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RESEARCH ARTICLE

A New Prospective of Colistin Resistance Profile of Some *Staphylococcus Species* Isolated from Iraqi Wound Fractured Foot

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ABSTRACT

Sulbactam belongs to β-lactamase inhibitors that are more commonly used for Gram-negative bacteria than Gram-positive bacteria. In this study, nine different species of *Staphylococcus* (*S. aureus*, *S. epidermidis*, *S.hominis*, *S. lentus*, *S. pseudintermedius*, *S. lugdunensis*, *S. haemolyticus*, *S. sciuri* , and *S. warneri*. were isolated from wounded-fractured feet of Iraqi patients. The spectrum of activity of sulbactam and colistin against all isolates was evaluated. Surprisingly, among *S. aureus* isolates, 5 (12) isolates were sensitive to colistin, whereas, all bacterial isolates were completely resistant to sulbactam. Here, our study revealed that combining colistin and sulbactam against colistin-sensitive *Staphylococcus* may not enhance the susceptibility of bacterial species, as the MIC values of the combination remain unchanged and even increased in some species, indicating the potential activity of colistin alone. Although, the MIC results showed there was no combination effect of colistin and sulbactam, a time killing assay was performed resulting in complete inhibition of colistin-sensitive *Staphylococcus* species, except for *S. aureus* and *S. aureus* standard strain (NCTC 13656). To further examine the effect of colistin, a study of colistin's autolysis activity was performed by increasing the membrane's permeability and leakage of intracellular fluids. Leakage of divalent positive charge (Ca^{2+} and Mg^{2+}) from sensitive colistin-treated isolates was measured by cytochrome C assay, which resulted in a remarkable reduction in cations on the cell surface. To our knowledge, this is the first study to identify the potential activity of colistin against clinically isolated *Staphylococcus* species other than *S. aureus* which showed a pattern of sensitivity against colistin alone.

Keywords: Beta-lactamase, Cations, Cytochrome C, Gram-positive bacteria, Polymyxin E

Introduction

Staphylococci belong to the *Staphylococcaceae* family. They are Gram-positive and appear in isolated or in clusters resembling grape clusters.[1] Catalase-positive, facultative anaerobes, non-motile, sugar fermenters, and non-spore-forming are capable of growing in high amounts of NaCl and pigment producers, which can differ for each species. Most notably, *Staphylococcus aureus* appeared with golden yellow colonies, due to their ability to ferment mannitol salt in media.[2,3] Based on their capacity to produce coagulase, *Staphylococci* are classified as either coagulase-positive (CoPS) or coagulase-negative (CoNS).[4] There are currently more than 80 species and subspecies in the genus, many of which are found on people's skin and mucous membranes,[5] including *S. aureus*, *S. epidermidis*, *S. saprophyticus*, *S. pseudintermedius*, *S. lugdunensis*, *S. haemolyticus*, and *S. warneri*. They cause a wide range of illnesses, from minor infections like skin and soft tissue infections (SSTI) to serious infections like bloodstream infections (BSIs), bacteremia, endocarditis, osteomyelitis, and pneumonia. *Staphylococci* are significant pathogens in humans, causing opportunistic infections and a wide spectrum of life-threatening systemic diseases.[6–8] In coagulase-positive *Staphylococci*, *S. aureus* is the most prevalent species and is the main pathogen linked to nosocomial human infections.[9]

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Sulbactam is a derivative of penicillin that is extensively used as an inhibitor of lactamases.[10] It resembles clavulanic acid in structure.[11] It can be attached to the active sites of β-lactamase antibiotics, protecting them from hydrolysis and restoring antibiotic action.[12] Although sulbactam individually was not usable for medical treatment, the ampicillin-sulbactam combination continues to be utilized for *Acinetobacter baumanii* infectious disease therapy.[13] Surprisingly, sulbactam can link to the penicillin-binding proteins (PBP) of *Acinetobacter* spp[14] and prevents the hydrolysis of Ambler class A penicillinases (such as TEM-1, TEM-2, and SHV-1) irreversibly.[15]

Colistin referred to as Polymyxin E, is classified as an antibacterial drug within the polymyxin antibiotic category. A basic search revealed that Colistin was discovered in 1947 from *Paenibacillus* bacteria, Polymyxins were banned for clinical usage in 1970. Already listed as an essential medicine by the WHO for its importance for human health, it received FDA approval in 1959. For decades, Colistin has been used to treat Gram-negative infections.[16] It is a bactericidal, narrow-spectrum molecule that targets the majority of Gram-Negative Bacteria (GNB) but is inefficient toward Gram-positive bacteria, anaerobes, and mycoplasma.[17] The lipopolysaccharide (LPS) of the GNB membrane is the primary attack of polymyxins.[18] The action mechanism of colistin involves initial binding to the lipopolysaccharide present in the outer membrane, followed by an electrostatic reaction between the alpha and beta-diaminobutyric acid of colistin and the phosphate groups in the lipid region A of the lipopolysaccharide (LPS). This process in competition replaces the divalent cations (Ca^{2+} and Mg^{2+}) from the phosphate groups in the membrane lipids.[19] The eventual breaking of the membrane culminates in the discharge of intracellular components and subsequent bacterial demise. However, the precise mechanisms underlying this cell death process remain inadequately comprehended.[20]

Antimicrobial resistance (AMR) is not a new phenomenon, but it is currently a serious health issue worldwide.[21] AMR refers to the capacity of microorganisms to neutralize the effects of antimicrobial drugs. This occurs when an antibiotic becomes ineffective in inhibiting bacterial growth.[22] As a result of the worldwide spread of MDR pathogenic bacteria, traditional antibiotics are becoming ineffective in the therapeutic treatment of patients with infections.[23] Excessive utilization of antimicrobial drugs can lead to the development of antimicrobial resistance in several harmful bacteria, including *Staphylococcus* spp.[24] The mechanism of action of antimicrobial resistance involves methicillin-resistant *S. aureus* (MRSA) encoding by *mecA* or *mecC* genes that are responsible for resistance to β-lactam antibiotics.[25,26] In addition to vancomycin-resistant *S. aureus* (VRSA) encoded *vanA* or other van resistance genes.[27] According to different studies, colistin combined with another antibiotic is a more effective treatment option for severe infections than using only one antibiotic alone, for instance.[28]

Several studies showed that using colistin with other antibiotics is more efficient for serious infection treatment than using an individual antibiotic as monotherapy. The study of Souli *et al.*,[28] reported that the potential action of the combination of colistin and imipenem was synergistic for 50% of isolates, or indifferent (50%) toward colistin-susceptible blaVIM-1-type metallo-β-lactamase-producing (MBL) *Klebsiella pneumoniae* strains, whereas, it was antagonistic (55.6%), and slightly synergistic (11%) against colistin-resistant strains.[29] Recently, colistin is reported to drive the *in vitro* activity of drugs such as isoniazid and amikacin against *Mycobacterium tuberculosis*.[30] Indicating the extensive use of colistin as a synergistic therapy against a variety of bacterial infections. In this study, we aimed to investigate whether the antibacterial effect of sulbactam against clinically isolated *Staphylococcus* spp. could be enhanced by colistin.

Materials and methods

Specimen collection and identification

One hundred and four (104) samples were collected from fracture wounds of infectious Iraqi patients under ethical approval licensed by Al- Kadhimiya Teaching Hospital (ID: 22/5205 on 13/10/2022). A total of 39 samples were identified as *Staphylococcus ssp.* using traditional morphological assays, and the Vitek2 system (Data not shown). *S. aureus* was characterized by its typical colonial appearance (displaying golden-yellow colonies), other *Staphylococcus* spp. were culturally and morphologically identified as Coagulase-Negative *Staphylococci* (CNS) forming non-golden yellow colonies with red zones.[31] The isolates were routinely cultured on Mannitol salt agar plates and inoculated in Mueller-Hinton medium (MH) for growth assay. All experimental assays were done in triplicate. The reference strain *S. aureus* NCTC13656 was purchased from Culture Collection Company/ UK Health Security Agency.

Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) determination

The MIC for each anHtibiotic was determined by the broth microdilution method as described by the Clinical and Laboratory Standards Institute.[32] Serial

concentrations of colistin or/and sulbactam (0.5–64 μg ml^{-1}) were prepared. Overnight cultures of Staphylococcus spp. and reference strain were grown in MH broth and the growth was adjusted to 5×10^5 in pre-warmed media. Different concentrations of colistin and sulbactam were added to the bacterial-containing plate. The plate was then incubated at 37°C for 24 hr. MICs were determined visually after the addition of the resazurin dye. The dye color changing from blue to pink indicates the presence of living cells. The MICs and MBCs were determined by plating directly the content of wells with MICs and the concentrations higher than the MICs values. The lowest concentration of the drug that completely inhibits the growth of the organism is referred to as MIC, and the lowest concentration of the drug that is bactericidal toward bacterial cells is defined as MBC.

The in vitro effects of colistin/sulbactam synergism

The synergism assays of colistin/sulbactam against colistin-sensitive *Staphylococcus* spp. were performed according to Mataraci and Dosler.[33] The assay was conducted using microtiter 96-well plates containing the bacterial suspensions at a final inoculum of 5×10^5 CFU ml^{-1}. Twofold concentrations of colistin and sulbactam were added, and the plates were incubated at 37°C for 24 hr. The reference strain *S. aureus* (NCTC 13656) was used as a positive control. The fractional inhibitory concentration (FIC) Index was calculated according to the formulas: $FIC_{sulbactam} = MIC_{colistin+sulbactam}/MIC_{sulbactam}$, $FIC_{colistin} = MIC_{colistin+sulbactam}/ MIC_{colistin}$, FIC Index $= FIC_{sulbactam} + FIC_{colistin}$. FIC Index values were explained as follows:

FICI $\leq$0.5 indicated synergism
0.5 < FICI$\leq$1 indicated an additive effect
1 < FICI$\leq$2 indicated the irrelevant effect
FICI >2 indicated an antagonistic effect.[34]

The time-kill assay

To study the effect of either colistin alone or in combination with sulbactam on the growth of colistin-sensitive *Staphylococcus* spp., time-killing assays were done according to Si W *et al.*,[35] An overnight culture of single colonies was adjusted to approximately 5×10^5 CFU mL^{-1} before the addition of colistin at concentrations of 0 or $^1/_2$ MIC (64 μg ml^{-1}), in the presence or absence of $^1/_2$ MIC (64 μg ml^{-1}) sulbactam. The samples were taken at different time intervals (0, 2, 4, and 24 h), each of which was serially diluted, spread on drug-free plates, and incubated at 37 °C for 24 h. A similar assay was performed without drugs as a control, and the standard strain *S. aureus* NCTC13656 was used as a positive control. The colonies for each dilution were counted, and each assay was performed in three biological replicates.

Autolysis assay

The autolysis assay was performed as described previously[36] with some modifications. Briefly, overnight cultures of colistin-sensitive *Staphylococcus* isolates and a standard strain were diluted in MH broth, and the growth was monitored to the mid-exponential phase (OD_{600} of 0.6). A 64 μg ml^{-1} colistin was added to the bacterial culture and incubated for 1 h. The exposed cells were washed twice in cold sterile distilled water and re-suspended in the same volume of 0.05 M Tris-HCl, pH 7.2, containing 0.05% Triton X-100, before being incubated at 30 °C. Bacterial cultures alone were incubated and washed under identical conditions and were used as a control. The optical density (OD_{600}) was measured every 30 min, and the data were referred to as the percent loss of OD_{600} at the indicated times in comparison to the relative data at time zero. All assays were done in triplicate.

Overall cell surface charge determination

In order to determine the total positive cell surface charge, the overnight cultures of colistin-sensitive *Staphylococcus* species were adjusted to the mid-exponential phase in pre-equilibrated Muller Hinton broth, and colistin was added and incubated for several time intervals (1, 2, and 4 h). Afterward, the growth was monitored to the mid-exponential phase, and the cells were harvested and washed twice with MOPS buffer (20 mM, pH 7.0), and the colistin-treated cultures were adjusted to an OD_{600} of 0.6 with the same buffer. The positively charged cytochrome c was added to a final concentration of 0.125 mg ml^{-1}, and incubated for 10 min at room temperature. The mixture was centrifuged and the supernatant was collected to quantify the unbound cationic cytochrome c at the absorbance A_{530} nm. Similar cultures of colistin-sensitive *Staphylococcus* species were used without colistin treatment as a control, and a reference strain as a positive control. All assays were done in triplicates.[35]

Results and discussion

Identification of clinically isolated staphylococcus species

A total of 104 clinical samples were collected from wound-fractured feet of Iraqi patients. Around 39

Table 1. Minimum inhibition concentration (MIC) and minimum bacterial concentration (MBC) of sulbactam and colistin against all isolates of *Staphylococcus* species.

		Sulbactam		Colistin	
No.	*Staphylococcus* species	MIC (μg ml^{-1})	MBC (μg ml^{-1})	MIC (μg ml^{-1})	MBC (μg ml^{-1})
1	*S. aureus*	None	None	1	2
2	*S. aureus*	None	None	None	None
3	*S. aureus*	None	None	None	None
4	*S. aureus*	None	None	None	None
5	*S. aureus*	None	None	0.5	1
6	*S. aureus*	None	None	1	2
7	*S. aureus*	None	None	None	None
8	*S. aureus*	None	None	2	4
9	*S. aureus*	None	None	None	None
10	*S. aureus*	None	None	2	4
11	*S. aureus*	None	None	None	None
12	*S. aureus*	None	None	None	None
13	*S. epidermidis*	None	None	0.5	1
14	*S. epidermidis*	None	None	0.5	1
15	*S. epidermidis*	None	None	0.5	1
16	*S. epidermidis*	None	None	0.5	1
17	*S. epidermidis*	None	None	0.5	1
18	*S. epidermidis*	None	None	0.5	1
19	*S. epidermidis*	None	None	2	4
20	*S. epidermidis*	None	None	4	8
21	*S. warneri*	None	None	1	2
22	*S. warneri*	None	None	0.5	1
23	*S. warneri*	None	None	0.5	1
24	*S. warneri*	None	None	2	4
25	*S. warneri*	None	None	0.5	1
26	*S. hominis*	None	None	0.5	1
27	*S. hominis*	None	None	0.5	1
28	*S. hominis*	None	None	0.5	1
29	*S. hominis*	None	None	2	4
30	*S. lentus*	None	None	None	None
31	*S. lentus*	None	None	0.5	1
32	*S. lentus*	None	None	0.5	1
33	*S. lentus*	None	None	1	2
34	*S. haemolyticus*	None	None	2	4
35	*S. haemolyticus*	None	None	None	None
36	*S. haemolyticus*	None	None	1	2
37	*S. lugdunensis*	None	None	0.5	1
38	*S. sciuri*	None	None	0.5	1
39	*S. pseudintermedius*	None	None	None	None
40	*S. aureus* (NCTC13656)	None	None	None	None

isolates out of 104 were identified as *Staphylococcus* species by Vitek2 compact system. In general, 12 (31%) isolates belong to *S. aureus*, 8 (20%) isolates belong to *S. epidermidis*, 5 (13%) *S. warneri*, 4 (10%) isolates of *S. hominis*, and *S. lentus*, 3 (8%) isolates belong to *S. haemolyticus,* 1 (2%) isolate belongs to *S. sciuri*, and 1 (3%) isolate belongs to *S. lugdunensis*, and S. *pseudintermedius* .Table S1 and Fig. S1.

Antimicrobial effect of colistin and sulbactam

The microtiter dilution assay was performed with sulbactam alone and with the induction of Colistin against *Staphylococcus* species, as shown in Table 1. According to the Clinical and Laboratory Standards Institute (CLSI), the susceptibility of sulbactam was determined as susceptible $\leq$4 μg ml^{-1} ; intermediate 8 μg ml^{-1} and resistant $\geq$16 μg ml^{-1}. In this study, all *staphylococcus* spp. were resistant to sulbactam, where the MIC and MBC values were zero. In contrast, the MIC values of colistin are between 0.5 and 64 μg mL^{-1}. Among 39 *staphylococcus* bacterial isolates, 29 have the values of MICs to colistin. (n=28, 71.8%) with MICs of $\leq$2 μg ml^{-1}, (n=10, 25.6%) with no MIC value, (n=1, 2.56%) with MICs of 4 μg ml^{-1} as shown in Table S1 and Fig. S2.

The rate of colistin-resistance (MICs > 2 μg ml^{-1} or have no MIC value) among all obtained isolates was determined in 11 isolates (11/39; 28.2 %), that has no MIC of which 7 were *S. aureus* (7/12; 58.3%), 1 was *S. lentus* (1/4; 25%), 1 was *S. haemolyticus* (1/3; 33.3%), 1 was *S. pseudintermedius* (1/1; 100%), and that has

Table 2. Illustrates the fraction inhibition concentration of colistin-sulbactam combination toward colistin-sensitive *Staphylococcus* species.

Staphylococcus species	Colistin MIC (μg ml^{-1})	Colistin + Sulbactam MIC (μg ml^{-1})	Colistin + Sulbactam FIC (μg ml^{-1})
S. aureus	1	0.5	0.5
S. aureus	1	16	16
S. aureus	1	16	16
S. epidermidis	0.5	0.5	1
S. epidermidis	0.5	0.5	1
S. epidermidis	0.5	0.5	1
S. epidermidis	0.5	0.5	1
S. warneri	0.5	0.5	1
S. warneri	0.5	0.5	1
S. warneri	0.5	0.5	1
S. warneri	0.5	0.5	1
S. hominis	0.5	0.5	1
S. hominis	0.5	0.5	1
S. hominis	0.5	2	4
S. hominis	2	0.5	0.25
S. lentus	0.5	0.5	1
S. lentus	0.5	0.5	1
S. lentus	0.5	0.5	1
S. haemolyticus	1	8	8
S. lugdunensis	0.5	0.5	1
S. sciuri	0.5	2	4
S. aureus (NCTC13656)	None	None	None

MICs of 4 μg ml^{-1} of which 1 was *S. epidermidis* (1/8; 12.5%), 0 was *S. warneri* (0/5; 0%), 0 was *S. hominis* (0/4; 0%), 0 was *S. lugdunensis* (0/1; 0%), 0 was *S. sciuri* (0/1, 0%). The rate of colistin-susceptible (MICs$\leq$2 μg ml^{-1}) among all obtained isolates was determined in 28 isolates (71.8%), of which 7 were *S. epidermidis* (7/8; 87.5%), 5 were *S. warneri* (5/5; 100%), 4 was *S. hominis* (4/4; 100%), 5 was *S. aureus* (5/12; 41.7%), 3 was *S. lentus* (3/4; 75%), 2 was *S. haemolyticus* (2/3; 66.7%), 1 was *S. lugdunensis* (1/1; 100%), 1 was *S. sciuri* (1/1; 100%), 0 was *S. pseudintermedius* (0/1; 0%). Table S2. The reference strain *S. aureus* (NCTC13656) was used as a positive control, which shows a pattern of complete resistance to colistin and sulbactam. The above results indicate that some clinical *Staphylococcus* species are sensitive to colistin. Table 1, Fig. S3.

The synergistic antibacterial activity of colistin in combination with sulbactam

Here, we attempt to investigate whether colistin can induce the activity of sulbactam. The synergism effect of colistin and sulbactam was investigated using 96-well microtiter plates, as shown in Table 2. The MICs of colistin were not decreased in the presence of sulbactam in 8 out of 9 isolates of colistin-sensitive *Staphylococcus* (*S. aureus*, *S. epidermidis, S. warneri, S. hominis*, *S. lentus S. haemolyticus*, *S. lugdunensis*, and *S. sciuri*). The MICs of colistin were the same as MICs of synergism, except for *S. aureus*, *S. hominis*, *S. haemolyticus*, and *S. scuri* which exerted an increase and decrease in the MIC values. Table 2, Fig. S4. The increase in the MIC values may be due to the interference of sulbactam with colistin and decreasing the potential binding with teichoic acid of colistin-sensitive *Staphylococcus* species. Table S3 Fractional inhibitory concentration index FIC could not be determined, as the FIC of sulbactam is 0, hence the result is an error. In addition, *S. aureus* (NCTC13656) was completely resistant to the combination of colistin and sulbactam. (Data not shown).

The time-killing activity of colistin-sulbactam combination

According to MIC results and to unequivocally determine whether colistin alone or with sulbactam can beat the colistin-sensitive *Staphylococcus* species, time-killing assays were performed with colistin in the absence and presence of sulbactam against *Staphylococcus* species that were sensitive to colistin. Fig. 1. The results showed that the treatment with colistin alone did not completely inhibit the growth of colistin-sensitive *Staphylococcus* species after 24 h, when compared to the control. Whereas, with the addition of $^1/_2$ MIC (64 μg ml^{-1}) sulbactam, the growth was decreased from 1 to 2 $\log_{10}$ in *S. aureus*. *S. epidermidis*, *S. warnri*, *S. haemolyticus*, and *S. sciuri* after 2hr exposure, Fig. 1a, b, e, c, and f. Interestingly,

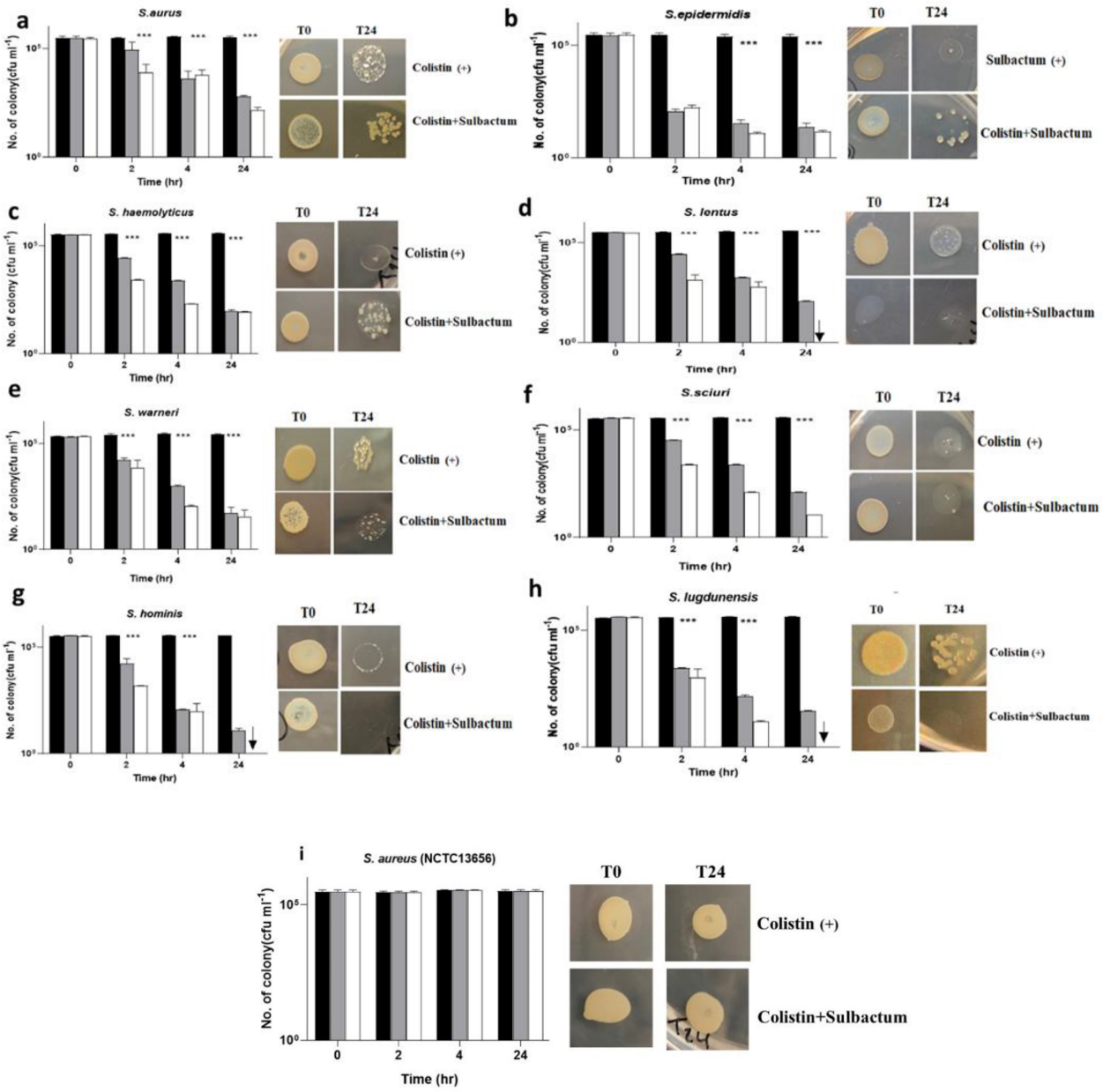

Fig. 1. Time-killing assay of colistin-sulbactam combination against eight colistin-sensitive *Staphylococcus* species. The bar shape refers to the number of colonies over time. The black bar refers to the control (without colistin or sulbactam); the grey bar refers to the colistin alone; the blank bar refers to the addition of colistin and sulbactam. a: *S. aureus*; b: *S. epidermidis;* c: *S. haemolyticus*; d: *S. lentus;* e: *S. warneri;* f: *S. sciuri;* g: *S. hominis;* h: *S. lugdunensis;* i: *S.aureus* (NCTC13656). All experiments were done in triplicate. The data shown are the mean and SD from three independent biological replicates. Statistical analysis was done by GraphPad Prism, the significance was done by two-way ANOVA (****P*< 0.001), and referred to by asterisks.

the colistin-sulbactam combination exerts a high synergistic effect against, *S. lentus, S. hominis* and *S. lugdunensis* resulting in complete growth inhibition, as shown in Fig. 1d, g, and h. In contrast, *S. aureus* (NCTC13656) doesn't show any decrease in the number of colonies after treatment with colistin Fig. 1i. The results here showed that colistin may slightly induce the activity of sulbactam, leading to partial or complete inhibition of colistin-sensitive *Staphylococcus* isolates.

Colistin-autolysis induction in colistin-sensitive staphylococcus species

According to the above results and to clarify whether colistin can induce the sensitivity of

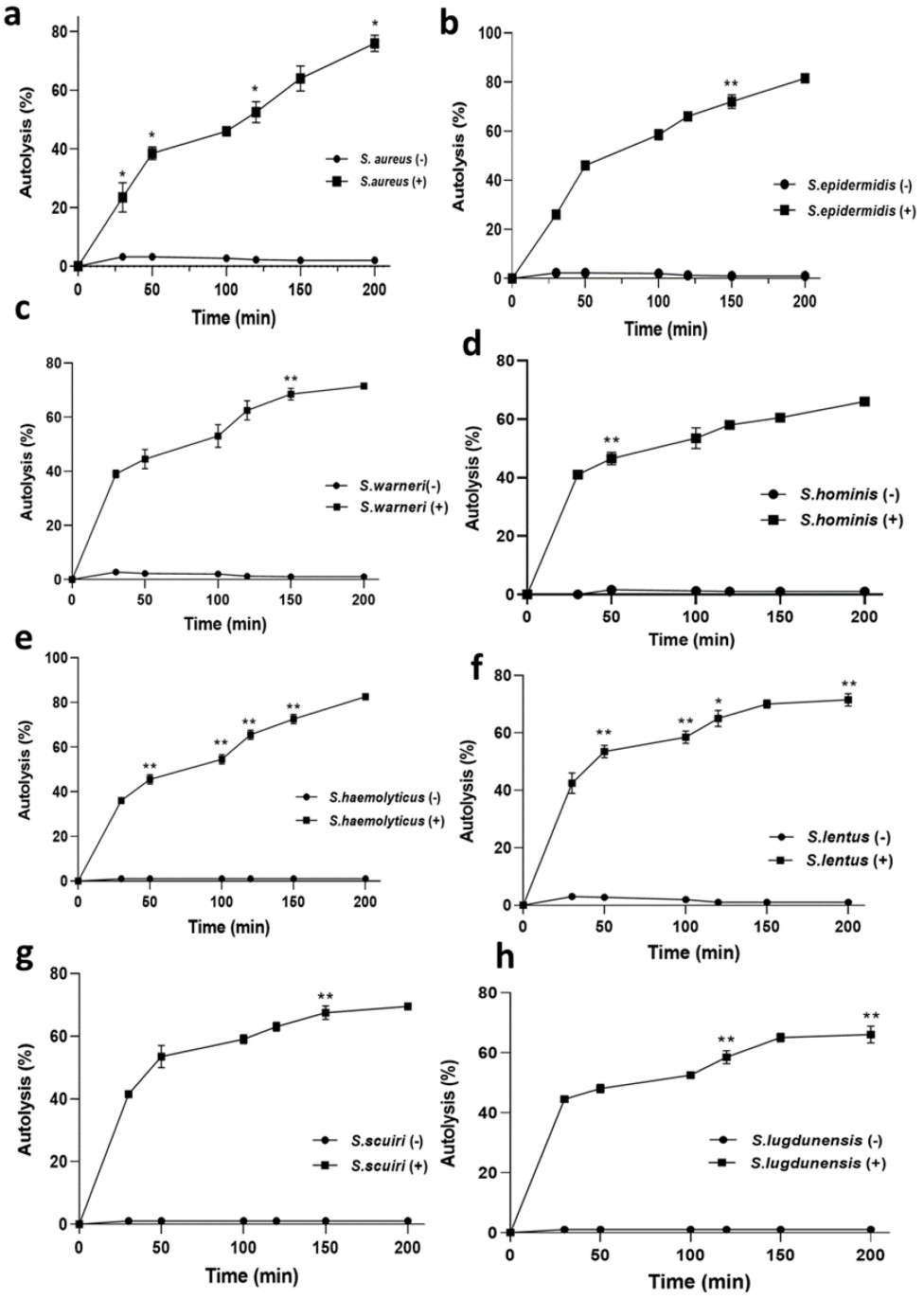

Fig. 2. Triton X-100 autolysis assay of colistin-sensitive *Staphylococcus* species: a: *S. aureus*; b: *S. epidermidis*; c: *S. warneri*; d: *S. hominis*; e: *S. haemolyticus*; f: *S. lentus;* g: *S. sciuri*; h: *S. lugdunensis*. The autolysis assays were repeated three times. The data shown represent the mean and SD from three biological replicates, and the statistical significance was determined by two-way ANOVA (*P<0.05. **P<0.01). The significance was indicated by asterisks.

colistin-sensitive *Staphylococcus* species to sulbactam, Triton X-100 autolysis activity assays were conducted. Fig. 2. The exposure of eight isolates to colistin (64 μg ml^{-1}) and sulbactam led to a significant increase in autolysis after 150 min exposure time in comparison to the control. The autolysis profile of colistin-sensitive *Staphylococcus* species was variable within species, where *S. haemolyticus* was significantly susceptible to colistin with a lysis percentage of 82.5 %, followed by *S. epidermidis* (81.5 %). Unlike *S. haemolyticus* and *S.epidermidis*, the colistin autolysis activity was slightly less effective in

S. aureus, S. warneri, and *S. lentus*, as the former showed only 76 % and 71.5 % for both species. In contrast, *S. scuiri*, *S. hominis*, and *S. lugdunensis* exhibit only 69.5 % and 66 % of total lysis after 150 min exposure, compared to the control, Fig. 2. However, no autolysis can be observed with *S. aureus* (NCTC13656) strain (Data not shown). Taking together, the results indicate that colistin exerts activity towards colistin-sensitive *Staphylococcus* species with slight synergism with sulbactam.

The contribution of colistin to decrease the overall-cell surface charge in colistin-sensitive staphylococcus species

To further investigate the ability of colistin to attenuate the bacterial cell membrane and its potential action is not coincidental, the quantity of bound cytochrome c was evaluated. The results showed that in the presence of colistin and sulbactam, the quantity of bound cationic cytochrome c was greater than that of the control, and increased gradually when the cells were incubated with colistin for 4 hr. Fig. 3. The efficiency of colistin as a cationic antimicrobial peptide relies on the fact that colistin can displace calcium and magnesium and makes the bacterial membrane more vulnerable to other peptides or drugs. This was illustrated with *S. warneri, S. epidermidis*, and *S. aureus* where the percentage of cytochrome c was increased by incubation time (4 h). Fig. 3b, c, and d. Whereas, *S. hominis*, *S. lugdunensis*, *S. sciuri* and *S. lentus*, the amount of cytochrome c decreased after 4 hr incubation, Fig. 3a, e, f, and g. These results indicate that colistin-sulbactam rapidly renders the cell membrane in colistin-sensitive species, and leads to leakage of positive charged ions. In addition, two hours of incubation with colistin were adequate to decrease the amount of positive cell surface charge in *S. hominis S. lugdunensis, S. sciuri*, Fig. 3a, e, and f. In addition, the reference strain *S. aureus* (NCTC 13656) showed a slight change in the amount of bounded cytochrome c after 4 h incubation (Data not shown).

Discussion

The ability of *Staphylococcus* species to acquire resistance to antibiotics and the emergence of antimicrobial resistance (AMR) *S. aureus*, raise a significant challenge to attenuate their pathogenicity toward human and public health. *Staphylococcus* is a major human pathogen and the main causative agent in hospitals and community-acquired infections. The extensive use of different types of antibiotics such as macrolides, tetracycline, and chloramphenicol resulted in the rapid development of antimicrobial resistance strains that can persist, proliferate, and survive in different environments.[37,38] Therefore, a combination of many drugs has been widely used against multidrug resistance Gram-negative bacteria, such as the combination of colistin and sulbactam against carbapenem resistant *Acinetobacter baumanii*.[39] Many studies revealed that colistin alone is able to inhibit and restrain the pathogenicity of opportunistic *A. baumannii* as well as is used to treat *E.coli* O157:H7 infections.[19,40] It was reported that the synergism between colistin and rifampin, fosfomycin, carbapenem, tigecycline, and trimethoprim-sulfamethoxazole was extensively used for multidrug resistant *A. baumannii* (MDR-AB) infection treatment.[41] In addition, it was observed that the combination of colistin and bacteriocins (Nisin A and Pediocin PA-1/AcH) was used against Gram-negative bacteria (GNB)[40] Sulbactam represents one of the most common semisynthetic β-lactamase inhibitors, which is typically used in combination with ampicillin or cefoperazone.[15,42] It is known that cefoperazone is sensitive to β-lactamase, although, in combination with sulbactam, the antibacterial spectrum of cefoperazone is expanded, and protection against β-lactamase hydrolysis is obtained.

The combination of cefoperazone-sulbactam is a wide-spectrum antibiotic, that is used to cure several acute bacterial infections, and multidrug-resistance organisms, such as extended-spectrum β-lactamase-producing Enterobacteriaceae and carbapenem-resistant A. baumannii.[43,44] The use of colistin with sulbactam has been widely reported against MDR *A. baumannii*.[42] Although, their synergistic effect against Gram-positive bacteria was not evaluated, as long as colistin is not active against Gram-positive bacteria. In our study, we attempt to examine the activity of colistin against nine different *Staphylococcus* species including *S. aureus*. The MIC results showed that among 12 isolates of *S. aureus*, five showed to be colistin–sensitive, which encouraged us to examine the synergism effect with sulbactam. The sulbactam alone was insufficient to exert a bactericidal effect against *Staphylococci*, hence, all *Staphylococcus* isolates were resistant, and the MIC values for sulbactam were zero. As shown in Table 1, in the addition of sulbactam to colistin no synergistic effect against colistin-sensitive *Staphylococcus* species was showed. This may be due to the narrow spectrum activity of sulbactam, and the development of *Staphylococcus* species resistance if sulbactam is used as a monotherapy.[45] Generally, most pathogens have several mechanisms to confer resistance toward antimicrobial drugs. The production of β-lactamases is the most common mechanism of antibiotic resistance, which destroys

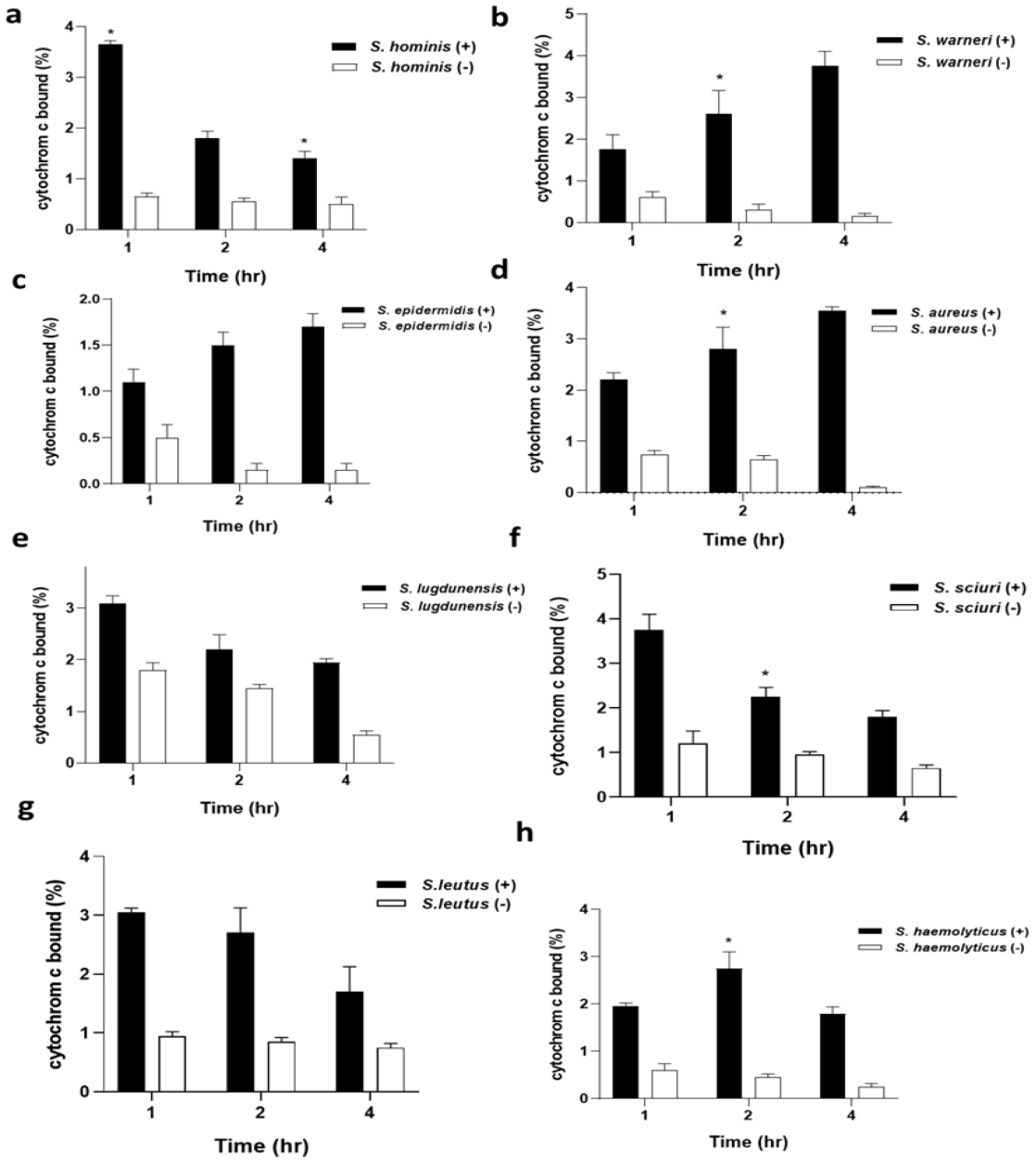

Fig. 3. Detection of cationic cytochrome c binding to whole *Staphylococcus* species cells in the presence or absence of colistin. a: *S. hominis*; b: *S. warneri*; c: *S. epidermids*; d: *S. aureus*; e: *S. lugdunensis*; f: *S. sciuri;* g: *S. lentus*; h: *S. haemolyticus*. The autolysis assays were repeated three times. The data represent the mean and SD from three biological replicates, and the statistical significance was determined by a *t*-test (**P*<0.05). The significance was indicated by asterisks. The black bar refers to the presence of colistin. The blank bar refers to the control (without colistin).

penicillins and cephalosporins by hydrolyzing the β-lactam nucleus. In this study, we showed that all *Staphylococcus* species were resistant to sulbactam. This is mainly due to the fact that sulbactam belongs to the class A β-lactamase inhibitor with potential activity against A. baumannii. The antibacterial activity of sulbactam against A. baumannii isolates was reported in the study of Ku and Yu,[15]. They mentioned that the potential action of sulbactam is mediated by the inhibition of penicillin-binding proteins (PBPs), mainly PBP1 and PBP3, and disruption of bacterial cell wall synthesis.[46,47] Most notably, sulbactam has a great affinity for PBP1a, PBP1b, and PBP3 in A. baumannii, but not for PBP2, which is the key enzyme essential in cell wall biosynthesis.[48]

In S. aureus and S. epidermidis, PBP2a, a variant type of transpeptidase, is expressed as another

mechanism of β-lactam resistance in addition to β-lactamase production.[49] In Methicillin resistant-*Staphylococcus*, the mecA encoding gene is acquired as an alternative transpeptidase penicillin-binding protein (PBP2a), except for ceftobiprole and ceftaroline, where PBP2a exhibits a very low affinity for almost all β-lactam antibiotics.[50] This suggests that sulbactam may increase the activity of beta-lactam antibiotics against MRSA.[51] Here, the efficiency of colistin alone or in combination with sulbactam against colistin-sensitive *Staphylococcus* species over time demonstrated that the bactericidal effect of both drugs was effective mainly against *S. hominis*, *S. lentus*, and *S. lugdunensis* after 24 hr. Notably, using colistin alone exerts a bacteriostatic rather than bactericidal effect, whereas, in the presence of sulbactam, bacterial growth is inhibited over time. However, the MICs of both drugs have not significantly changed.

Most of the *S. aureus* isolates in the current study were resistant to colistin alone or in combination with sulbactam. Treating *S. aureus* infections can be difficult due to the potential resistance to numerous medicines. *S. aureus* can develop resistance to almost all antibiotics that have been used in medical practice. The development of antibiotic resistance in *S. aureus* can occur through both genetic inheritance and acquisition. Inherited resistance encompasses genes that are inherently present on chromosomes. They provide characteristics such as reduced membrane permeability, increased expression of efflux pumps, and enzymatic inactivation of antibiotics. Acquired resistance encompasses the occurrence of genetic mutations and the transfer of genes between strains through mobile genetic components.[52]

The previous study indicated that the combination of colistin and sulbactam may be an appropriate treatment for a multi-drug resistant (MDR) isolate of *A. baumannii*. The minimum inhibitory concentrations (MICs) of colistin and sulbactam were determined to be 0.5 μg ml^{-1} and 128 μg ml^{-1}, respectively.[39] Hence, the effect of colistin and sulbactam against *Staphylococci* is unknown; we hypothesized that colistin may enhance the activity of sulbactam toward some colistin-sensitive *Staphylococcus* isolates over time. The time killing assay showed a significant inhibition of some species after 4 h incubation, Fig. 1. This might be due to the ability of colistin to increase the permeability of the cell wall, allowing other antibiotics to enter the Gram-positive bacterial cell wall by active transport.[53] Moreover, the effect of colistin on the *Staphylococcus* cell surface was investigated to illustrate the increased susceptibility to sulbactam. Based on our results, the disruption of the cell surface of colistin-sensitive *Staphylococcus* species by colistin increases the entrance of sulbactam into the cells and induces the leakage of Mg^{2+} and Ca^{2+} ions to enhance sulbactam antibacterial activity.

The ability of colistin to disrupt the bacterial cell surface was supported by our results Fig. 2. Treatment of colistin-sensitive *Staphylococcus* species with colistin increases the autolytic activity and reduces the positive charge on the bacterial cell surface, and cellular concentration of $Mg^{2+,}$ Ca^{2+}, Mn^{2+}, and Zn^{2+}.Therefore, it enhances the susceptibility of *Staphylococcus* species to sulbactam.[36] This explains the lethal effect of colistin against Gram-positive bacteria. The autolytic activity of colistin is due to the electrostatic interaction between the positively charged colistin and teichoic acids, which leads to disrupt the cell surface of Gram-positive bacteria. In addition, colistin exerts an oxidative damage cellular death pathway in Gram-positive bacteria, by displacing divalent cations from teichoic acids on the cell surface, destroying the cell wall and leading to cell death. In addition, colistin exerts an oxidative damage cellular death pathway in Gram-positive bacteria, by displacing divalent cations from teichoic acids on the cell surface, destroying the cell wall, and ultimately causing cell death.[54]

Furthermore, the treatment of bacterial cells with an effective non-ionic detergent (Triton X-100), increases the permeability of the cell membrane by dissolving the lipid and hydrolyzing the hydrogen bonds in lipid bilayers and cell lysis.[55,56]

In this study, the interaction of cationic Cytochrome C with bacterial cells exposed to colistin in comparison to the control was measured, and the binding of Cytochrome C that interacts electrostatically with the negatively charged bacterial cells was quantified. In the presence of colistin, the colistin-sensitive *Staphylococcus* species exert more efficient binding of Cytochrome C, suggesting that colistin confers more anionic-charge, in comparison to the control Fig. 3. These results indicate that colistin could attenuate the negative charge of bacterial cell membrane, and make the bacterial cells more susceptible to other antibiotics.[36] Our results are in agreement with the study of Mohapatra *et al.*[57] who stated that exposure of *Staphylococcus* cells to colistin resulted in high negative charges and displacement of the membrane-stabilizing cations such as Ca^{2+} and Mg^{2+}. The cell membrane of *Staphylococcus* species contains different types of phospholipids and glycolipids, such as phosphatidylglycerols (PG), lysyl-phosphatidylglycerols (Lys-PG) monoglycosyldiacylglycerols (MGDG), diglycosyldiacylglycerols (DGDG), and cardiolipins (CL). Many external factors, such as pH, temperature, and antibiotics, can affect the lipid composition of the membrane.[58] Basically, *Staphylococcus* cell wall is susceptible to cationic antimicrobial drugs, due to the anionic overall surface charge

conferred by teichoic acids. In addition, the negative and positive charges of the membrane are balanced by the proportion of lipid class Lys-PG and lipid PG. When Lys-PG increases, the resistance to positively charged antimicrobial peptides (CAMPs) increases. Antimicrobial resistance patterns of *Staphylococcus* isolates are associated with the differences in PG and Lys-PG contents. In the present study, when a particular isolate has a lower amount of positively charged lipids (such as Lys-PG), it would be more susceptible to colistin. Therefore, *Staphylococcus* species were more susceptible to colistin.[59]

Conclusion

The emergence of multidrug-resistant pathogens led to the development of new strategies to overcome their pathogenicity. The antimicrobial effect of sulbactam towards Gram-positive bacteria was never reported earlier. Hence, all *Staphylococcus species* have a robust mechanism to overcome different types of antibiotics. The ability of colistin to attenuate different *Staphylococcus species* has been reported in this study, Table 1. Although, some species exert a strong resistance profile, others were either slightly inhibited or completely inhibited in the presence of colistin and sulbactam together, Fig. 1. The potential action of colistin towards some species may be due to the fact that colistin renders the cytoplasmic membrane, resulting in the leakage of ions and paving the way for other antibiotics' entrance. A. *baumanni* was showed to be a good example of colistin's mechanism. Hence, some *Staphylococcus species* exert similar patterns when exposed to TritonX-100 and the release of unbound cytochrome C, Figs. 2 and 3. Our hypothesis concludes that despite of weak potential synergism of colistin and sulbactam against *Staphylococcus species*, a profound mechanism of colistin could attenuate the resistance ability of some species other than *S. aureus*, considering the source of isolation, resistance acquisition, and the genetic variations that take place during infection. Hence, the ability of colistin to restrain different species is not coincidental. The occurrence and fast dissemination of *Staphylococcus species* in patients with different diseases increase the risk of infection and impede treatment strategies. Colistin's treatment of different *Staphylococcus species* isolates from patients with different diseases could be another option to render the resistance ability not only for *Staphylococcus species* but for other gram-positive bacteria.

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Authors' declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for republication, which is attached to the manuscript.
- Author(s) sign on ethical consideration's approval.
- No animal studies are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at the University of Baghdad and under the Declaration of the Higher Ministry of Health, and approved by the Institutional Review Board of Al-Kadhimiya Teaching Hospital (protocol code 22/5205, date of approval: 13/10/2022).

Authors' contribution statement

Conceptualization, H.H. A., methodology, Z.M.F and H.H.A., software:, H.H.A., validation, H.H.A and Z.M.F., formal analysis, Z.M.F and H.H.A., investigation, Z.M.F., resources, Z.M.F and H.H.A., data curation, H.H.A., writing—original draft preparation, Z.M.F., writing—review and editing, H.H.A., visualization, Z.M.F., supervision, H.H.A., project administration, H.H.A., funding acquisition, Z.M.F.

Journal declaration

Second author (H.H.A) is an editor for the journal but did not participate in the peer review process other than as an author. The authors declare no other conflict of interest.

Supplementary materials

Supplementary materials are available at the following link https://bsj.uobaghdad.edu.iq/cgi/viewcontent.cgi?filename=0&article=5207&context=home&type=additional&preview_mode=1

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منظور جديد في صورة مقاومة الكوليستين لبعض انواع المكورات العنقودية المعزولة من جرح كسر القدم لمرضى عراقيين

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الخلاصة

السلباكتام ينتمي إلى مثبطات بيتا لاكتاماز التي تستخدم بشكل اكثر شيوعاً للبكتريا السالبة لصبغة غرام من البكتريا الموجبة لصبغة غرام. في هذه الدراسة، تسع انواع مختلفة من المكورة العنقودية (*S. aureus, S. epidermidis, S. warneri, S. lentus, S. Pseudintermedius, S. lugdunensis, S. haemolyticus, S. sciuri, and S. warneri*) تم عزلها من جروح أقدام مرضى عراقيين مكسورة. تم تقييم نطاق النشاط من السلباكتام والكوليستين ضد جميع العزلات. ومما يدعو للدهشة، كانت 5(12) من عزلات ال *S. aureus* حساسة للكوليستيسن، بينما كانت جميع العزلات البكتيرية مقاومة بالكامل للسلباكتام. هنا، كشفت دراستنا أن دمج الكوليستين والسلباكتام ضد المكورة العنقودية الحساسة للكوليستين قد لا يعزز الحساسية من الأنواع البكتيرية، حيث ان قيمة الحد الأدنى للتركيز المثبط (Mic) للمزيج لم تتغير بل أزدادت في بعض الأنواع، مما يشير إلى النشاط المحتمل من الكوليستين بمفرده. على الرغم من ان نتائج الحد الأدنى للتركيز المثبط Mic أضهرت عدم وجود تأثير المزيج من الكوليستين والسلباكتام، فقد تم اجراء أختبار قتل الوقت الذي اظهر تثبيط كامل من الأنواع المكورة العنقودية الحساسة للكوليستين بإستثناء ال *S. aureus* و*S. aureus* السلالة القياسية (NCTC 13656). ولغرض زيادة فحص تأثير الكليستين، تم إجراء نشاط التحلل الذاتي للكوليستين بوساطة زيادة نفاذية الغشاء والتسرب من السوائل الداخل خلوية. تم قياس تسرب الشحنة الموجبة ثنائية التكافؤ (Ca^{2+} and Mg^{2+}) من العزلات الحساسة المعالجة بالكوليستين بواسطة فحص السايتوكروم C، مما أدى إلى إنخفاض ملحوظ في الكاتيونات عل سطح الخلية. على حد علمنا، هذه هي أول دراسة لتحديد النشاط المحتمل من الكوليستين ضد أنواع المكورات العنقودية المعزولة سريرياً بخلاف ال *S. aureus* التي أظهرت نمط من الحساسية ضد الكوليستين بمفرده.

الكلمات المفتاحية: بيتا لاكتاميز، كاتيونات، سايتوكروم C، البكتريا الموجبة لصبغة غرام، البوليميكسين E.