Research article

Antibacterial activity of klebocin against methicillin resistance *Staphylococcus aureus*

Alyaa Razooqi Hussein[1], Zainab Zamel Khalaf[2]

[1,2]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

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Corresponding author: **Alyaa Razooqi Hussein.** Email:alyatiba@yahoo.co.uk, alyaa.a.hafedh@gmail.com

ABSTRACT

Introduction and Aim: Bacteriocins are antimicrobial peptides that have bactericidal and/or bacteriostatic activity against other bacteria. The aim of this study was to assess the antibacterial efficiency of Klebocin a *K. pneumoniae* bacteriocin, against biofilm formation by clinical isolates of methicillin resistant *Staphylococcus aureus* MRSA.

Materials and Methods: *S. aureus* isolated from clinical samples was identified according to vitek 2 system Antibiotic susceptibility test was performed according to disc diffusion method. Vitek 2 compact system was also used to detect MRSA strains. Agar well diffusion method was used to evaluate the antibacterial activity of klebocin from *K. pneumoniae* towards 11 strains of *S. aureus* by the microtiter plate method throughout the initial and final steps of biofilm development.

Results: Our finding shown that crude extract of klebocin was active against all the tested pre-formed biofilm of *S.aureus* isolates and the percentage of inhibition ranging from 78.9-88.5%. Also the results of the current study demonstrated that all matured bacterial biofilm was inhibited in at a percentage ranging from 41.2% - 91.2%.

Conclusion: This finding proved that klebocin had antibacterial activity against planktonic cells of *S.aureus* in addition to antibiofilm activity against premature and mature biofilm of MRSA. Also klebocin had broad spectrum activity effecting Gram +ve bacteria and its biofilm production.

Keywords: Antibacterial activity; klebocin; MRSA.

INTRODUCTION

Staphylococcus aureus is a major pathogenic bacterium that causes a diversity of clinical presentations such as superficial skin and soft tissue infection, sepsis, pneumonia, and endocarditis in humans and animals (1). The bacterium is widespread in hospitals as well as the community, and treatments for infections are limited due to the prevalence of multi-drug resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) (2) which is a global health concern.

Microorganisms are known to form biofilms, a process whereby they attach themselves to any surface and grow, facilitating their growth and survival. The biofilm matrix is mainly composed of proteins, exopolysaccharides, nucleic acids, lipids and other metabolites (3). Biofilm microorganisms are resistant to the immune system of the host as well as antibiotic therapy (4). As a result, biofilms on the faces of medicinal apparatus such as indwelling vascular tubes, prosthetic junctions, and cardiac pacemakers can be excessively hard to remove, necessitating invasive therapies for instance device elimination (5). *Staphylococcus aureus* exhibits a strong capacity to attach to abiotic or biotic surfaces and form biofilms, which lead to frequently recurrent or long-lasting chronic infections (3). As a result, innovative antimicrobial drugs with a variety of bactericidal mechanisms, as well as alternative therapeutic techniques, are critical in the fight against MRSA infections that develop biofilms.

Bacteriocins are antimicrobial peptides produced by ribosomes that are generally stable across a wide temperature and pH range, have low toxicity, and have high antibacterial activity when used in therapeutically adequate doses (6). These peptides are generally regarded as safe, and continue to pique the curiosity of many experts due to their prospective usage in the pharmaceutical and food industries. Bacteriocins are produced by bacteria to help them compete for resources and habitat with other bacteria. Bacteriocins are susceptible to both antibiotic-sensitive and antibiotic-resistant bacteria because their methods of action are different from antibiotics and hence, bacteriocins are considered a viable alternative to antibiotics in treating bacterial infections, especially those caused by antibiotic-resistant bacteria (7). Klebocins are bacteriocins produced by *Klebsiella* spps that inhibit biofilm formation in different Enterobacteriaceae species (8). In addition, klebocins have been shown to have a broad range of antimicrobial activity against several pathogenic Gram positive and Gram negative organisms (9). In this study, we aimed to evaluate the antibacterial and antibiofilm activities of Klebocin produced from *K. pneumoniae* against MRSA strains.

MATERIALS AND METHODS

Sample collection

Fifty-seven clinical (wound swabs and urine) samples were collected, during September to November 2020 from hospitals in Baghdad, Iraq.

S. aureus isolation and identification

For isolation of *S. aureus* from wound samples, the wound swab was directly inoculated on mannitol salt agar medium, followed by incubation at 37°C for 18-24 hours. Typical colonies were picked and identified as *S. aureus* based on Gram staining and pattern of biochemical tests using standard procedures (10). The identity of the isolates was further confirmed using the Vitek2 system (Biomerieux -France).

Antibiotics susceptibility test for *S .aureus*

Kirby-Bauer disk diffusion method was used to fix the isolates' susceptibility. Bacteria were inoculation on Mueller Hinton agar and antibiotic disks were placed on medium surfaces using sterile forceps after turbidity was corrected to the McFarland standard of 0.5. The antibiotics used were gentamicin (10µg), chloramphenicol (30µg), ciprofloxacin (5µg), cefotaxime (30µg), and tetracycline (10µg). After a 24-hour period of incubation at 37°C. According to CLSI (2019) recommendations, the inhibition zones were measured in millimetres, and the results were classified as S, I, or R by way of sensitive, intermediate, or resistant respectively. Each isolate was deemed methicillin-resistant when the minimum inhibitory concentration (MIC) breakpoint of oxacillin was >2 mg/L and cefoxitin was >4 mg/L (11).

K. pneumoniae isolation and identification

The urine samples were directly cultured on MacConkey agar and then incubated at 37°C for 18-24 hours. Lactose fermenter (mucoid) colonies were chosen and recultivated using the streaking method on new sterile MacConkey agar plates to achieve pure, well-isolated colonies. Biochemically tests for confirmation of isolates included colony morphology and further classification included tests like indole, methyl red, vogus proskauer, and citrate utilization test, tripleSugar iron, motility, and urease tests by standard procedures (10).

Ability of the *K. pnuemoniae* isolates to produce klebocin was tested by Agar well diffusion method. Briefly, each isolate grown overnight in Trypticase soy broth was inoculated to wells on Mueller Hinton agar (Himedia). After 24 hours clear zones (indicative of bacteriocin production) that appeared around the wells were measured. *K. pneumonia* K42 strain, a klebocin producer was used as a positive indicator strain.

Klebocin extraction

K.pneumonia strains positive for klebocin production by well diffusion assay were further subjected to klebocin extraction following the extraction procedure described earlier (12). To obtain the crude extract, the isolate was first inoculated onto Luria Bertani (LB) broth containing 5% glycerol and incubated overnight in a shaker incubator. Once the cells reached a density of $3x10^8$, Mitomycin at a concentration of 2µg/ml was added and incubated further for 3 hours with shaking. After 3 hours the mixture was centrifuged in a cooling centrifuge (Beckman Coulter, Germany) at 5000g for 30 mins. to separate out the supernatant containing klebocin. The crude klebocin extracted was estimated for its protein concentration using bovine serum albumin as the standard (13).

Biofilm formation assay

The ability of clinical *S. aureus* isolates to form biofilm was assessed using the microtiter plate method described by Atshan *et al*., (14). Bacteria were grown in Tryptic soya broth (TSB) having 1% glucose in 96-well microtiter plates and incubated at 37°C for 24 hours aerobically. Afterward the non-attached cells were rinsed three times with distilled water, and the adherent bacterial cells in each well fixed for 20 minutes with 200 µl of absolute ethanol. The plates were let down and dehydrated overnight following which the adherent cells were stained with 200µl of crystal violet (0.1% conc.) for 15 minutes. After the time period the plates were rinsed with distilled water to remove the excess dye and the colour developed read at an O.D of 490nm in an ELISA reader. The assay was repeated three times, with sterile TSB serving as a negative control. Based on the OD values the biofilm formation was categorized as absent, weak, moderate and high.

Anti-biofilm activity of klebocin

The inhibitory effect of klebocin extract on biofilm formation was tested on *S. aureus* isolated in this study. The method employed for the study of antibiofilm activity was based on the method described by Harry and Walker (15). To the biofilm matrix formed by each isolate of *S. aureus* in wells, 100 µl of klebocin extract was added and incubated at 37°C for 24h. After incubation period the cells were rinsed, stained with crystal violet and the absorbance read at 490 nm in an ELISA reader. The biofilm inhibition percentage for calculated using the formula:
Biofilm inhibition (%) = (Control OD- Test OD / Control OD) ×100

RESULTS

In this study, only 11 clinical samples confirmed positive for the presence of *S.aureus.* Results for antibiotic susceptibility test for the 11 isolates showed susceptibility to antibiotics chloramphenicol (100%),

gentamicin (89%), ciprofloxacin (78%), cefotaxime and tetracycline (0%) as shown in Table (1). Among the 11 isolates 9 (81.8%) were observed to be methicillin-resistant.

Table 1. *S. aureus* antibiotics susceptibility test

Isolate	CIP	CTX	C	CN	TE
S1	S	R	S	S	R
S2	S	R	S	S	I
S3	R	R	S	S	R
S4	S	R	S	S	R
S5	R	R	S	R	R
S6	S	R	S	S	R
S7	S	R	S	S	I
S8	S	R	S	S	R
S9	S	R	S	S	I

CIP: Ciprofloxacin; CTX: Cefotaxime; C: Chloramphenicol. CN: Gentamicin; TE: Tetracycline; R: resistant. I: intermediate; S: sensitive

All the *S. aureus* strains positive for methicillin resistance (n=11) were observed to be biofilm formers. Among them 6 (54.54%) isolates were strong and 5 (45.45%) moderate biofilm formers (Table 2). For further experimental studies only those that were strong biofilm formers were selected.

K. pneumoniae isolates from samples were tested for their inhibition of the methicillin-resistant *S. aureus* strains. Three isolates of *K. pneumoniae* (K1, K2, and K5) inhibited growth of *S. aureus* tested. The zone of inhibition produced by the *Klebsiella* isolates K1, K2 and K3 on planktonic cells of *S.aureus* isolates are presented in Table 3.

Table 2: Biofilm formation by *S. aureus*

***S. aureus* isolate**	**O.D**	**Biofilm type**
S. aureus 1	0.328	Strong
S. aureus 2	0.360	Strong
S. aureus 3	0.234	Moderate
S. aureus 4	0.363	strong
S. aureus 5	0.751	strong
S. aureus 6	0.238	Moderate
S. aureus 7	0.397	Strong
S. aureus 8	0.294	Strong
S. aureus 9	0.270	Moderate
S. aureus 10	0.215	Moderate
S. aureus 11	0.252	Moderate

The maximum inhibition was by *K. pneumoniae* strain K1 which inhibited the growth of 6 *S. aureus* isolates, followed by K3 (5 isolates) and K2 (3 isolates). The three strains were selected for crude klebocin extraction. The protein concentrations in klebocin crude extracts from the three ranged from 36.31 to 165.43 µg/ml. These crude extracts were observed to inhibit the biofilm formation by the 11 methicillin-resistant *S. aureus* strains tested. A representative plate indicating the inhibitory effect of klebocin on *S. aureus* is shown in Fig. 1.

Table 2: Growth inhibition of *S.aureus* planktonic cells by *K.pneumoniae* strains

K.pneumoniae strains	Zone of inhibition (mm) for MRSA positive *S.aureus* strains										
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
K1	25	-	-	30	15	-	-	-	10	10	15
K2	23	-	-	20	-	-	-	-	-	-	10
K3	10	10	-	20	20	-	-	-	-	-	15

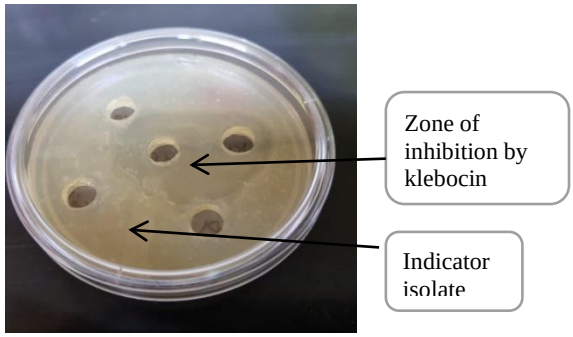

Fig. 1: Antibacterial activity of klebocin against on *S. aureus*

Table 4: The inhibition activity of klebocin crude extracts on *S. aureus* attached and mature biofilms

Isolate	**Control O.D.**	**Klebocin inhibition on formation**			
		Pre-mature biofilm		**Mature biofilm**	
		O.D.	**%**	**O.D.**	**%**
S. aureus 1	0.323	0.065	79.8	0.082	74.6
S. aureus 2	0.332	0.070	78.9	0.089	73.1
S. aureus 4	0.317	0.063	80.1	0.155	51.1
S. aureus 5	0.822	0.094	88.5	0.072	91.2
S. aureus 7	0.344	0.057	83.4	0.202	41.2
S. aureus 8	0.281	0.056	80.0	0.106	62.2

O.D. Optical density

The activity of klebocin on pre-+mature biofilm formed by these strains showed klebocin to be effective for all strains tested with inhibition ranging between 78.9%-88.5%. While the highest effectiveness was against strain *S. aureus* 5, the lowest was seen for *S. aureus* 2 (Table 4). Similarly the activity of klebocin on matured biofilm showed an inhibition percentage ranging from 41.2 to 91.2. As seen from Table4, klebocin showed highest inhibition for biofilm formation by strain *S. aureus* 5 and the least for *S. aureus* 7.

DISCUSSION

Studies based on antibiotics susceptibility testing have shown the antibiotics amikacin, chloramphenicol, nitrofurantoin, penicillin G, and teicoplanin to be most effective against methicillin-resistant *Staphylococcus aureus* (MRSA) infections (16). However, in this study antibiotic resistance typing showed mixed results for *S. aureus* with isolates being highly susceptible to chloramphenicol and resistant to cefotaxime. Recent studies have shown that the antimicrobial proteins (bacteriocins) produced by bacteria to have a bactericidal and bacteriostatic effect on a broad spectrum of Gram +ve and Gram-ve bacteria (16, 17). The bacteriocin klebocin produced by *Klebsiella* strains has been shown to have antibacterial activity against pathogenic bacterial species, molds and yeasts (16,17,18). Crude klebocin extracted from clinical isolates of *Klebsiella pneumonia* was demonstrated to inhibit biofilm formation in different Enterobacteriaceae species (12). Our studies with klebocin on pathogenic *S. aureus* MRSA strains isolated from clinical samples showed that klebocin was effective in inhibiting the growth and bio-film formation by this pathogen. MRSA isolates have been shown to develop biofilms (19,20) formation which is an important aspect of many *Staphylococcus aureus* infections. Further, biofilm formation by strains could be weak, moderate or strong (20). All strains of *S. aureus* included in this study were observed to be either moderate or strong biofilm formers. This assumes significance as these strains were isolated from clinical cases and clinical isolates have been demonstrated to form robust biofilms impacting adhesion and pathogenesis (20). The antibiofilm activity of klebocin against mature biofilm of *S. aureus* observed in this study is in accordance with an earlier study by Okuda *et al.,* (21) who studied the effect of bacteriocins on MRSA. The authors based on their mode-of-action studies, further suggest that bacteriocins form pores on biofilm cells, which helps treat MRSA biofilm infections (21). The inhibition for biofilm formation exhibited by klebocin in this study could probably be due to similar mode of action, which renders it useful as an alternative to antibiotics and combat *S. aureus* pathogenesis. Our results were in consistence with Kazim *et al., (*22) who revealed that *Pseudomonas flourescence* Levan has antibacterial activity against *Staphylococcus aureus*, *E. coli* and *Listeria*, but it had no action counter to *Salmonella typhi*. Also, our finding was in accordance with results observed by Abed and his co-authors (23). They used plant essential oils to preserve yogurt from spoilage via different kinds of microorganisms and to prevent the transmission of pathogenic microorganisms. In the light of the information presented thus far, it was necessary to evaluate klebocin's effectiveness against a wide variety of dangerous pathogenic bacteria such as *Brucella melitensis* (24), *Proteus vulgaris* (25) and others.

CONCLUSION

Klebocin demonstrated antibacterial action against planktonic *S. aureus* cells as well as antibiofilm activity against MRSA biofilms, both premature and mature. klebocin produced by a Gram -ve bacteria has shown to inhibit biofilm forming activity of Gram +ve bacteria and therefore we conclude that klebocin has a broad-spectrum activity, and thus could be a used as a feasible alternative to antibiotics.

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CONFLICT OF INTEREST

The authors disclose no competing interests.

REFERENCES

1. Tong, S. Y. C., Davis, J. S., Eichenberger, E., Holland, T. L., Fowler, V. G. *Staphylococcus aureus* infections: Epidemiology, pathophysiology, clinical manifestations, and management. Clinical Microbiology Reviews. 2015;28(3): 603-661.
2. Boucher, H. W., Corey, G.R. Epidemiology of methicillin resistant *Staphylococcus aureus*. Clinical Infectious Diseases. 2008; 46(Suppl. 5): S344-S349.
3. Sugimoto, S., Iwamoto, T., Takada, K., Okuda, K., Tajima, A., Iwase, T., *et al*., *Staphylococcus epidermidis* Esp degrades specific proteins associated with *Staphylococcus aureus* biofilm formation and host pathogen interaction. Journal of bacteriology. 2013; 195(8):1645-1655.
4. Kania, R.E., Lamers, G.E., Vonk, M. J., Dorpmans, E., Struik, J., Tran Ba Huy, P., *et al*., Characterization of mucosal biofilms on human adenoid tissues. The Laryngoscope.2008; 118(1):128-134.
5. Donlan, R.M. Biofilm formation: a clinically relevant microbiological process. Clinical Infectious Diseases. 2001; 33:1387-1392.
6. Chikindas, M.L., Weeks, R., Drider, D., Chistyakov, V.A., Dicks, L.M. Functions, and emerging applications of bacteriocins. Current opinion in biotechnology. 2018; 49:23-28.
7. Cotter, P.D., Ross, R.P., Hill, C. Bacteriocins-a viable alternative to antibiotics? Nature Reviews Microbiology. 2013; 11(2):95-105.
8. Cascales, E., Buchanan, S., Duché, D., Kleanthous, C., Lloubès, R., Postle, K. Colicin biology. Microbiology and molecular biology reviews. 2007; 71(1):158-229.
9. AL-Charrakh, A.H., Yousif, S.Y., AL-Janabi, H.S.

Antimicrobial spectrum of the action of bacteriocins from Klebsiella isolated from Hilla- Iraq. Iranian Journal of Microbiology. 2011;.2(5):1-11.
10. Forbes, B. E., Sahm, D. F., Weissfeld, A. S., Trevino, E. A. Bailey & Scott's Diagnostic Microbiology, 11th ed. Toronto: 2007; 288-302;The C.V. Mosby Company.
11. Akbari-Ayezloy, E., Hosseini-Jazani, N., Yousefi, S., Habibi, N. Eradication of methicillin resistant *S. aureus* biofilm by the combined use of fosfomycin and beta-chloro-L-alanine. Iranian Journal of Microbiology. 2017; 9 (1):1-10.
12. Khalaf, Z. Z., Hussein, A. R. Antibiofilm activity of klebocin crude extract against some species of Enterobacteriaceae. Iraqi Journal of Science. 2018; 59 (4A):1826-1835.
13. Bradford, M.M. 1976. .Adye binding assay for protein . *Anal Biochem*; 72: 248-254.
14. Atshan, S.S., Shamsudin, M.N., Sekawi, Z., Lung, L.T.T., Hamat, R.A., Karunanidhi, A. Prevalence of adhesion and regulation of biofilm-related genes in different clones of *Staphylococcus aureus*. Journal of BioMedicine and Biotechnology. 2012; 3: 10-17.
15. Harry, C.O., Walker, D. Cytotoxic activity of colicin E1, E3 and E9 against *E. coli* BW25113 in the planktonic and biofilm states. The International Journal of Current Research and Academic Review. (2013);1(2): 55-71.
16. Pato, U., Riftyan, E., Ayu, D. F., Jonnaidi, N. N., Wahyuni, M. S., Feruni, J., *et al.*, Antibacterial efficacy of lactic acid bacteria and bacteriocin isolated from Dadih's against *Staphylococcus aureus*. Food Science and Technology, Campinas. 2022; 42: e27121.
17. Musleh, R.M., AL-Jumaily, E.F. Omar-Zahid, L.A. Purification of klebocin extracted from local isolate *Klebsiella pneumonia* (k64). Iraqi Journal of Biotechnology. 2010; 9(2): 239-247.
18. Beenken, K. E., Mrak, L.N., Griffin, L.M., Zielinska, A.K., Shaw, L.N., Rice, K. C., *et al.*, Epistatic relationships between sarA and agr in *Staphylococcus aureus* biofilm formation. PLoS One. J. pone. 2010;5: 1- 13.
19. Ben Abdallah, F., Lagha, R., Ahmed, G. Biofilm Inhibition and Eradication Properties of Medicinal Plant Essential Oils against Methicillin-Resistant *Staphylococcus aureus* Clinical Isolates. Pharmaceuticals. 2020; 13: 369.
20. Velázquez-Suárez, C., Cebrián, R., Gasca-Capote, C., Sorlózano-Puerto, A., Gutiérrez-Fernández, J., Martínez-Bueno, M., *et al.*, Antimicrobial Activity of the Circular Bacteriocin AS-48 against Clinical Multidrug-Resistant *Staphylococcus aureus*. Antibiotics. 2021; 10: 925.
21. Okuda, K., Takeshi, Z., Shinya, S., Tadayuki, I., Akiko, T., Satomi, Y., *et al.*, Yoshimitsu M. Effects of bacteriocins on methicillin-resistant *Staphylococcus aureus* Biofilm. Antimicrobial Agents and Chemotherapy. 2013; 57 (11): 5572-5579.
22. Kazim**,** A. R., Hussein, A. R., Radif, H. M. The Role of Levan Produced from Pseudomonas fluorescens as Antipathogenic Substances and Phagocytic Enhancer against Some of Bacterial Isolates. Journal of Global Pharma Technology.2019; 11(5) :253-259.
23. Abed, I. J., Hussein, A. R., Abdulhasan, G. A. Dubaish, A. N. Microbiological Effect of Lemongrass Cymbopogon Citratus and Spearmint Mentha Spicata Essential Oils as Preservatives and Flavor Additives in Yogurt. Iraqi Journal of Science. 2022; 63(7): 2839-2849.
24. Awadh, H.A., Hammed, Z.N., Hamzah, S.S., Saleh, T.H., Al-Rubaii, B.A.L. Molecular identification of intracellular survival related *Brucella melitensis* virulence factors. Biomedicine (India). 2022; 42(4):761-765**.**
25. Abdul-Gani, M.N., Laftaah, B.A. Purification and characterization of chondroitinase ABC from *Proteus vulgaris*, an Iraqi clinical isolate. Current Science. 2017;113(11):2134-2140.