Isolation of Lytic Bacteriophage Cocktail Against Methicillin-resistant *Staphylococcus Aureus* Isolated From Human Skin Infections

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a *S. aureus* that resistant to β-lactam antibiotics (e.g., Cefoxitin and Oxacillin). MRSA has a tremendous capacity to develop resistance to other classes of antibiotics and forming a real threat to patients. The process of exploring a new tactic of non-antibiotic treatments has become an urgent need. A bacteriophage is one of the possible treatments that strongly suggested. Bacteriophages are viruses that infect bacteria as a natural host with a bactericidal capability against multidrug-resistant bacteria that do not respond to conventional antibiotics. The current study investigates the lytic efficacy of phage-cocktail in vitro, specifically against S. aureus isolated from skin infections and find out the possible association of phage-antibiotic resistance. A total of 43 isolates of Methicillin-resistant staphylococcus aureus were isolated from skin infections. The isolates are distributed as (10 isolates of burn, 4 isolates of diabetic foot ulcer, 7 isolates of surgical wounds, 3 isolates of pressure ulcer, and 19 of skin and soft tissue infection). The isolates exhibited variant antibiotic susceptibility against 12 antibiotics (Cefoxitin FOX, Vancomycin VAN, Oxacillin OX, Rifampin RA, Chloramphenicol C, Nitrofurantoin F, Clindamycin DA, Azithromycin AZM, Amikacin AK, Trimethoprim-sulfamethoxazole SXT, Ciprofloxacin CIP, and Gentamicin CN). A bacteriophage cocktail was isolated using a phage-enrichment technique, high titer phage lysate (5*109 pfu/ml) was obtained and investigated against 43 MRSA isolates. The phage-cocktail showed high specificity to S. aureus but variable susceptibility to 43 MRSA isolates. It was observed that there was no association ($p > 0.05$) between phage and antibiotic resistance of (FOX, OX, VAN, RA, C, F, and DA) where the significant association was observed ($p < 0.05$) with (AZM, AK, SXT, CIP, and CN). Significantly, the more antibiotic-resistant isolates exhibited more sensitivity to phage-cocktail, which represents a promising alternative to antibiotics that do not affect with increasing antibiotic resistance.

Keywords: MRSA, Phage-antibiotic susceptibility, Phage-cocktail, Wound infections.

INTRODUCTION

Staphylococcus aureus is a gram-positive pathogen associated with manifold infections. It has numerous defense mechanisms, and virulence factors made it able to invade the host body and evade the immune system. *S. aureus* is able to infect normal individuals and cause progressive fatal infections in contrast to other pathogens such as candida or pseudomonas that mostly affect immune-compromised individuals. Toxic shock syndrome (TSS) appearance 30 years ago and continuing evolution of virulence and antibiotic resistance are examples of percussive ability to change its epidemiology and clinical manifestations.[1] MRSA is one of the major nosocomial pathogens that is classified as hospital-associated, community-associated, and livestock-associated MRSA according to the differences in risk and virulence factors as well as in antibiotic resistance.[2] MRSA represents a formidable threat with high morbidity and mortality. It affects a different part of the human body and causes various infections such as bacteremia, endocarditis, osteomyelitis, and skin and soft tissue infection SSTI. A wide range of virulence factors is expressed by *S. aureus*, including capsule and protein, to evade the immune system and hyaluronidase to invade tissues in addition to hemolysis and leukocidins.[3] Wounds are a fertile niche for the growth of pathogenic bacteria as it contains an aggregation of protein exudate and accumulation of necrotic tissues. With the emergence of new strains of *S. aureus*, it was recorded increased numbers of skin infections over the last years with increasing antibiotic resistance by MRSA.[4]

The mounting threat of antibiotic resistance by multidrug resistant bacteria (superbugs) calls scientists to scan for alternatives to antibiotics. Bacteriophages are one of the under experience alternatives. Bacteriophages are viruses that infect and kill bacteria. They are an obligate intracellular parasite that multiplies and lyse bacteria to release new progeny.[5]

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Bacteriophages have many advantages over antibiotics that make it one of the best possible strategies to mitigate the challenge of increasing resistance to antibiotics.[6] This study aims to isolate bacteriophages against MRSA associated with human skin infections and assess bacteriophage host range and bacterial phage-susceptibility in vitro.

MATERIALS AND METHODS

Study Population

One hundred patients with different types of skin infections were targeted in the study; swabs were collected from different resources in Al-Muthanna Province south of Iraq (Al-Hussein Teaching Hospital, Samawa Women and Children Hospital, and outpatient clinics). Swabs were collected with irrespective of age and sex and according to specific criteria, from patients before antibiotic therapy and from wounds that were failed to heal over a long period, deteriorating or clinically infected, which can be predicted from fever, pain, redness, and drainage.

Study Preparation

For open wounds and surgical infection, the superficial area was cleaned with sterile saline, superficial exudates, and overlying debris were removed. A swab was gently rolled over the surface of the wound approximately five times, focusing on an area where there is evidence of pus or inflamed tissue. For burn wounds, different areas were swabbed because the microorganisms may not be distributed evenly. Pus was aspirated from lesion by syringe and needle at the time of incision, debridement of an infected wound, or drainage.[7]

Bacterial Isolation

Wound samples were collected using swab with a stuart transport medium (specimen can be stored in the sampling tube for 72h at 4°C). Swabs were cultured in blood agar for the recovery of potential pathogens, including S. aureus. Gram-positive cocci, Beta-hemolytic, and catalase-positive colonies transferred to mannitol salt agar. Mannitol fermenter and coagulase-positive colonies are diagnosed as *S. aureus.*[8] Antibiotic disc diffusion test was performed to detect MRSA that resistant to Cefoxitin (Fox).[10] MRSA isolates were confirmed by VITEK 2 Compact and MRSA Chromogenic agar. MRSA isolates were kept as master stock in 4ml of BHI broth with 20% glycerol at -20C.

Antibiotic Susceptibility Test

The antibiotic susceptibility of Methicillin-resistant *S. aureus* isolates was determined by the disk diffusion method of[9] against 12 antibiotics, and the result was interpreted according to CLSI (2017).[10]

Phage Isolation

Phage isolation was performed using bacteriophage enrichment protocol with modification in the sample and bacterial inocula size and in lysate processing.[11] 100 ml of untreated sewage water were collected at different times in well-closed containers. Sewage water first was filtered through filter paper and was kept overnight at 4°C with a few drops of chloroform.

Overnight supernatant was centrifuge 6000 rpm for 15 min to remove bacteria and debris. Centrifuged and filtered sewage water was collected in 250 ml flask, 100 ml of 2X BHI broth (supplemented with 2 mM CaCl2) was added with 100 μL of overnight MRSA isolate and incubated overnight 37°C with agitation. The enriched sample was obtained, 100mL of culture was transferred to centrifuge tubes (class or solvent-resistant plastic) with two drops of chloroform of each tube, centrifuged at 6000 rpm for 15 min, the supernatant was collected using a disposable pipet and filtered through 0.45μm and 0.22μm Millipore syringe filter, respectively. The lysate was checked for the presence of bacteriophage using the spot test.[12]

Phage Purification, Propagation, and Enumeration

The purification and propagation of bacteriophage were done by serial plaques picking and culturing tell homogenized plaques morphology was obtained.[11] The enumeration of bacteriophage in the crude lysate was determined by the double-agar-overlay technique.[13]

Bacteriophage Stock

Two methods were used to store bacteriophage stock. The first method was to preserve bacteriophage within infected *S. aureus* cells. In brief, 5 mL of overnight MRSA culture at 37 °C in BHI broth supplemented with (10 mM of MgSO4 and 10 mM of CaCl2) was obtained and then infected with bacteriophage at multiplicity of infection (M.O.I.) between 0.1 and 0.5 and kept 15 min without shaking to allow phage adsorption then 15% of glycerol was added and stored at -80 °C for long storage. The second method was achieved by picking from two to four homogenized plaques by sterile cotton swab and suspended in 1 ml of SM buffer (100 mM NaCl, 8 mM MgSO4·7H2O, and 50 mM Tris–HCl, pH 7.5) then centrifuged at 10,000 rpm for 10 min and two drops of chloroform was added and kept at 4°C for the purpose of short term storage.[14]

Bacteriophage Host Range and Bacterial Susceptibility

Bacteriophage cocktail was tested against different hosts, including (*Staphylococcus aureus, Staphylococcus saprophyticus, Staphylococcus epidermidis, Escherichia coli, and Pseudomonas aeruginosa*) as well as MRSA isolates were tested to phage susceptibility by Spot test. Briefly, an overnight culture of the tested host was streaking on nutrient agar and left inverted 10 min at room temperature to dry, the plate was divided into four parts by marker, and one drop of phage-cocktail was dropped in three parts, and one part was left as a control.[15]

STATISTICAL ANALYSIS

Statistical analysis was perform using the Chi-Square test of Independence SPSS Statistics (version 23) to estimate the significant association between phage and antibiotic resistance.[33]

RESULTS AND DISCUSSION

During a period from January to April 2019, a total of 100 swabs of different types of skin infections were processed

to identify MRSA. 60 out of 100 samples were diagnosed as *S. aureus* and 43 (70%) out of 60 *S. aureus* isolates were identified as MRSA according to the cultural properties and biochemical tests confirmed by VITEK 2 Compact and MRSA Chromogenic agar (Condalab, CATALOGUE NUMBER: 1423) (Figure 1).

The obtained percentage of MRSA was close to Al-Khafaji[17] and Montazeri *et al.*,[18] but less than Sarhan *et al.*,[19] and more than Al-Azawi,[20] which perhaps attributed to the different number of patients and limited sources of infection. MRSA was the main causative agent of wound infections in Erbil City, north of Iraq.[21] *P. aeruginosa* and MRSA are the most prevalence pathogens in wound infections as well as the dermatological infections by MRSA were expected to rise sharply over the next years.[22,23] In a previous study by Al-saadi, and Kadhim, it was revealed that the incidence of MRSA in Al-Hussain Teaching Hospital of Al-Samawa City was 30% as the most prevalence diagnosed pathogen isolated from walls,

floors, and burn infections.[24] The percentages of diagnosed isolates are shown in (Figure 2) related to the site and number of infections, MRSA infected wounds of Skin and soft tissue infection SSTI was the highest at 19.44% while pressure ulcer infection was the lowest at 3.4% which may due to the different numbers of patients included in the study.

MRSA isolates exhibited diverse antibiotic susceptibility patterns (Figure 3). The antibiotic profile of MRSA was determined using the disk diffusion method, according to CLSI (2017) [10]. The detection of methicillin-resistance *S. aureus* by resistance to Cefoxitin using the disc diffusion method was 100% accurate.[25] Cefoxitin has higher sensitivity than Oxacillin to detect MRSA, and thus it can be used as a surrogate for the Oxacillin.[26]

The isolation of lytic bacteriophage against MRSA found too complicated, and many modifications had been needed to detect lytic phages. Mattila *et al.*,[11] mentioned the isolation of MRSA phages were more difficult in
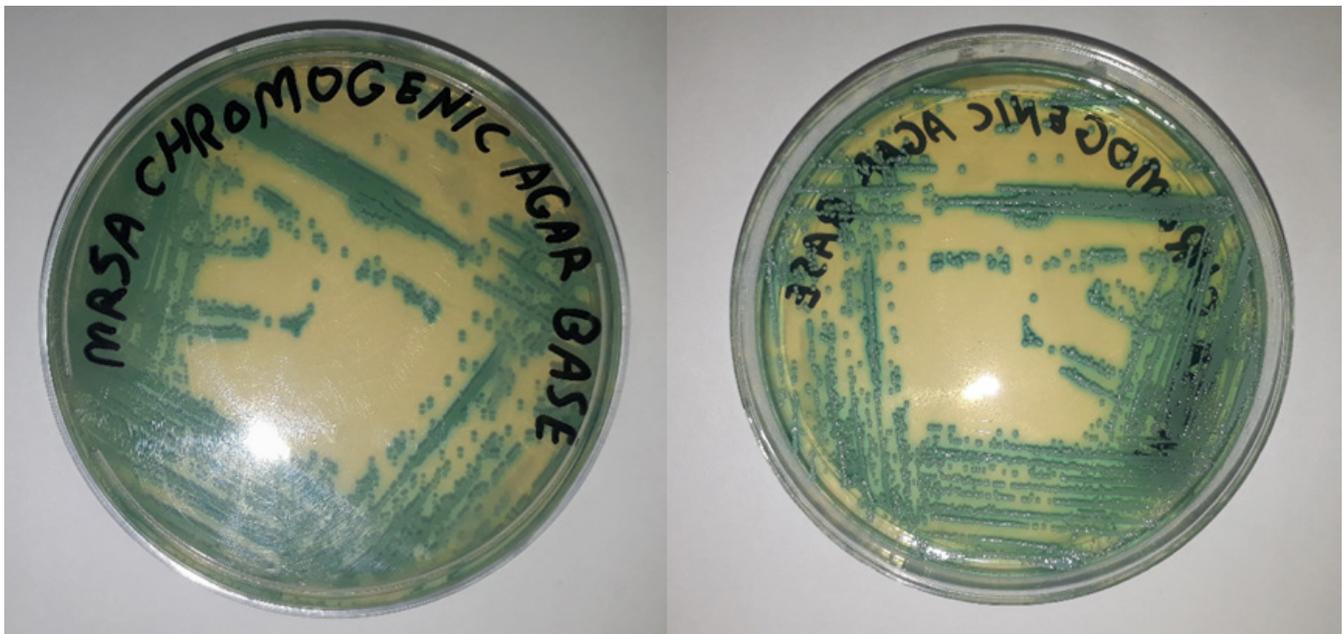

Figure 1: The alpha-glucosidase produced by *S. aureus* cleaves the chromogenic substrate and gives a greenish color to the *S. aureus* colonies. The Cefoxitin inhibits the growth of *S. aureus* sensitive to methicillin and selectively grow MRSA.
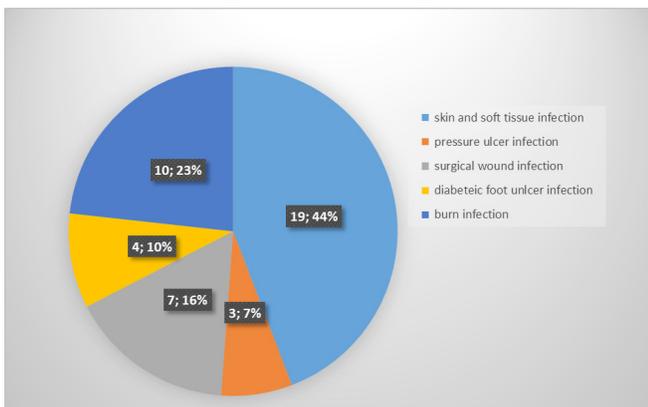

Figure 2: Percentage of MRSA isolates from clinical samples of different wound infections
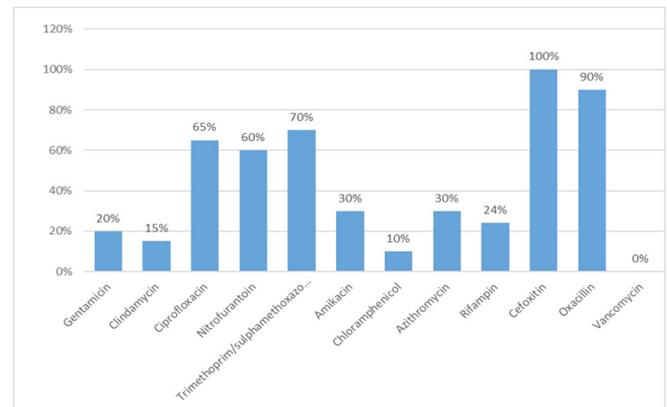

Figure 3: Antibiotic susceptibility profile of 43 MRSA isolates against 12 antibiotics used to MRSA treatment.

comparison with other pathogens (*P. aeruginosa, Salmonella, Klebsiella pneumonia, Acinetobacter baumannii*, extended spectrum beta-lactamase *E. coli*, and vancomycin resistant *Enterococcus*). It can be attributed to the existence of the vast majority of *S. aureus* phage as temperate (lysogenic) non-virulence (lytic) phages.[27] In a previous study by Alsaffar and Jarallah,[32] it was recommended to use more than one bacterial isolate as a phage host to increase the probability of phage retrieval from sewage samples. More than one type of lytic bacteriophages (cocktail) had been isolated against MRSA by picking up more than one morphological type of plaques and mixing together to produce a bacteriophage cocktail. A phage cocktail has more capability to infect multiple host strains.[16] The resistance to bacteriophages can be reduced by utilizing of phage mixture (cocktail) that targets various host cell receptors, and the possibility to develop resistance against phage-cocktail remains less than single phage.[28] The propagation, purification, and enumeration of bacteriophage were achieved using a double-agar overlay method (Figure 4) until high titer phage-cocktail lysate ($5*10^9$ pfu/ml) had been obtained. Bacteriophage cocktail showed high specificity to

S. aureus (Figure 5 and Table 1), while different susceptibility was revealed by MRSA isolates (Figure 6).

The co-therapy of phage and antibiotics had been noted to decrease resistant mutants and increase treatment effectiveness to treat MRSA that do not respond to conventional antibiotics.[29] The resistance to phages and antibiotics are evolving independently, and the exploitation of phage-antibiotic combination represents a promising treatment strategy.[30,31] No association ($p > 0.05$) was found between phage and antibiotic resistances of (Rifampin RA, Chloramphenicol C, Nitrofurantoin F, and Clindamycin DA). At the same time, it was a significant association ($p < 0.05$) between phage and antibiotic resistance of (Azithromycin AZM, Amikacin AK, Trimethoprim-sulfamethoxazole SXT, Ciprofloxacin CIP, and Gentamicin CN) as shown in (Table 2).

When isolates become more resistant to antibiotics, they become more sensitive to phage in case of (AZM, Ak, SXT, and CIP) (Figures 7, a, b, c, and d) except for CN. The Gentamicin-resistant isolates became more resistance to phages, as shown in (Figure 7, e). Statistically, it was revealed that more antibiotic-resistance was exhibited by phage-sensitive isolates ($p < 0.05$), as shown in (Figure 8 and Table 3), where isolates that have resistance to more than six types of antibiotics were more sensitive to bacteriophage. The pre-exposure of *S.*
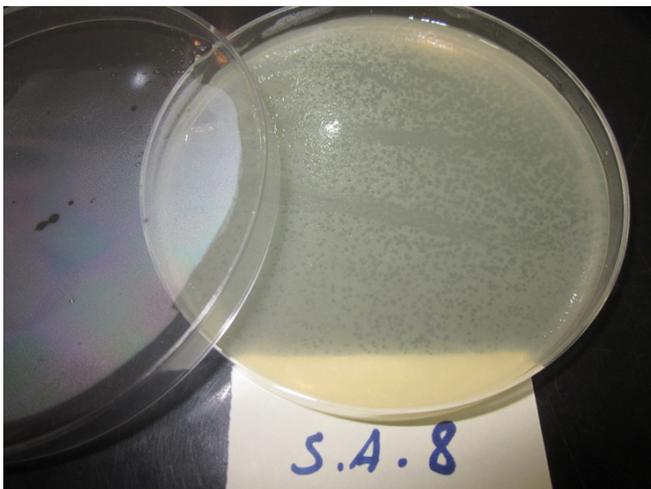

Figure 4: The double-agar overlay method shows bacteriophage plaques formed on MRSA plates after 24h at 37 °C incubation.
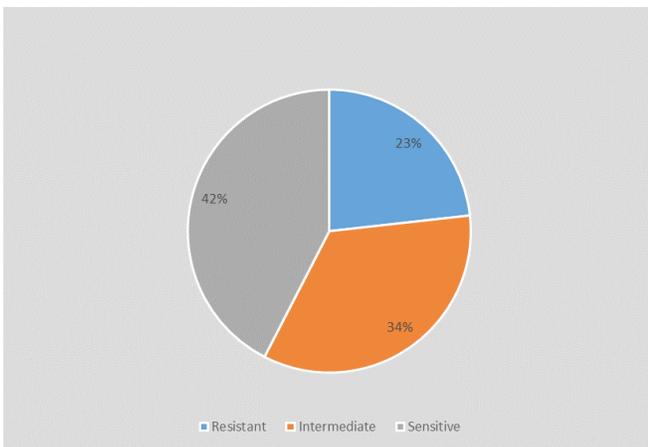

Figure 6: The susceptibility percentage of MRSA isolates to a bacteriophage cocktail regardless of isolates sources.
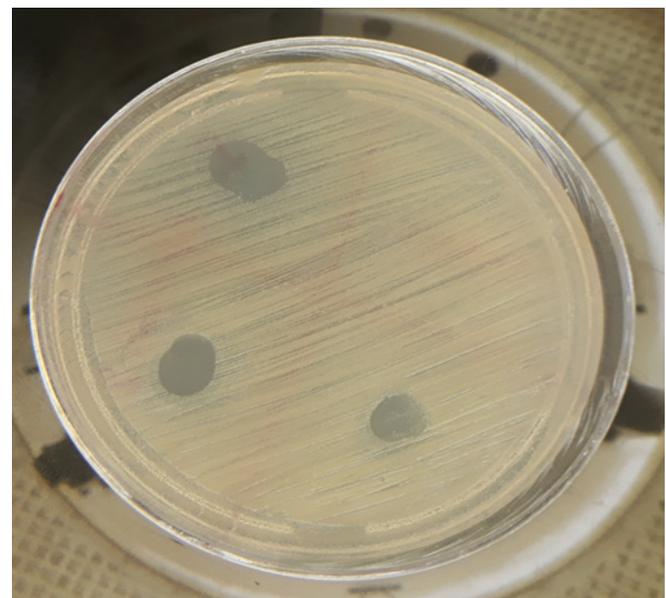

Figure 5: Spot test shows MRSA bacteriophage cocktail with high specificity to MRSA.

Table 1: The host range of Bacteriophage cocktail against five bacterial species using Spot test technique.

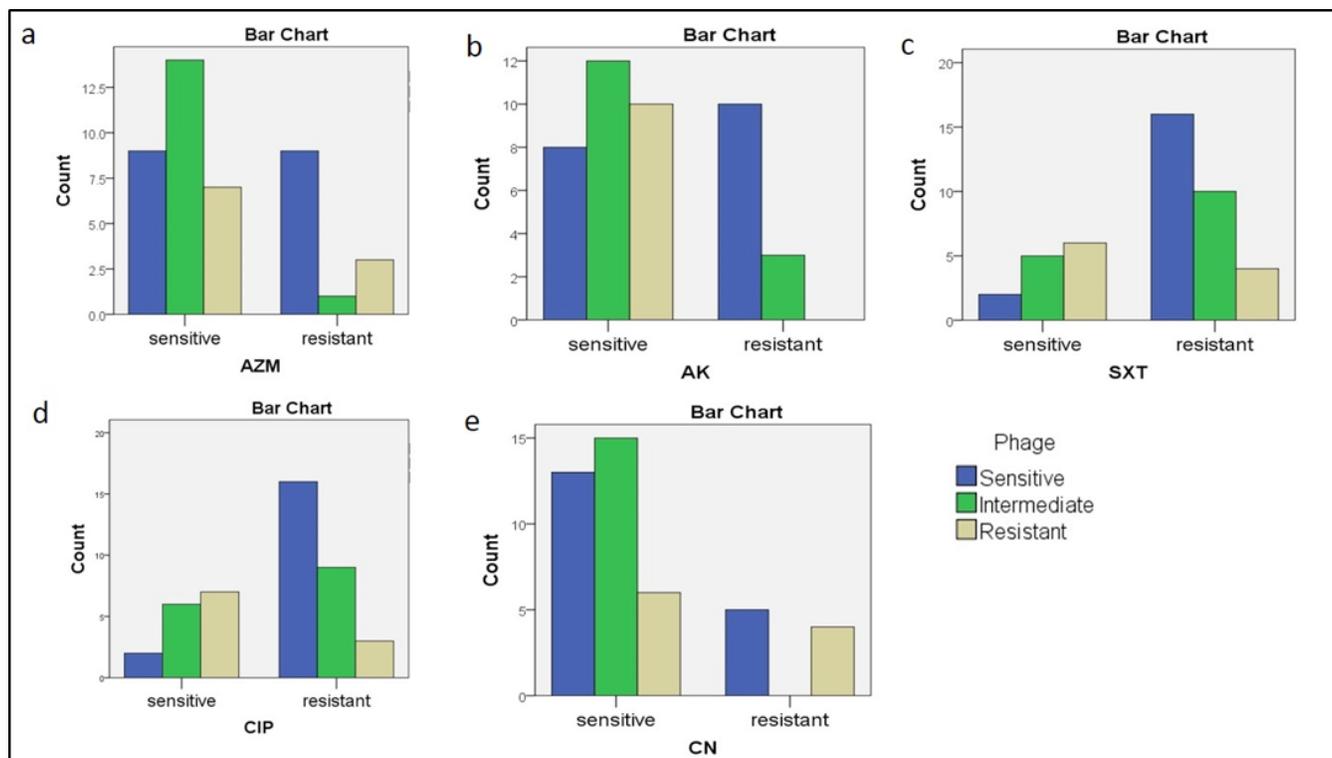| Bacterial host	Susceptibility to phage-cocktail
S. aureus	+
S. saprophyticus	-
S. epidermidis	-
E. coli	-
P. aeruginosa	-

Figure 7: The significant association between phage and antibiotic resistance of (AZM, AK, SXT, CIP, and CN) where isolates tend to be more sensitive to phage at more antibiotic resistance except of CN-resistant isolates.

Table 2: The correlation between antibiotic and phage susceptibility of 43 MRSA isolates.

antibiotics		Phage-cocktail susceptibility			Pearson Chi-square
Sensitive (no. 18) (42%)		Intermediate (no. 15) (35%)	Resistance (no. 10) (23%)	*p*	
RA	Sensitive	12	11	10	0.125
	Resistant	6	4	0	
AZM	Sensitive	9	14	7	0.026
	Resistant	9	1	3	
C	Sensitive	16	14	9	0.905
	Resistant	2	1	1	
AK	Sensitive	8	12	10	0.005
	Resistant	10	3	0	
SXT	Sensitive	2	5	6	0.025
	Resistant	16	10	4	
F	Sensitive	5	7	5	0.403
	Resistant	13	8	5	
CIP	Sensitive	2	6	7	0.006
	Resistant	16	9	3	
DA	Sensitive	14	15	8	0.152
	Resistant	4	0	2	
CN	Sensitive	13	15	6	0.035
	Resistant	5	0	4	

Table 3: Significant association between phage susceptibility and antibiotic resistance of 43 MRSA isolates where more phage-sensitive isolates exhibit the highest resistance to antibiotics (more than six antibiotics).

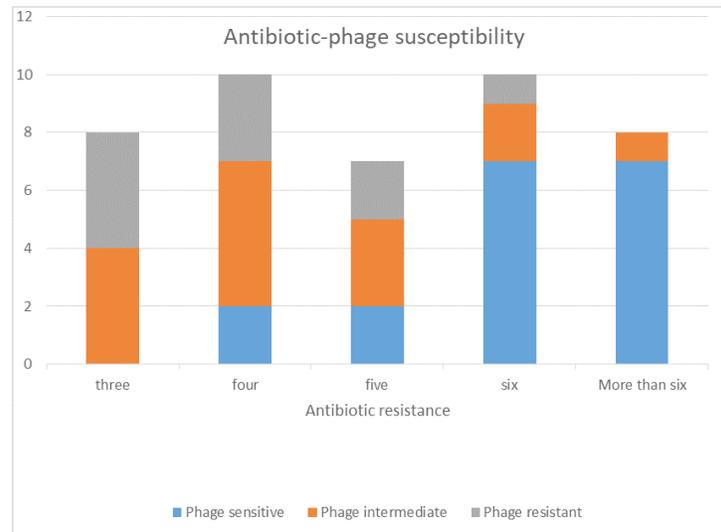|	Phage susceptibility			*p value*
Antibiotic resistance	sensitive	intermediate	resistant	
three	0	4	4	
four	2	5	3	
five	2	3	2	
six	7	2	1	
More than six	7	1	0	*0.014*

Figure 8: The distribution of phage susceptibility of MRSA isolates related to antibiotic sensitivity, where sensitivity to phage-cocktail positively correlated with increasing antibiotic resistance.

aureus to antibiotics primed them to be more sensitive to lytic bacteriophages.[34]

In the current study, MRSA was isolated from clinical samples where pre-exposure to antibiotics is highly weighted in contrast to wild isolates, where there was a chance of possible mutations to resist antibiotics which made them more sensitive to phages. The direct negative interaction had been proven between phage and antibiotics against resistant bacteria. The study found that the resistance to antibiotics by MRSA isolates was high in contrast to its relatively low capacity to resist phage-cocktail, which points to the non-convergent mechanisms of phage-antibiotic resistance of MRSA isolates of this study as confirmed by Allen *et al.*[35] The study of phage-antibiotic synergy is needed to be evaluated against MRSA as well as the genetically-based investigation is required to understand the nature of possible random or nonrandom synergism between both of phages and antibiotics to eradicate planktonic and biofilm state of MRSA. The fact of association between phage and antibiotics is yet unclearly, but it can be attributed to the effect of cumulative mutations that makes bacteria resistant to an antibiotic but sensitive to phage and vice versa.[35]

CONCLUSION

MRSA isolates exhibited variable multidrug resistance ability against 12 antibiotics used to treat MRSA wound infections. The isolation of MRSA specific bacteriophages was somewhat difficult and more than one phage enrichment round was needed. MRSA phage-cocktail was an effective bactericidal agent against MRSA isolates in vitro. MRSA isolates exhibited different percentages of antibiotic-phage resistance, where more antibiotic-resistant isolates were more phage-sensitive. The effectiveness, safety, and efficacy of bacteriophages are required to be evaluated and deeply studied in vivo.

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