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Comparative study between Pure Bacterocin and Vancomycin on Biofilms of MRSA isolated from medical implants

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Abstract:
Thirty swabes of medical implants were collected from Al-Yarmouk's hospital which were cultured on manitole agar to isolate *Staphelococcus aureus* . Only four samples gave positive results with this media. It was used ten types of antibiotics to test the sensitivity of this bacterium against them. All isolates of *S. aureus* were recorded as multidrug resistant and were considered as MRSA. One pledge alternative therapy is the utilize of certain pure bacterocin MIC (32.5 to 62.5 µg/ml) and it was compared with vancomycin (200-400 µg/ml) with average of (8 – 15) mm diameter of inhibition zones recpectively. The first reduction of biofilm formation ability has been proved in catheters when treated by pure bacterocin. The test shows the highest effective bacterocin against biofilm, while Vancomycin is significantly lower; this suggests that new bacteriocins are highly potent against biofilm of bacteria especially in treatment of resistance bacterial infections by forming stable pores on biofilm causing reduction of biofilm formation percentage to about 70- 85 %, while vancomycin has less reduction percentage against biofilms with an average values of about 40 -52 %. The disruption of MRSA biofilms is specific with lower concentration of (Vancomycin and Bacitrocin) and has effected on formation of MRSA's biofilms using microtiter plate method and ELISA reader machine. This study show using bacterocin is the best choice to reduce biofilm that formed by microbial pathogens which is important to control the infections-biofilm associated in patients with medical artifacts.

Key words: MRSA, Bacterocin, pathogenic bacteria, Vancomycin

INTRODUCTION

MRSA isolates have a gene which is acquired from other bacteria have antibiotics resistant to all beta-lactam, *S. aureus* is an opportunistic human pathogen which is widely distributed. It causes skin infections and actually life-threatening complication such as syndrome toxic shock which is the causative agent. MRSA is a major concern in hospital environments because it represents a symptomatic carrier for a highest danger factors which expose patients to a long hospitalization intervals. Some pathogenic traits are relevant to biofilm production of *S. aureus*, which increases resistance to therapeutic treatments and to the host's mechanisms defense (1). The composed of biofilm is adhering cells to hold gather and a surface by a polymer array, the components corer of the matrix are proteins and (eDNA) Extracellular, Community-associated MRSA, are outside originated hospitals, while these have generally been simpler to treat, some have animate into hospitals and have become resistant to drugs increasingly other than beta-lactams (2).

Biofilms of *S. aureus* are treated commonly by antibiotics protein which considerd as a synthesize inhibiter that targets the cell membrane, cell wall, (DNA, RNA) synthesis as well as inhibiter or like Cu2+ antimicrobials membrane breakage lead subsequent cell lysis (3). Usage of antibiotics for the corrective of infections has been proposed to be answerable for the appearance of multidrug-resistant bacteria (4).

These strains, habit antibiotics' resistant to many and also ß-lactam seminal resistance to antibiotics, shows a particular ability to assail in the hospitals of most Iraqi cities (5). Dangerous factors in the hospital are acquired of MRSA encompass prolonged hospitalization that bides in an adsorbent care unit, chronic diseases such as chronic malignancy ,renal failure and longer exposure to antibiotics, surgery and contact with a patient who is colonized or infected with MRSA (6). Antimicrobial peptides are bacteriocins or proteins ribosomally safe to eukaryotic cells which are non-toxic. Bacteriocins are released by different varieties of bacteria and archea,which they produce natural protien that effects against other strian and the producer has a specific immunity action (7). Bacteriocins produced by both G-ve and G+ve bacteria, which have inhibitory effects towards sensitive strains. It has a useful use in health care products and cosmetics for acne treatment and also being used in inhibition of caries dental in toothpaste diseases (8).

Staphylococcin Bac188 are known to produce a wide variety of inhibitory substances which effect against the biofilm of multidrug resistant bacteria. The mechanisms of biofilm's resistance antibiotic include the presence of persisted cells, micro environments within the biofilm that prevent the antibiotic efficacy and antibiotic access reduced to bacteria within a biofilm (9). Cell of microorganism in a biofilm exhibit resistance to the host's immunity and treatments by antibiotic. Biofilms formation on surfaces of devices medical, such as catheters vascular, joints prosthetic and pacemakers cardiac, represent accusative agent to make the eradicate and invasive procedures difficult (10). In a biofilm formation, there were embedded macromolecules (in extracellular matrix), such as (polysaccharides, proteins, nucleic acids, and lipids). Bacterial cells in a biofilm exhibit resistance to the host's immunity and antibiotic treatments ,therefore, once biofilms form on surfaces of medical implants such as indwelling vascular catheters, prosthetic joints, and cardiac pacemakers. An antimicrobial agent that capable of reducing the bacteria in biofilms efficiently, would be considered agood drug in treating chronic and biofilm-associated infections (11). The aim's study is using bacteriocins, that form stability pores on the membrane of cells target, against MRSA biofilms and to debar or cure biofilm-associated infections.

MATERIAL AND METHODS

Isolates and Culture media

- **Confirmation of Bacteria**

Thirty swaps were collected from medical implants from (Al-Yarmouk Teaching Hospital/Baghdad/Iraq), cultured on mannitol salt agar and incubated at 37C° for 24 hours. The selection of isolates is depending on the colonie's morphology, Gram stain reactions and biochemical characteristics (Coagulase, Catalase reagent and Oxidase reagent). For screening strain that produces biofilm using tryptic soy broth (TSB) with 1 % (Glucose) cultured supplemented at 37 °C over night. For inoculum standard, there were homogenized MRSA cells in solution saline (NaCl 0.85 %) and the suspending was diluted to 0.5× 10^8 CFU·mL−1 using a O.D (9).

- **Confirmation of MRSA and Antibiotic Resistance Testing**

All isolates of *S. aureus* which recorded any level of Methicillin's resistance, were cultured and grew on (MHA) plates at 37 C for

18 hours in the found of the following antibiotics: Penicillin P (10µg), Cefotaxiitin CEF (30), Chloramphenicol C (30), Cefotaxime, CTX (30), Methicillin, ME (5) Oxacillin, OX(1), Erythromycin, E (15) Gentamycin ,CN (10) Tetracycline, TE (30) and Vancomycin, VA (30). The zone of inhibition (mm) around each disk was compared with a documented standard interpretive chart for Clinical and Laboratory Standards Institute (12) .

- **VITEK 2 System**

A specific number of the bacterial isolates were selected to confirm their identification susceptibility using the vitek 2 system.

Bacterocin activity assay

The production of Bacterocin was accomplished using all MRSA isolates(M1, M2, M3, and M4). M1 isolate gave the best production of bacteriocin using *Staphylococcus aureus* and *Escherichia coli* (Obtain from Department of Biology, College of Science, Baghdad. Iraq) as an indicator organism. The culture medium that was used for production of Bacterocin was liquid medium (TSB). *In vitro* antibacterial activity of Pure Bacterocin were examined for inhibitory activity against different strains of bacteria using the Agar Well Diffusion (AWD) assay. Measurement of Bacterocin activity was carried out by serial two-fold dilutions of it. These dilutions were used to detect the antibacterial activity of bacterocin against indicator bacteria by agar well diffusion assay. The arbitrary unit (AU) was defined as the reciprocal of the highest dilution producing a clear zone of growth inhibition of the test isolate. AU was calculated as: (1000 / 100) × D, where 1000: constant, 100: volume of supernatant in a well (µl) and D: the dilution factor (13).

Extraction and Purification of Bacterocin

MRSA (M1) growth as (2% of 6×10^8 cell/ml) was incubated under aerobic conditions at 37°C for 24 hours. Cells were harvested by centrifugation at 6000 rpm for 15 minutes. The bacterocin that was extracted, referred as crude, was heated at 80°C for 10 minutes, then cooled and centrifuged at 6000 rpm for 15minutes.The supernatant was mixed thoroughly with Ammonium sulphat at a ratio 70%.The mixture was centrifuged at 4000 rpm for10 minutes to achieve phase separation. Then the sediment was re-suspended in 20mM sodium citrate buffer (pH 7) and referred to as partial purified Bacterocin (PPB) (14). A first step Chromatography (DEAE-Cellulose) gel for Bacterocin purification was done. This gel was prepared according to the Pharmacia catalogue (Pharmacia Fine Chemical/ Sweden). (PPB) was loaded slowly over the DEAE-Cellulose. Eluted from the column (2 × 40) cm dimensions, the bacterocin was by 20mM sodium citrate buffer (pH 7)and flow rate was adjusted to give 40 ml per hour. 5 ml for each fraction was collected. Activity and absorbance at 280nm (15).

Estimation of protein by Lowry's method

Samples were analyzed for protein Bacterocin Using Lowry's method (16).

Minimum inhibitory concentration (MIC) assay

MIC assays were performed by microdilution in 96-well plates, with recommendations in accordance from the Clinical and Laboratory Standards Institute. A serial dilution was performed starting vancomycin with (200- 400)µg /ml and Bacterocin between (32.5-62.5) µg /ml both were two-fold higher supernatants on Mueller Hinton (MH) medium containing (5×10^5) CFU·mL−1 of (MRSA) per well. The performed with the following step controls: TSB medium without MRSA (negative control); TSB medium containing 5×10^5 CFU·mL−1 of MRSA (positive control). The plate was incubated for (24 hours at 37 °C) and revealed with 20 µL of Resazurin (0.01 %) for 30 min at 37 °C. At the same time 5 µL of each suspension (samples and control) was cultured on MH agar for 24 hours at 37°C with the optical density of (0.05 at 595) nm after which the inhibitory concentrations were classified as (bactericidal or bacteriostatic). (17)

Biofilm formation on a polystyrene plate

The study of strain to form biofilms following treatment with (Pure Bacterocin and Vancomycin) according to the methodology proposed by (18). In briefly, MRSA was cultured with 1 % Glc in 5 mL of TSB for 18 h at 37 °C and treated with pure Bacterocin and Vancomycin of the MIC or control medium. At first the cultures were diluted as (1:100) in the same medium, 200 µL of these dilutions were inoculated in a 96-well plate, then plates were incubated at 37°C for 24 hours. After incubation the plates were washed twice with phosphate-buffered saline (PBS), dried for 1 hours at 65 °C, then 1 % of crystal violet was added and the plates were incubated for a further 30 min at 25 °C. Each well was washed twice with PBS, 200 µL PBS is obtained using following formula:

Biofilm degree = Mean OD 630 of tested bacteria - Mean OD 630 of control.

Statistical analysis

The data of this study are compared using Statistical Analysis System - SAS (19) and the least significant difference (LSD) .

RESULT AND DISCUSSION

Only four isolates gave positive results on manitol salt agar, which they were *S.aureus* then they were tested by biochemical raction Coagulase, Catalase reagent and Oxidase reagent) so they gave positive results with them and with gram stain too.

The results of MRSA clinical isolations is showed highly significant differences ($P<0.01$) among clinical samples. The isolates showed multi-resistant to Methicillin, Peicillin G, Cefoxitin, Erythromycin, Oxacillin, Chloramphenicol and Tetracyclin, while they are sensitive to vancomycin.(table 1), these results agree with Al-Dahbi (20) who reports of *S. aureus* strains which exhibit resistance to these antimicrobial agents.

Table (1) Antibiotic susceptibility test of *S. aureus*

Antibiotic	Resistance No. %	Intermediate No. %	Sensitive No. %
Methicillin	4 (100)	0 (0.00)	**0 (0.00)**
Penicillin G	3 (75)	1 (25)	**0(0.00)**
Cefoxitin	2 (50)	1 (25)	**1 (25)**
Erythromycin	3 (75)	0 (0.00)	**1 (25)**
Oxacillin	2 (25)	0 (0.00)	**2 (25)**
Chloramphenicol	3 (75)	0 (0.00)	**1 (0.00)**
Tetracyclin	3 (75)	1 (25)	**1 (25)**
Clindamycin	4 (100)	0 (0.00)	**0 (0.00)**
Gentamycin	3(75)	0 (0.00)	**1 (25)**
Vancomycin	1 (25)	0 (0.00)	**3 (75)**

Vitek 2 system

The results of identification using this system give confirmation of positive results for MRSA and as a selected organism with a probability 98-99%. Edward *et al*. (21) show that out of *S. aureus* isolates have (Oxacilin-sensitive) and (cefoxitin-resistant) profile with a sensitivity that reaches to 88.7% and a specificity reaches up to 99.5% for MRSA isolates.

Production Bacterocin

Bacterocin was heated to denaturant any proteases and heat-sensitive proteins. Ammoinum sulphat extraction exhibited complete recovery of Bacterocin activity, extraction by ammonium sulphate 70%. These results indicated that the Bacterocin by MRSA were, under the present condition. carried a negative charge opposite to ion exchange charge and binding with exchanger resin. Fractions which have activity are collected and

concentrated. The specific activity of Bacterocin reached 1000 AU/mg with 2.8 purification fold and Bacteriocin yield (34) % respectively. (Figure 1)

The result show inhibition zone diameter reached (15 and 14) mm respectively by well diffusion assay (Figure 2). The best concentration of of Bacterocin and Vancomycin is shown in (Figure 3)

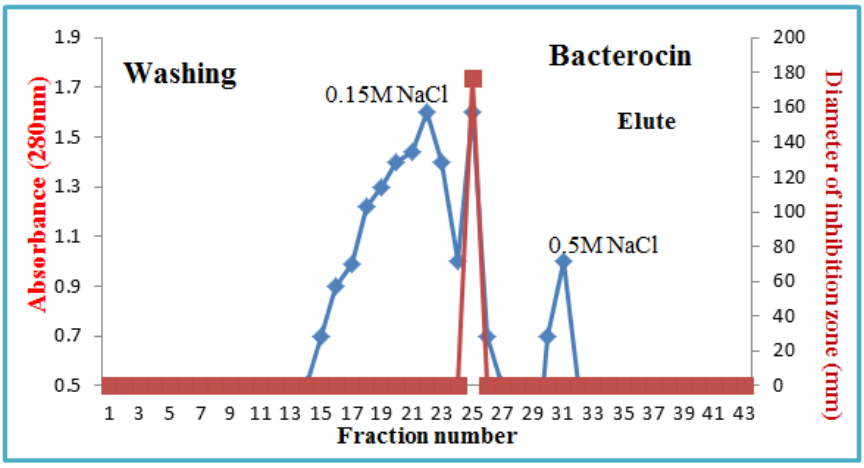


Figure 1: Ion exchange chromatography for Bacteriocin column (2×40cm) equilibrated with Tris-HCl buffer (10 mM , pH 7), eluted with Tris – HCl buffer with NaCl (0.15 -0.75 M) in flow rate 0.8 ml /min., 5ml for each fraction.

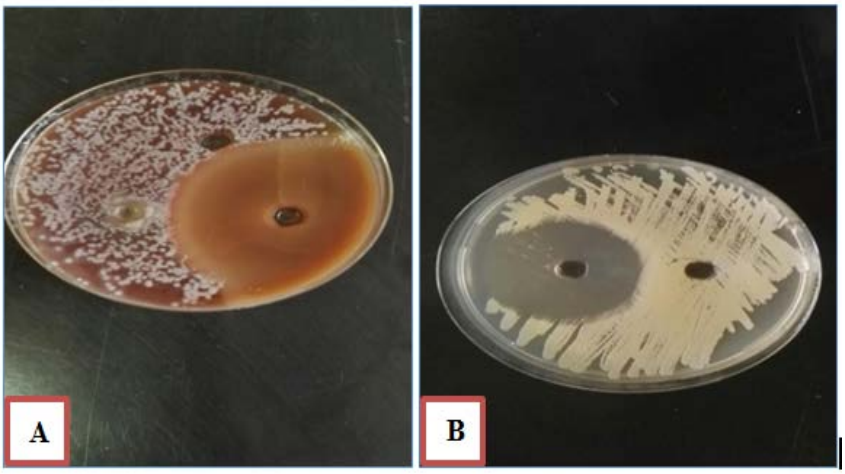


Figure 2: Inhibitory effect by WDA (A) Pure Bacterocin against MRSA in mannitol salt agar 24-48 hrs at 37 °C (B) Perception 70% ammonium sulphat aganist *E.coli* O157:H748 hrs at 37°C in Sorbitol agar.

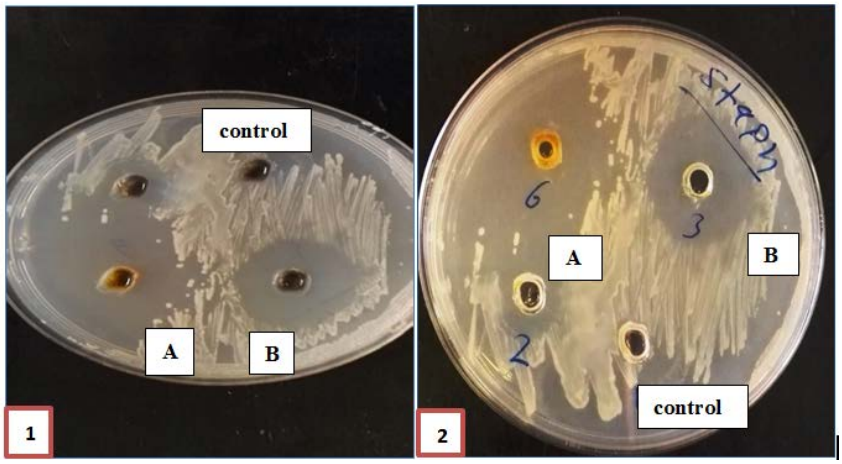


Figure 3: comparison between Vancomycin (A- 400, B- 200 µg /ml) Purified bacterocin (A- 32.5, B-62.5 µg /ml) on Muller Hinton agar for 24-48 hours at 37°C against *E .coli* by WAD.

Biofilm Assay Method

Antimicrobial susceptibility tests revealed that Pure Bacterocin has stronger inhibitory effect on MRSA growth MIC (62.5 µg /ml) than of vancomycin with (400µg /ml) (Figure4) and (Figure 5). The possibility of MRSA species to damp pathogens of clinical samples is importance such as *S. aureus* had been assessed before. Okuda *et al*. (22) showed that *L. fermentum* (CMUL054) and *L. plantarum* were acted against *S.aureus* In this study , it has shown the effect of antibiotic bactericidal activity against growth of MRSA. . The production of MRSA biofilm (M1 , M 2 , M 3 and M4) were selected to be testing with the effect of bacterocin following the procedure of Dunne (23). Tissue culture containing biofilms of MRSA were incubated with concentration of M1 Bacterocin (32.5 µg/ml)and vancomycin (400 µg/ml) for over night. The absorbance of bacterial biofilm before treatment was 0.86 while after Bacterocin – treated was 0.09 and 0.43 when treated vancomycin (Table 2).

The O.D of Anti-MRSA M3 biofilm was handled with vancomycin for overnight,the result show minimal change and remaining around 0.43nm, contempt the actuality that the biofilms were treated with (400 µg/ml) comper treat bacterocin 0.09 nm, an analogous experiment was flume, but with incubation of the biofilms with the 3 anti-microbials medium (TSB). More same results when the assay was flume with TSB rather than (PBS). Bacterocin decline the O.D read of biofilms to near background by overnight , while vancomycin had small or no effects even after 24 hours of incubation.The exact of (Lysostaphin) mechanism act against MRSA biofilms unclear remains. It has been shown that, even if an antimicrobial agent can kill some of the bacteria enclosure in the biofilm, the biofilm is not disruptive unless the extracellular matrix is also trashed (24).

The staphylococcal may include components of cell walls extracellular biofilm matrix, (polyglycines) including, that by lysostaphin might be potency, thus, disrupting the extracellular biofilm matrix. Others have been found in matrix of biofilms the extracellular *P. aeruginosa* extracellular (DNA) is required for biofilm formation (25).

Table (2) MRSA isolates Pre-treatment and Post – treatment Bacterocin and Vancomycin

MRSA isolates	Pre-treatment ($O.D_{630}$)	Post – treatment Bacterocin	Post –Vancomycin
MRSA 1	0.66	0.13	0.24
MRSA2	0.72	0.18	0.55
MRSA 3	0.86	0.09	0.43
MRSA 4	0.55	0.21	0.94

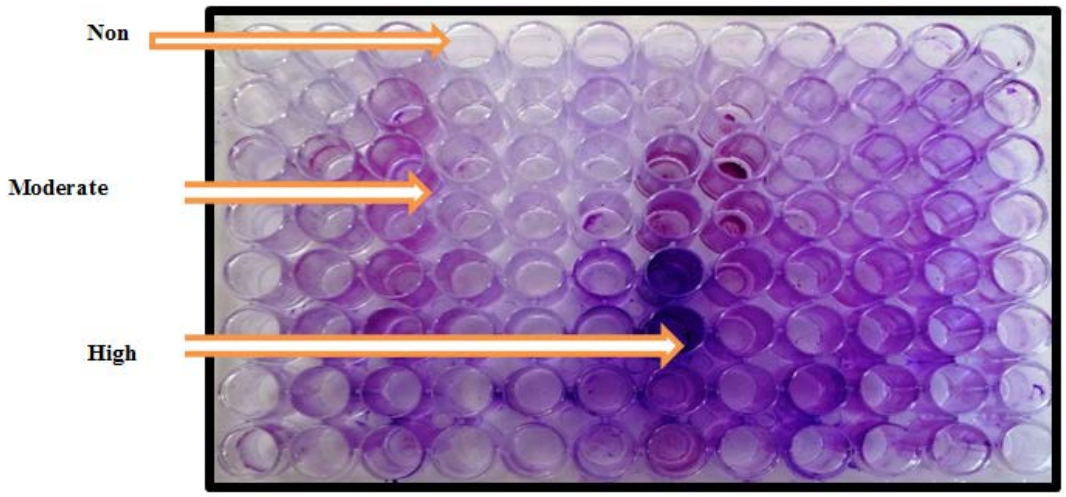
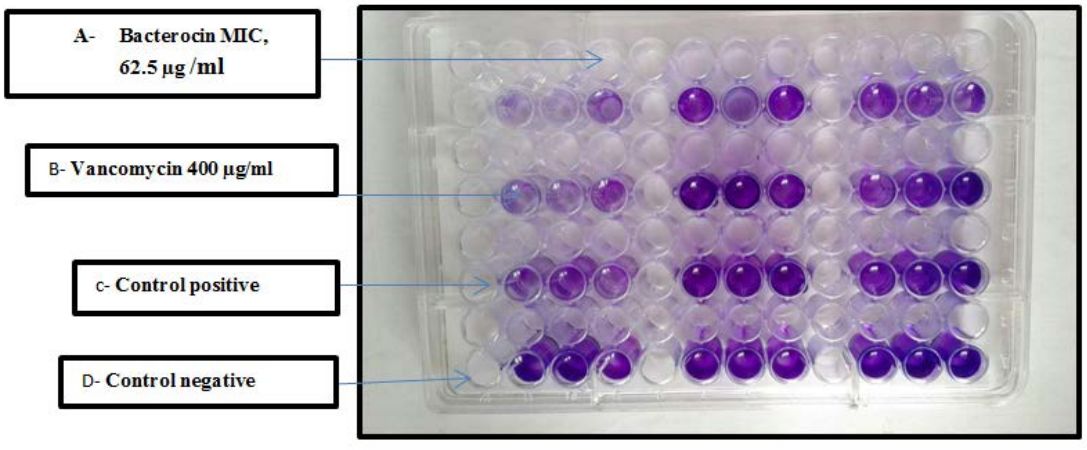


Figure (5) screening of Biofilm producer by TCP method: high, moderate and non slime producer differentiation with crystal violet staining in 96 well tissue culture plate



Figurs (6): A-Bacteriocin - 62.5 µg /ml B- Vancomycin400µg/ml
C-culture wells were inoculated with 10^8 CFU of disrupted biofilms of (MRSA3) (as indicated).D- culture Broth without indicated MRSA

At first, the MICs of Bacterocin and vancomycin by the micro-liquid dilution method against MRSA were determined. After the addition of vancomycin which have mode a bacteriostatic of action, killed the cells quickly. Incubation times for complete bactericidal action for Bacterocin showed activity against MRSA, Vancomycin showed that its killing rate is less than that of Bacterocin.

The ability of Bacteriocin to depend on the biofilms of the multiplicate S. *aureus* strains was tailored by ocularly comparing the variation of safranin stain or Gram stain density between Bacteriocin and Vancomycin treated and untreated biofilms.

CONCLUSION

Thus, we suggests that the effects of pure bacteriocins against most isolate of MRSA. The tested bacteriocins showed the highest bactericidal activation against MRSA biofilm material and suggest that bacterocin from MRSA attacks biofilm cells are more effective than (Vancomycin) using although is widely used at (first-line therapy) for difference MRSA infections.

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