of pro-inflammatory cytokines and the critical osteoclast-activating molecule receptor activator of NF-κB ligand (RANK-L) (Marriott et al., 2004; Somayaji et al., 2008; Claro et al., 2013). Second, *S. aureus* invasion of osteoblasts results in cell death (Alexander et al., 2003; Young et al., 2011). However, the specific staphylococcal factors that trigger osteoblast cell death or induce osteoclastogenesis are unknown. Furthermore, it has yet to be determined if these *in vitro* phenomena are responsible for pathogen-mediated bone destruction *in vivo*.

In the beginning of antibiotic era when antibiotic resistance was not reported, osteomyelitis caused by *S. aureus* could not be cured in all the (100%) cases. The possible reasons for the failure may be biofilm formation; low antibiotic levels achieved in bone tissue and reduced activity of antibiotics in bone and its marrow (Stengel et al., 2001; Lazzarini et al., 2005). In contrast to antibiotics, phages are known to penetrate the biofilm as they propagate in their bacterial host.

Many phages produce depolymerases that hydrolyze biofilm extracellular polymers and they can penetrate the inner layers of the biofilm by degrading components of the biofilm exopolymeric matrix. There is a report suggesting that the phages can enter the macrophages to kill the intracellular form (Broxmeyer et al., 2002). It is known that there is a decrease in lysis of the bacterial cells when they are in dormant state (Matsuzaki et al., 2005; Heilmann et al., 2012). This might be the reason for isolation of *S. aureus* till the third dose of the phage, *i.e.* up to 6th day of the therapy. However, single dose of the phage cocktail has been found to be effective in acute bacterial infection, *e.g.* septicaemia in mouse burn model caused by *Pseudomonas aeruginosa* (our unpublished data) as bacteria actively multiply in blood circulation.

CONCLUSIONS

S. aureus was the most frequent isolated bacteria from bone infection in all age patients. Sewage water was observed to be the best environmental source to get lytic phages with aggressive infective qualities and the isolated phages show high degree of lysis.

In vivo study showed that treatment with bacteriophage 3×10^8 PFU/ml intramuscularly was highly effective and infected rabbit cured with the first period of treatment in comparison with ceftaroline 40 mg/kg. All infected animals treated with phage showed no toxicological effect, and no other complications like anorexia or body weight loss when compared with Ceftaroline.

Radiographic finding showed that bone changes remained even when treatment course with antibiotic ended. All radiographical features of osteomyelitis had disappeared completely in case of treatment with specific phage intramuscularly.

ACKNOWLEDGEMENT

Medicine, Baghdad University, Baghdad, Iraq.

CONFLICT OF INTERESTS

There exists no conflict of interest.

AUTHORS' CONTRIBUTION

All the authors have contributed equally in terms of giving their technical knowledge to frame the article.

REFERENCES

•Aghazadeh M, Rahbar M, Monnavar MK, Moghadam FS (2009). Sensitivity pattern of methecillin resistant and methicillin sensitive *Staphylococcus aureus* isolates, against several antibiotics including tigecycline in Iran: A hospital study. Pak. J. Med. Sci. 25(3): 443-446.

•Aidan JS, Kuang Y, Kurimoto M, Yamamichi K, Iwano H, Tanji Y (2009). Isolation from sewage influent and characterization of novel *Staphylococcus aureus* bacteriophages with wide host ranges and potent lytic capabilities. Appl. Environ. Micobiol. 75(13): 4483–4490. https://doi.org/10.1128/AEM.02641-08

•Alexander EH, Rivera FA, Marriott I, Anguita J, Bost KL, Hudson MC (2003). *Staphylococcus aureus* induced tumor necrosis factor related apoptosis inducing ligand expression mediates apoptosis and caspase-8 activation in infected osteoblasts. BMC Microbiol. 3: 5. https://doi.org/10.1186/1471-2180-3-5

•Al-Geobory HA (2011). Comparative study between Methicillin resistant *Staphylococcus aureus* (MRSA) and Methicillin sensitive *Staphylococcus aureus* (MSSA) and detect the antimicrobial effects of some plant extracts on them, M.Sc. thesis. College of Science, Baghdad University, Iraq.

•AL-Jundiy AS (2005). Immunological study on TSS-1 toxin extracted from *Staphylococcus aureus* Isolated from infected wounds, Ph. D. thesis. College of Science, Al-Mustansyria University.

•Alt V, Bitschnau A, Osterling J, Sewing A, Meyer C, Kraus R, Meissner SA, Wenisch S, Domann E, Schnettler R (2006). The effects of combined gentamicin- hydroxyapatite coating for cementless joint prostheses on the reduction of infection rates in a rabbit infection prophylaxis model. Biomaterials. 27(26): 4627-4634. https://doi.org/10.1016/j.biomaterials.2006.04.035

•Ayliffe G (1997). The progressive intercontinental spread of methicillin- resistant *Staphylococcus aureus*. Clin. Infect. Dis. 24(1): 74-79. https://doi.org/10.1093/clinids/24.Supplement_1.S74

•Borysowski J, Gorski A (2008). Is phage therapy acceptable in the immunocompromised host? Int. J. Infect. Dis. 12(5): 466– 471. https://doi.org/10.1016/j.ijid.2008.01.006

•Boucher H, Miller LG, Razonable RR (2010). Serious infections caused by methicillin-resistant *Staphylococcus aureus*. Clin. Infect. Dis. 51(2): S183-S197. https://doi.org/10.1086/653519

•Brady RA, Leid JG, Kofonow J, Costerton JW, Shirtliff ME (2007). Immunoglobulins to surface associated biofilm immunogens provide a novel means of visualization of methicillin-resistant *Staphylococcus aureus* biofilms. Appl.

Environ. Microbiol. 73(20): 6612-6619. https://doi.org/10.1128/AEM.00855-07

•Broxmeyer L, Sosnowska D, Miltner E, Chacón O, Wagner D, McGarvey J (2002). Killing of *Mycobacterium avium* and *Mycobacterium tuberculosis* by a mycobacteriophage delivered by a nonvirulent Mycobacterium: a model for phage therapy of intracellular bacterial pathogens. J. Infect. Dis. 186(8): 1155-1160. https://doi.org/10.1086/343812

•Brüssow H, Desiere F (2001). Comparative phage genomics and the evolution of Siphoviridae: insights from dairy phages. Mol. Microbiol. 39(2): 213-222. https://doi.org/10.1046/j.1365-2958.2001.02228.x

•Bubeck WJ, Schneewind O (2008). Vaccine protection against *Staphylococcus aureus* pneumonia. J. Exp. Med. 205(2): 287-294. https://doi.org/10.1084/jem.20072208

•Catry B, Van Duijkeren E, Pomba MC, Greko C, Moreno MA, Pyörälä S, Ruzauskas M, Sanders P, Threlfall EJ, Ungemach F, Törneke K, Munoz-Madero C, Torren-Edo J (2010). Scientific Advisory Group on Antimicrobials (SAGAM). Reflection paper on MRSA in food- producing and companion animals: epidemiology and control options for human and animal health. Epidemiol. Infect. 138(5): 626-644. https://doi.org/10.1017/S0950268810000014

•Ce´dric J, Caillon J, Batard E, Le-Mabecque V, Amador G, Ge Y, Biek D, Potel G (2010). Evaluation of the in vivo efficacy of intramuscularly administered ceftaroline fosamil, a novel cephalosporin, against a methicillinresistant Staphylococcus aureus strain in a rabbit endocarditis model. J. Antimicrob. Chemother. 65(10): 2264-2265. https://doi.org/10.1093/jac/dkq328

•CDC (2005). Health care associated methicillin resistant *Staphylococcus aureus* (HAMRSA). Available at: http://www.cdc.gov/ncidod/dhqp/armrsa.html

•Chandan K, Mishra RR, Saraf SK, Kumar M, Srivastav AK, Nath G (2016). Phage therapy of staphylococcal chronic osteomyelitis in experimental animal model. Indian J. Med. Res. 143(1): 87-94. https://doi.org/10.4103/0971-5916.178615

•Chihara S, Segreti J (2010). Osteomyelitis. Dis. Mon. 56(1): 5-31. https://doi.org/10.1016/j.disamonth.2009.07.001

•Chomel BB, Sun B (2011). Zoonoses in the Bedroom. Emerg. Infect. Dis. 17(2): 167-172. https://doi.org/10.3201/eid1702.101070

•Claro T, Widaa A, McDonnell C, Foster TJ, O'Brien FJ, Kerrigan SW (2013). *Staphylococcus aureus* protein a binding to osteoblast tumour necrosis factor receptor 1 results in activation of nuclear factor kappa B and release of interleukin-6 in bone infection. Microbiol. 159(1): 147–154. https://doi.org/10.1099/mic.0.063016-0

•CLSI (2012). Clinical Laboratory Standards Institute: Performance standards for antimicrobial susceptibility testing; Twenty-second Informational Supplement. CLSI document M100-S22. CLSI, Wayne, PA.

•Dajcs JJ, Thibodeaux BA, Hume EB, Zheng X, Sloop GD, O'Callaghan RJ (2001). Lysostaphin is effective in treating methicillin-resistant *Staphylococcus aureus* endophthalmitis in the rabbit. Curr. Eye Res. 22(6): 451-457. https://doi.org/10.1076/ceyr.22.6.451.5486

•Deresse D, Solomon G, Yihdego D (2012). Antibiotic-resistance Staphylococcus aureus isolated from cow'smilk in the Hawassa area, South Ethiopia. Annls. Clin. Microbiol. Antimicrobiol. 11(26): 1-6.

•Dias RS, Eller MR, Duarte VS, Pereira ÂL, Silva CC, Mantovani

HC, Oliveira LL, Silva E, de AM, Paula SOD (2013). Use of phages against antibiotic-resistant *Staphylococcus aureus* isolated from bovine. J. Anim. Sci. 91(8): 3930-3939. https://doi.org/10.2527/jas.2012-5884

• Eid AJ, Berbari EF (2012). Osteomyelitis: a review of pathophysiology, diagnostic modalities and therapeutic options. J. Med. Liban. 60(1): 51-60.

• El-Kamel AH, Baddour MM (2007). Gatifloxacin biodegradable implant for treatment of experimental osteomyelitis: *in vitro* and *in vivo* evaluation. Drug Deliv. 14(6): 349-356. https://doi.org/10.1080/10717540601098716

• Elliot I, Skerrtt G (2010). Small animal radiography and MRI. Wiley Blackwell, Oxford, UK.

• EUCAST (2009). Breakpoint tables for interpretation of MICs and zone diameters. The European Committee on Antimicrobial Susceptibility Testing. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/EUCASTbreakpointsv1.0

• Felten A, Grandry B, Lagrange PH, Casin I (2002). Evaluation of three techniques for detection of low-level methicillinresistant Staphylococcus aureus (MRSA) a disk diffusion method with cefoxitin and moxalactam, the Vitek 2 system, and the MRSA screen latex agglutination test. J. Clin. Microbiol. 40: 2766–2771. https://doi.org/10.1128/JCM.40.8.2766-2771.2002

• Fernande CJ, Lorna AF, Collignon P (2005). Cefoxitin resistance as a surrogate marker for the detection of methicillin-resistant *Staphylococcus aureus*. J. Antimicrob. Chemother. 55: 506–510. https://doi.org/10.1093/jac/dki052

• García P, Madera C, Martinez B, Rodríguez A, Suárez JE (2009). Prevalence of bacteriophages infecting *Staphylococcus aureus* in dairy samples and their potential as biocontrol agents. J. Dairy Sci. 92: 3019-3026. https://doi.org/10.3168/jds.2008-1744

• García P, Madera C, Martínez B, Rodríguez A (2007). Biocontrol of *Staphylococcus aureus* in curd manufacturing processes using bacteriophages. Int. Dairy J. 17: 1232-1239. https://doi.org/10.1016/j.idairyj.2007.03.014

• Garcia-Arias M, Balsa A, Mola EM (2011). Septic arthritis. Best Pract. Res. Clin. Rheumatol. 25: 407–421. https://doi.org/10.1016/j.berh.2011.02.001

• Górski A, Borysowski J, Miedzybrodzki R, Weber- Dabrowska B (2007). Bacteriophages in medicine. In: Mc Grath S, van Sinderen D, eds. Bacteriophage: Genetics and Microbiology. *Caister Academic Press*, Norfolk, UK. Pp. 125-158.

• Gorwitz RJ, Jernigan DB, Powers JH, Jernigan JA, (2006). Convened experts' meeting on management of MRSA in the community. Strategies for clinical management of MRSA in the community: Summary of an experts' meeting convened by the Centres for Disease Control and Prevention. Available at http://www.cdc.gov/ncidod/dhqp/ar_mrsa_ca.html (Accessed 2 Feb 2010).

• Heilmann S, Sneppen K, Krishna S (2012). Coexistence of phage and bacteria on the boundary of self-organized refuges. Proc. Natl. Acad. Sci. U.S.A. 109: 12828-12833. https://doi.org/10.1073/pnas.1200771109

• Jaime M, and Tiffany F, (2003). Isolation of *E. coli* bacteriophage. Phage Method. 2: 266-276.

• Jassim SAA, Abdulamir AS, Abu Bakar F (2012). Novel phage-based bio processing of pathogenic *E. coli* and its biofilms. World J. Microbiol. Biotechnol. 28: 47-60. https://doi.org/10.1007/s11274-011-0791-6

• Jorge LS, Chueire AG, Rossit AR (2010). Osteomyelitis: a current challenge. Braz. J. Infect. Dis. 14(3): 310- 315. https://doi.org/10.1590/S1413-86702010000300020

• Kalmus P, Aasmäe B, Kärssin A, Orro T, Kask K (2011). Udder pathogens and their resistance to antimicrobial agents in dairy cows in Estonia. Acta. Vet. Scand. 53: 54. https://doi.org/10.1186/1751-0147-53-4

• Kealy JK, Mcallister H, John PG (2011). Diagnostic radiography and ultrasonography of small animals, 5th adition. Pp. 433-436.

• Krogstad P (2009). Osteomyelitis. In: Feigin RD, Cherry JD, Demmler-Harrison GJ, Kaplan SL, editors. Textbook of pediatric infectious diseases. 6th ed. Saunders Elsevier, PA, USA. Pp. 725-742.

• Kwiatek M, Parasion S, Mizak L, Gryko R, Bartoszcze M, Kocik J (2012). Characterization of a bacteriophage, isolated from a cow with mastitis, that is lytic against *Staphylococcus aureus* strains. Arch. Virol. 157: 225–234. https://doi.org/10.1007/s00705-011-1160-3

• Kysela DT, Turner PE (2007). Optimal bacteriophage mutation rates for phage therapy. J. Theoret. Biol. 249(3): 411–421. https://doi.org/10.1016/j.jtbi.2007.08.007

• Lawton EM, Ross RP, Hill C, Cotter PD (2007). Two-peptide lantibiotics: a medical perspective. Mini Rev. Med. Chem. 7: 1236-1247. https://doi.org/10.2174/138955707782795638

• Lazzarini L, Lipsky BA, Mader JT (2005). Antibiotic treatment of osteomyelitis: what have we learned from 30 years of clinical trials? Int. J. Infect. Dis. 9: 127-138. https://doi.org/10.1016/j.ijid.2004.09.009

• Lee JH (2003). Methicillin (Oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. Appl. Environ. Microbiol. 69(11): 6489-6494. https://doi.org/10.1128/AEM.69.11.6489-6494.2003

• Li B, Jiang B, Boyce BM, Lindsey BA (2009). Multilayer polypeptide nanoscale coatings incorporating IL-12 for the prevention of biomedical device-associated infections. Biomaterials. 30: 2552-2558. https://doi.org/10.1016/j.biomaterials.2009.01.042

• Loeffler A, Pfeiffer DU, Lindsay JA, Magalhaes RJ, Lloyd DH (2011). Prevalence of and risk factors for MRSA carriage in companion animals: a survey of dogs, cats and horses. Epidemiol. Infect. 139(7): 1091-1028. https://doi.org/10.1017/S095026881000227X

• Longping L, Zhiying Z (2014). Isolation and characterization of a virulent bacteriophage SPW specific for *Staphylococcus aureus* isolated from bovine mastitis of lactating dairy cattle. Mol. Biol. Rep. 41: 5829-5838. https://doi.org/10.1007/s11033-014-3457-2

• Mansura SM, Azhar S, Azharuddin S and Tambe S (2015). Harnessing the power of bacteriophage for pathogen reduction in wastewater. Int. J. Curr. Microbiol. Appl. Sci. 2: 152-161.

• Marriott I, Gray DL, Tranguch SL, Fowler VG Jr, Stryjewski M, Scott Levin L, Hudson MC and Bost KL (2004). Osteoblasts express the inflammatory cytokine interleukin-6 in a murine model of *Staphylococcus aureus* osteomyelitis and infected human bone tissue. Am. J. Pathol. 164: 1399–1406. https://doi.org/10.1016/S0002-9440(10)63226-9

• Marwa B, Sabri MSC, Ahmed SA (2014). *In vitro* study on using bacteriophages in the treatment of pathogenic *Escherichia coli* in Iraq. Iraqi J. Med. Sci. 12(2): 131-138.

• Mathews CJ, Weston VC, Jones A, Field M, Coakley G (2010). Bacterial septic arthritis in adults. Lancet, 375: 846–855.

https://doi.org/10.1016/S0140-6736(09)61595-6

• Matsuzaki S, Rashel M, Uchiyma J, Ujihara T, Kuroda M, Ikeuchi M, Fujieda M, Wakiguchi J, Imai S (2005). Bacteriophage therapy: a revitalized therapy against bacterial infectious diseases. J. Infect. Chemother. 11(5): 211–219. https://doi.org/10.1007/s10156-005-0408-9

• Middleton JR, Fales WH, Luby CD, Oaks JL, Sanchez S, Kinyon JM, Wu CC, Maddox CW, Welsh RD, Hartmann F (2005). Surveillance of *Staphylococcus aureus* in veterinary teaching hospitals. J. Clin. Microbiol. 43(6): 2916-2919. https://doi.org/10.1128/JCM.43.6.2916-2919.2005

• Morgan M (2008). Methicillin resistant *Staphylococcus aureus* and animals: zoonosis or humanosis? J. Antimicrob. Chemother. 62(6): 1181-1187. https://doi.org/10.1093/jac/dkn405

• NCCLS (2000). National Committee for Clinical Laboratory Standards. Approved Standard M7-A5: Methods for Dilution Antimicrobial Susceptibility Test for Bacteria that Grow Aerobically, 5th Ed.

• Niu YD, McAllister TA, Xu Y, Johnson RP, Stephens TP, Stanford K (2009). Prevalence and impact of bacteriophages on the presence of *Escherichia coli* O157:H7 in feedlot cattle and their environment. Appl. Environ. Microbiol. 75(5): 1271-1278. https://doi.org/10.1128/AEM.02100-08

• Nuh KILI (2004). A comparison between Medetomidine-Ketamine and Xylazine- Ketamine anaesthesia in rabbits. Turk. J. Vet. Anim. Sci. 28: 921-926.

• Oot RA, Raya RR, Callaway TR, Edrington TS, Kutter EM, Brabban AD (2007). Prevalence of *Escherichia coli* O157 and O157:H7-infecting bacteriophages in feedlot cattle feces. Lett. Appl. Microbiol. 45: 445–453. https://doi.org/10.1111/j.1472-765x.2007.02211.x

• Rabillard M, Souchu L, Niebauer GW, Gauthier O (2011). Haematogenous osteomyelitis: clinical presentation and outcome in three dogs. Vet. Comp. Orthop. Traumatol. 24: 146-150. https://doi.org/10.3415/VCOT-10-07-0116

• Rich M, Roberts L (2004). Methicillin-resistant *Staphylococcus aureus* isolates from companion animals. Vet. Rec. 154(10): 310.

• Sandeep K (2006). Bacteriophage precision drug against bacterial infections. Curr. Sci. 90(5): 361–363.

• Sanders ER (2012). Aseptic laboratory techniques: Plating methods. Microbiology, immunology and molecular genetics, University of California, Los Angeles. https://doi.org/10.3791/3064

• Santos TM, Ledbetter EC, Caixeta LS, Bicalho ML, Bicalho RC (2011). Isolation and characterization of two bacteriophages with strong in vitro antimicrobial activity against *Pseudomonas aeruginosa* isolated from dogs with ocular infections. Am. J. Vet. Res. 72: 1079-1086. https://doi.org/10.2460/ajvr.72.8.1079

• SAS (2012). Statistical analysis system, User's Guide. Statistical version 9.1. SAS. Inst. Inc., Cary., N.C., USA.

• Sener M, Kazimoglu C, Karapinar H, Gunal I, Afsar I, Karatas Sener AG (2010). Comparison of various surgical methods in the treatment of implant-related infection. Int. Orthop. 34: 419-423. https://doi.org/10.1007/s00264-009-0750-0

• Shasha SM, Sharon N, Inbar M (2004). Bacteriophages as antibacterial agents. Harefuah. 143(2): 121–125.

• Shi D, Hao Y, Zhang A, Wulan B, Fan X (2010). Antimicrobial resistance of *Staphylococcus aureus* isolated from bovine mastitis in China. Transbound. Emerg. Dis. 57: 221-224. https://doi.org/10.1111/j.1865-1682.2010.01139.x

• Shi S, Zhang X (2012). Interaction of *Staphylococcus aureus* with

osteoblasts. Exp. Ther. Med. 3(3): 367-370.

• Skov R, Smyth R, Clausen MAR, Larsen N, Frimodt-Møller B, Olsson-Liljequist, Kahlmeter G (2003). Evaluation of a 30 mg cefoxitin disc on Iso-Sensitest agar for detection of methicillin resistant Staphylococcus aureus. J. Antimicrob. Chemother. 52: 204–207. https://doi.org/10.1093/jac/dkg325

• Somayaji SN, Ritchie S, Sahraei M, Marriott I, Hudson MC (2008). *Staphylococcus aureus* induces expression of receptor activator of NF-kappaB ligand and prostaglandin E2 in infected murine osteoblasts. Infect. Immunity. 76: 5120–5126. https://doi.org/10.1128/IAI.00228-08

• Sowmya N, Savitha S, Mallure S, Mohanakrishnan K, Sumathi G, Arumugam P (2014). A two year study of spectrum of bacterial isolates from wound infections by aerobic culture and their antibiotic pattern in a tertiary care center. Int. J. Curr. Microbiol. Appl. Sci. 3(8): 292-295.

• Stapleton PD, Shah S, Ehlert K, Hara Y, Taylor PW (2007). The beta-lactam- resistance modifier (-)- epicatechin gallate alters the architecture of the cell wall of *Staphylococcus aureus*. Microbiol. 153: 2093-2103. https://doi.org/10.1099/mic.0.2007/007807-0

• Stengel D, Bauwens K, Sehouli J, Ekkernkamp A, Porzsolt F (2001). Systematic review and meta-analysis of antibiotic therapy for bone and joint infections. Lancet Infect. Dis. 1: 175-188. https://doi.org/10.1016/S1473-3099(01)00094-9

• Sulakvelidze A, Kutter E (2005). Bacteriophage therapy in humans. In: Kutter E, Sulakvelidze A, editors. Bacteriophages: Biology and Application. CRC Press, Boca Raton, FL. Pp. 381-436.

• Sulakvelidze A, Alavidze Z, Morris GJ Jr (2001). Bacte-riophage therapy. Antimicrob. Agents Chemother. 45(3): 649-659. https://doi.org/10.1128/AAC.45.3.649-659.2001

• Swenson JM, Fred CT, Cefoxitin Disk Study Group (2005). Results of disk diffusion testing with cefoxitin correlate with presence of *mecA* in *Staphylococcus* spp. J. Clin. Microbiol. 43(8): 3813-3823. https://doi.org/10.1128/JCM.43.8.3818-3823.2005

• USEPA (200l). U.S. Environmental Protection Agency's (EPA). Method 1601: Male-specific (F+) and Somatic Coliphage in Water by Two-Step Enrichment Procedure. EPA- 821-R-01-030. Office of Water, Washington D.C.

• Vinodkumar CS, Kalsurmath S, Neelagund YF (2008). Utility of lytic bacteriophage in the treatment of multidrug- resistant *Pseudomonas aeruginosa* septicemia in mice. Indian J. Pathol. Microbiol. 5(3): 360– 366. https://doi.org/10.4103/0377-4929.42511

• Weese JS, Archambault M, Willey BM, Hearn P, Kreiswirth BN, Said-Salim B, McGeer A, Likhoshvay Y, Prescott JF and Low DE (2005). Methicillin-resistant *Staphylococcus aureus* in horses and horse personnel, 2000-2002. Emerg. Infect. Dis. 11(3): 430-435. https://doi.org/10.3201/eid1103.040481

• Wills QF, Kerrigan C, Soothill JS (2005). Experimental bacteriophage protection against *Staphylococcus aureus* abscesses in a rabbit model. Antimicrob. Agents Chemother. 49: 1220-1221. https://doi.org/10.1128/AAC.49.3.1220-1221.2005

• Yoon SJ, Choi YJ, Lee SY, Son JS, Jun SY, Kang SH (2010). Bacteriophage having killing activity specific to *Staphylococcus aureus*. US patent 8,071,352.

• Young AB, Cooley ID, Chauhan VS, Marriott I (2011). Causative agents of osteomyelitis induce death domain-

containing TNF-related apoptosis-inducing ligand receptor expression on osteoblasts. Bone. 48: 857–863. https://doi.org/10.1016/j.bone.2010.11.015

• Zeidan IA (2005). Genetic and bacteriologic study to *Staphylococcus aureus* isolated from clinical specimens and resistant to Vancomycin antibiotic. M.Sc. thesis. College of Science, Baghdad University.

• Zuluaga AF, Galvis W, Saldarriaga JG, Agudelo M, Salazar BE, Vesga O (2006). Etiologic diagnosis of chronic osteomyelitis: a prospective study. Arch. Int. Med. 166(1): 95–100. https://doi.org/10.1001/archinte.166.1.95