RESEARCH ARTICLE

Increased Antibacterial Activity of Ciprofloxacin by Combination with *Staphylococcus aureus* Siderophores

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ABSTRACT

This study is interested in using siderophore molecules produced from *Staphylococcus aureus* in combination with ciprofloxacin (CIP) against different multidrug resistant (MDR) bacterial isolates. Three types of siderophore have been obtained from *S. aureus*; hydroxamate, catecholate, and carboxylates(staphyloferrin).

Results have been shown an increase in CIP activity when combined with siderophores. Inhibition results were compared for each siderophore only, siderophore + (CIP), and CIP only.

CIP + hydroxamate and catecholate were recorded high inhibition zone (26 mm), while CIP + carboxylates from the same isolate were recorded larger zone (29 mm), whereas CIP + staphyloferrin has the maximum inhibition zone (36 mm). Markedly, the three siderophore types were isolated from CIP-resistant *S. aureus*.

Keywords: Ciprofloxacin, Siderophore, *Staphylococcus aureus.*

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INTRODUCTION

Siderophores are small molecules used for obtaining iron, which plays an important role in infection occurrence, thus considered virulence factors, especially for pathogenic bacteria like multi-drug resistant (MDR) *S.aureus* the major causative agent in nosocomial infections, MDR refers to the bacterial isolates exhibit resistance to one or more class of antimicrobial agents. Variations in management, medical care, random administration of antimicrobial drugs, and long treatment periods have led to emerging highly resistant isolates to all common antimicrobial drugs.[1,2] Broad-spectrum fluoroquinolones have been used in treating infections caused by gram-positive /negative bacteria by inhibiting cytoplasmic DNA gyrase or topoisomerase IV, and resistance is rising after replacing the amino-acids in the targeted enzyme; also the resistant bacteria can use efflux pumps that called quinolones resistant protective proteins (Qnr protective proteins) and producing aminoglycoside acetyltransferase.[3]

Siderophores use the "Trojan horse strategy" to form complexes with antibiotics and help select antibiotics to the resistant bacterial cells.[4] Trojan horse strategy; facilitating penetration to the bacterial cell leading to killing or inhibition; this mechanism is applied to overcome bacterial resistance. Many studies have reported the importance of this strategy in designing improved antimicrobial agents that can enter the bacterial cell and reduce its resistance.[4-6] Synthetic siderophore-drug complexes may be a promising solution for treating multidrug-resistant bacterial infections or some human diseases.[7] These conjugates have drawn attention in circumventing common antibiotic resistance mechanisms as outer membrane permeability barriers, enzymatic failure, or blocked diffusion.[8]

Aim of the Study

The aim is to study the increasing the antibacterial activity of the ciprofloxacin by combining the crude hydroxamate, catecholate, and staphyloferrin extracts of *S.aureus*.

METHODS

1-Bacterial Isolates

Fifty *S.aureus* isolates from different clinical sources in addition to healthy individuals (respiratory microbiota) were collected, isolated, and identified by standard diagnostic methods: Gram reaction, motility test, oxidase, catalase and coagulase tests, hemolysis type, fermentation of mannitol (mannitol salt agar 7.5% NaCl). Identification has been confirmed by VITEK 2 system (bio Mérieux).

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2-Antibiotic Susceptibility Test

The antibiotic susceptibility pattern of *S. aureus* isolates was drawn according to Clinical and Laboratory Standards Institute (CLSI, Kirby- bauer assay) that detected MDR- MRSA isolates. *S.aureus* ATCC 29213 was used as a control. Minimum Inhibitory concentration (MIC) was made for vancomycin to determine sensitive and resistant isolates.

3-Screening for the Siderophore Producing Isolates

S.aureus isolates have been tested for their ability to secrete siderophore molecules. Three diverse methods were used to detect the siderophore producer *S. aureus* isolates represented by growth on CAS- agar, O-CAS, and LB-2, 2'-Dipyridyl agar media.[9]

The producing isolates were subjugated to more specific tests to determine the highest producing isolate quantitatively, so the colored zone diameters of siderophores were measured(nm) on CAS-agar plates around the well containing bacterial suspension grown in LB-2,2'-Dipyridyl broth of chosen isolates.[10] The isolates with the largest zone were chosen, grown in Succinate Minimal Medium (SMM) incubated at 37°C /pH 7 with continuous shaking for 72–96 hrs. to choose the optimum production conditions.[11]

4-Extraction of Siderophores

The producing isolates were inoculated in SSM medium incubated at 37°C /pH 7 with continuous shaking for 72 hours, siderophores were extracted by cool centrifugation (4°C with 8000 rpm/min.for 10 minutes), pellet was discarded, and the supernatant was collected then 6M HCl was added for 2 hours. The supernatant was extracted with ethyl acetate (3:1) volume and shacked vigorously in a separation funnel, and the extraction was done twice, organic molecules have been collected and evaporated by a rotary evaporator at 38°C. The dried extract was weighted, and 1mg was taken and dissolved in 10 mL of PBS with a final concentration of 100 µg/mL. Productivity percentage was detected.[12] Quantitative and qualitative tests were applied to determine the types of siderophores.[13-17]

5- Preparation of:

Ciprofloxacin (CIP) Diluted Solution: Ciprofloxacin 2000 µg/mL was diluted to 1000µg/mL, then diluted by half dilution to 100 µg/mL stored at 4ºC.

Siderophore +CIP Combined Solution: CIP solution was combined with the extracted and dialyzed siderophores at ratio 1:1 in the same concentration of 100 µg/mL. Combined solutions were set for few minutes before use.

Filter Paper Discs: Filter papers (medium pore size) were cut to a small disc shape with a diameter of 6 mm and sterilized by autoclave, impregnated in solutions of extracted, dialyzed siderophores and CIP as well as a combination of siderophore + CIP. Discs were let to stand few minutes in the solutions, allowed to dry for 15 minutes, control discs emerged in sterile ddH_2O.

6-Antibacterial Activity Test of Siderophores

The test was applied on MDR bacterial isolates; *Enterobacter spp., Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Serratia marcescens, and S. aureus* from which the bacterial inoculum tubes were made and adjusted to the 0.5 McFarland turbidity standard (approx. cell density 1.5 x 10^8 CFU/mL). Disc diffusion method was used, and the inhibition zone (mm) results for siderophores alone, siderophore, CIP, and CIP alone were compared. Three solutions were prepared for siderophores, antibiotics, and combination (1:1) from the same concentration for each and distal water as control. Discs from filter paper were soaked in these solutions for several minutes, dried, and located on Mueller –Hinton agar cultured with the bacterial isolates under-study; plates were incubated at 37°C/ 24 hours, diameters of inhibition zones(mm) were measured.

RESULTS AND DISCUSSION

1-Identification of Bacterial Isolates

Conventional tests identified bacterial isolates; Gr+cocci, β-hemolytic on blood agar, coagulase & catalase, and oxidase-positive, ferment mannitol on mannitol salt agar (7.5% NaCl). Results of identification by VITEK 2 system were (92–98%) compared to *S. aureus* ATCC 29213.

2-Antimicrobial Susceptibility of *S. aureus*

Eight antibiotics from different seven classes which have usually been used in the treatment of *S. aureus* disorders were used for antibiotic susceptibility test; cefoxitin, ciprofloxacin, levofloxacin, gentamicin, meropenem, tigecycline, trimethoprim, and vancomycin (Table 1).

3-Detection of Siderophores

Eleven *S. aureus* isolates were the high siderophore producing detected by the three methods CAS, O- CAS, and 2,2'-Dipyridyl (Figure 1).

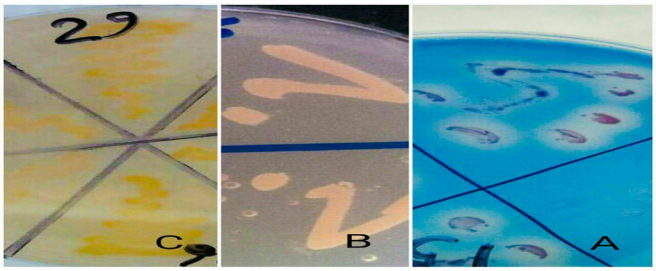

Figure 1: Siderophore Producing *S.aureus* Isolates: A, B reddish orange colonies on CAS agar and O-CAS agar C; Light orange on LB-2,2'-Dipyridyl agar with clear discoloration zone around colonies

Table 1: Results of susceptibility test of *S. aureus* isolates

Antibiotic	*FOX (30ug)*	*CIP (5ug)*	*LVX (5ug)*	*GM (10ug)*	*MEM (10ug)*	*TGC (15ug)*	*TMP (5 ug)*	*VA* (30 ug)*
Antibiotic Resistance%	68%		36%	28%	12%	4%	38%	66%

FOX; Cefoxitin, CIP; Ciprofloxacin, GM; Gentamicin; LVX; Levofloxacin, MEM; Meropenem, TGC; Tigeyclin, TMP; Trimethoprim, VA; Vancomycin

These isolates were chosen to grow in LB broth supplemented with Fe^{+2} chelator 2,2'-Dipyridyl, the supernatant was loaded into CAS agar wells. Variable measurements of orange-colored zone diameters were noticed on CAS agar plates, Presswood (2010) used LB-2,2'-Dipyridyl broth for cultivation *S. aureus* isolates; he observed that zone diameter after 18 hours was 10 mm and reached 14 mm after 24 hours.[12] Our results have shown that O-CAS is the best technique in siderophore detection on solid media.

Five isolates (5,16,20,27,34) revealed the highest zone diameter with (14,14,15,15,14) mm respectively. To get more precise results, the isolates were grown in SMM broth for 96 hours and examined for siderophore production percentage by CAS solution assay using spectrophotometer as shown in Figure 2.

Incubation was done with constant shaking to maintain constant aeration as the affinity of siderophores to iron would increase with the continuous oxygen supply, which acts as an electron donor.[4]

4-Extraction of Siderophores

Hydroxamate and catecholate were detected in the extract, while staphyloferrins were not extracted in ethyl acetate. Due to the high hydrophilicity of staphyloferrin siderophores, they would not be dissolving in ethyl acetate. Therefore, staphyloferrin was concentrated by dialysis bag by concentrating the SMM supernatant with sucrose. Radhakrishnan *et al*. (2014) have obtained hydroxamate siderophores from *Bacillus sp.* SD12 isolated from iron factory soil by extraction with ¼ volume of ethyl acetate twice.[18] Iraqi studies of khalaf and Jarjees (2011) and Khelkal (2016) have extracted catecholate siderophores from *S. aureus,* and *K. pneumoniae* isolates, respectively, using SMM and ethyl acetate.[19,20]

Drechsel *et al.* (1993) showed that staphyloferrin B purified from *S. hyicus* was a massively hydrophilic metabolite.[21] Also, in the culture fluid of several *Staphylococcus* strains, staphyloferrin A was detected as a vastly hydrophilic compound and an additional iron-regulated compound called staphyloferrin B.[4,22,23]

5-Determination of Siderophores Types

Quantitative and qualitative tests were applied to determine the types of siderophores (Table 2).

6-Determination of the Antibacterial Activity of Siderophores

To determine the antibacterial activity of siderophores, they were tested against different twelve MDR bacterial isolates MDR Isolates; *Enterobacter spp., Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Serratia marcescens,* and *S. aureus*. Six isolates with the highest MDR pattern% were chosen to be examined for antibacterial activity of different siderophore types (Figures 3-5).

In Figure 4, staphyloferrin in the dialyzed supernatant of isolate no. 27 revealed more effect than the extract in its antimicrobial activity and improved CIP effect with the largest zone diameter (29mm). This may be attributed to its chemical structure, which may contain active or potential groups more than hydroxamate or catecholate, or it may be the dominant siderophore type secreted by *S. aureus* isolates.

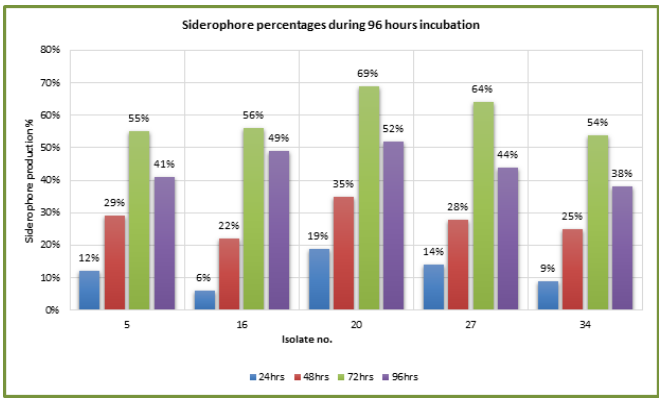

Figure 2: Siderophore detection by CAS quantitative assay

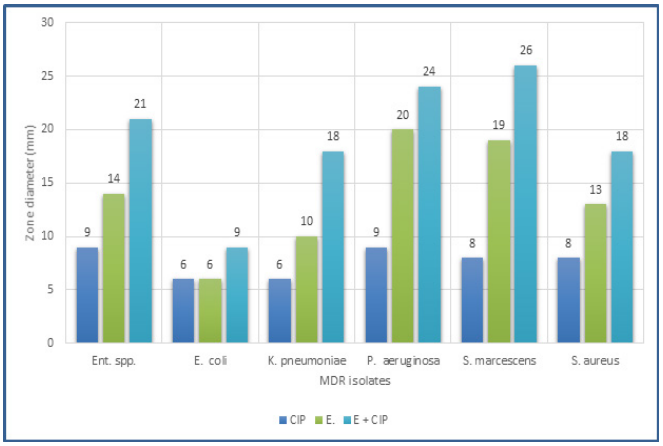

Figure 3: Antibacterial activity of the hydroxamate and catecholate extract of isolate no.27.

Table 2: Detection assays of siderophore types

		Detection in the supernatant	
Siderophores detection assays		*Isolate no. 20*	*Isolate no. 27*
Siderophores%	CAS solution assay	69%	64%
Hydroxymates	FeCl3 (420–450) nm	-	+
	Tetrazolium chemical assay	-	+
Catecholates	FeCl3 (495 nm)	-	+
Carboxylates (staphyloferrins)	Shenker(190–280) nm	+	+
	Vogel chemical assay	+	+

Note: +; siderophore producer, -; siderophore non-producer

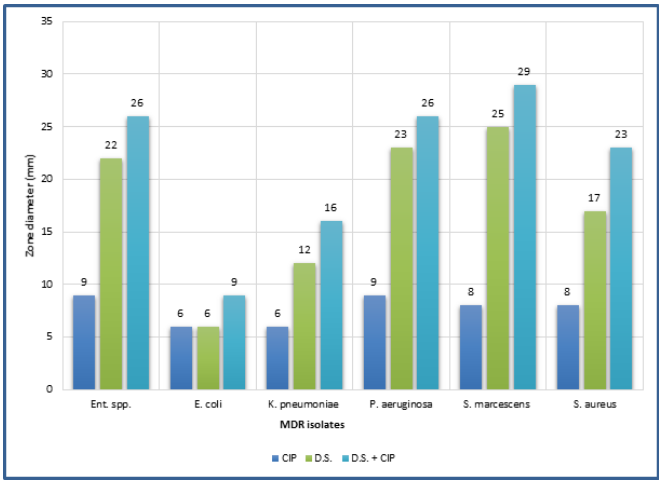

Figure 4: Antibacterial activity of the dialyzed staphyloferrin of isolate no. 27. Note: D.S.; dialyzed supernatant.

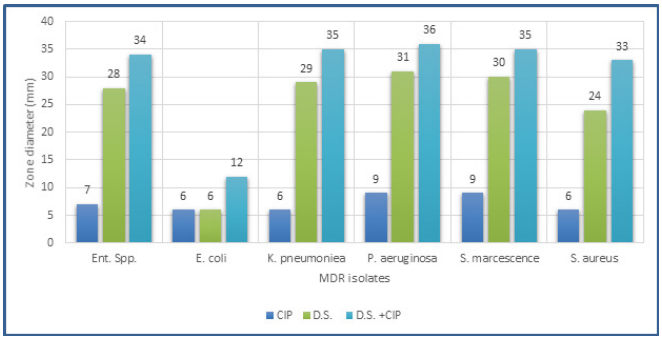

Figure 5: Antibacterial activity of the dialyzed staphyloferrin of isolate no. 20.
Note: D.S.; dialyzed supernatant.

Figure 5 shows the results of staphyloferrin in the dialyzed supernatant of isolate no.20 on MDR isolates. Actually, as for isolate no. 27, isolate no. 20 also showed prominent exceed of CIP effects when combined with the staphyloferrin against MDR isolates with the largest zone diameter (36 mm).

Some MDR isolates like *E. coli* and *S. aureus* showed high resistance against the effect of (CIP + siderophores). The antimicrobial activity of (CIP + siderophore) may be affected negatively by other possibly present metabolites secreted by *S. aureus* bacterial cells in the supernatant. Some secreted enzymes especially may cause denaturation to the siderophore structure or affect its combination with the CIP, according to siderophore-specific receptors that differ for each bacterial species. However, the immense diversity in the chemical structures of siderophore constitutes a valuable chemical collection for the design of specific siderophore -antibiotic conjugates. Maybe the combination of siderophore + CIP had high inhibitory effects on MDR isolates but incapable of passing in bacteria by diffusion.[24] A study revealed that structurally related fluoroquinolone-conjugates were synthesized by linking the carboxylic acid functionality of staphyloferrin A and its derivatives to the piperazinyl nitrogen of the parent drug via amide bond formation. The resulting siderophore–fluoroquinolone conjugates were screened against a panel of bacteria associated with infection in humans.[25]

CONCLUSION

Synergistic antibacterial effect has appeared between crude extract of *S.aureus* siderophores and ciprofloxacin.

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