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Synergistic Effect of Staphyloxanthin and some Antibiotics on Clinical Isolates of *Klebsiella pneumoniae*

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Abstract: *Klebsiella pneumoniae* is one of the main causes of UTIs around the world. The aim of the study to examine the impact of staphyloxanthin and combination with some antibiotics on *K. pneumoniae*.150 urine samples obtained from Baghdad hospitals. The collected samples were streaked onto macConkey agar, followed by biochemical tests; then, using VITEK-2-compact system; thirty isolates were confirmed as *K. pneumoniae*. In addition, 10 pre-isolated and identified *Staphylococcus aureus* (producers of staphyloxanthin) were taken. The staphyloxanthin pigment extracted, purified. Antibiotic susceptibility testing of (30) *K. pneumoniae isolate* was assessed using KB testing. MIC of antibiotics (meropenem and ciprofloxacin) and staphyloxanthin were assessed for isolates using broth microdilution method, the effect of combination of staphyloxanthin and antibiotics was also evaluated for the same tested isolates. The result exposed that Meropenem MIC was 2 µg/ml, while MIC for staphyloxanthin pigment was 250 mg/ml. Ciprofloxacin MIC was 1000 µg/ml regarding the synergistic effect of the (ciprofloxacin and meropenem) combined with the Staphyloxanthin their MIC decreased for all, the results showed. It was concluded staphyloxanthin and antibiotics (ciprofloxacin and meropenem) have impact on *K. pneumoniae.*

Keywords: Meropenem, staphyloxanthin, Ciprofloxacin, *Klebsiella pneumonia*.

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Introduction

Klebsiella pneumoniae is an opportunistic pathogen that can cause a wide range of infections (1). In humans, *Klebsiella* spp. is common commensals in the mouth, nasopharynx, and gastrointestinal tract (2). It is a Gram negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic rod-shaped bacillus (3). The colony's glistening, mucoid appearance on agar plates is due in large part to the polysaccharide capsule, whose considerable thickness is a critical factor in determining virulence and pathogenicity (4). The fact that *K. pneumoniae* is one of the "ESKAPE" diseases, the most prevalent MDR pathogens worldwide that include six bacterial pathogens raises concerns for public health (5). An infection-causing strain of *K. pneumoniae*, known as hypervirulent *K. pneumoniae* (hvKp), is frequently seen in communal settings and can affect relatively healthy people(6). The infection is often manifested in multiple organs. New methods to limit and restrict bacterial development must be developed as MDR strains of these bacteria have become a significant issue in the treatment of *Klebsiella* infections (7). Over many years, *K. pneumoniae* urinary tract infections have generally

increased in frequency, importance, and morbidity (8). The development of these bacteria as infections of the urinary tract and as causes of antibiotic resistance presents a difficult management and therapeutic challenge for the clinician(9). Many pigments (extracted from bacteria) have recently been used as an alternative to or in combination with anti- biotics.

Materials and methods

Isolation of *Klebsiella pneumoniae*

One hundred fifty urine specimens were collected from The Baghdad Medical City's Ghazi Hariri Hospital and cultivated on MacConkey agar, between August to December 2022. By subculturing on mac- Conkey agar and then incubating at 37°C for 18–24 hours, isolates were acquired from these labs.

Identification of *K. pneumoniae*

Culture media, including macConkey agar, and nutrient agar, were inoculated with the isolates and then incubated at 37 °C for 18 to 24 hours. Both morphologically and biochemically suspicious colonies were identified.

-Microscopic characteristics

Gram staining was used to investigate the suspicious colonies in order to determine the distinctive appearance of bacteria under a light microscope.

-Identification of *K. pneumoniae* by Vitek-2 compact system.

The initial biochemical tests, which showed the presence of *K. pneumonia* in the bacterial isolates, were followed by the use of the Vitek-2 device, to confirm isolates identification.

Screening of *Staphylococcus aureus* isolates for staphyloxanthin pigment production

In order to choose the isolate that produced the highest amounts of staphyloxanthin, *Staphylococcus aureus* isolates that were obtained from biology department/college of science/Baghdad university, and verified by the identification process, were examined in an agar culture for the formation of staphyloxanthin, as specified in the following sections:

-Preparation of inoculum

To create a *S. aureus* isolation inocu- lum, loopfuls of *S. aureus* growth from an overnight culture on nutrient agar were transferred to skim milk agar. The culture was kept at 37°C in an incubator for 72 hours.

-Cultivation method

Skim milk agar medium was prepared and autoclaved at 121˚C, for 15 min. The medium was then poured onto petri dishes, and each plate received a 1 ml inoculum of each *S. aureus* isolate. Following that, petri dish plates were cultured for 72 hours at 37 °C in an incubator. After incubation, the growth's appearance with color (yellow) indicates a successful outcome.

-Extraction of staphyloxanthin pigment

The pigment from *S. aureus* (STX) was extracted using methanol, with modification (10). Tryptic soy broth (TSB) was inoculated with a *S. aureus* 24-hour culture and 24 hr. incubation at 37 °C with shaking. The supernatants were removed after the bacteria were centrifuged at 10,000 rpm for 10 minutes. The pellets were resuspended since being extracted with methanol for 24 hours at 37 °C while being shaken. Following that, the extract was centrifuged for 10 minutes at 10,000 rpm to obtain the supernatants.

-Estimation Staphyloxanthin pigment UV-Vis spectroscopy

To estimation the Production of staphyloxanthin, a double-beam of UV-Visible spectrophotometer was used to

measure the absorbance at 460 nm for the quantitative assessment of the yellow pigment. As propose by Dong, et al, (11). An absorption spectrum with a single maximum between 600 and 460 nm best describes the pigments under examination (12).

Antibiotic susceptibility testing

It was done by using the modified Kirby-Bauer approach (13) toward the antibiotics: cefepime, ciprofloxacin, merop- enem, amikacin, tetracycline, augmentin, carbencillin and trimethoprim.

Determination of minimum inhibitory concentration (MIC)

The MIC values of the antibiotics (meropenem, ciprofloxacin) and staphylo- xanthin, as well as combination of meropenem with staphyloxanthin and Ciprofloxacin with staphyloxanthin were determined against *K. pneumoniae* isolates, by using Broth microdilution method (14).

-Determination of MIC of staphyloxanthin, meropenem, and combination of them against *K. pneumonia*

Mueller hinton broth contained of staphyloxanthin, meropenem and comb- ination of them. For 24 hours, the plates were incubated at 37°C. A 100 µl mixture of new bacterial suspensions devoid of staphyloxanthin and meropenem was also prepared, and both staphyloxanthin and Meropenem were also added to conduct the positive controls (compatible with the 0.5 McFarland standard).

-Determination of MIC of staphyloxanthin, ciprofloxacin and combination of them against *K. pneumonia*

Mueller hinton broth contained of staphyloxanthin, Ciprofloxacin and comb- ination of them. For 24 hours, the plates were incubated at 37°C. A 100 µl mixture of new bacterial suspensions devoid of staphyloxanthin and ciprofloxacin was also prepared, and both staphyloxanthin and ciprofloxacin were also added to conduct the positive controls (compatible with the 0.5 McFarland standard).

Results and discussion

Isolation and identification of *K. pneumoniae*

Eighty eight gram negative Bacterial isolates were gained from 150 urine specimens after cultivation on MacConkey's agar medium. Only gram-negative bacterial species can grow on MacConkey agar, which is a selective and differentiating agar that can further distinguish the gram-negative organisms based on their lactose metabolism. The results presented that 77 (51.3%) could ferment lactose (lactose fermenter), while 30 isolates (20%) of them characterized by large, mucoid, lactose fermenter with pink to red in color colonies on macConkey agar were *K. pneumoniae* (15). Oxidase and catalase test were performed. Findings showed that all bacterial isolates were catalase-positive and oxidase-negative. All isolates were found to be non-motile when motility was further examined. It is expected that the 30 bacterial isolates found to be *K. pneumoniae, which* were confirmed by Vitek -2 compact. Ballén, *et al.*, in their study, isolated *K. pneumoniae* in percentage 40% (16) and Hamad,, *et al*.,isolated *K. pneumoniae* in percentage 32%(17), while it was reported about 14.70 % by Lin *et al.*,(18).

Identification of *staphylococcus aureus* by Vitek -2 compact system

The Vitek-2 compact device was used to confirm the identification of the ten pre-isolates of *S. aureus* that grew on nutrient ager and developed yellow

pigment. The results showed that all the isolates were *Staphylococcus aureus*.

Screening of *staphylococcus aureus* isolates for staphyloxanthin production

The ten pre-isolates of *S. aureus* were subjected for screening practice that select the best staphyloxanthin producing isolate, to be used for further experiments in this study. The screening was achieved in skim milk agar, and, for more consistency, all isolates were grown under identical conditions for inoculum size, pH, cell count, incubation time, and shaking rate.

The results exhibited that S1 was the best isolate (the colour was darker) than one isolates, followed by the S4, (Figures 1, 2). While other isolate was not produce pigment. AL-Kazaz *et al.*,(2014) were found that the skim milk agar medium had the maximum creation of pigment for 72 hr. at 37˚C (19).

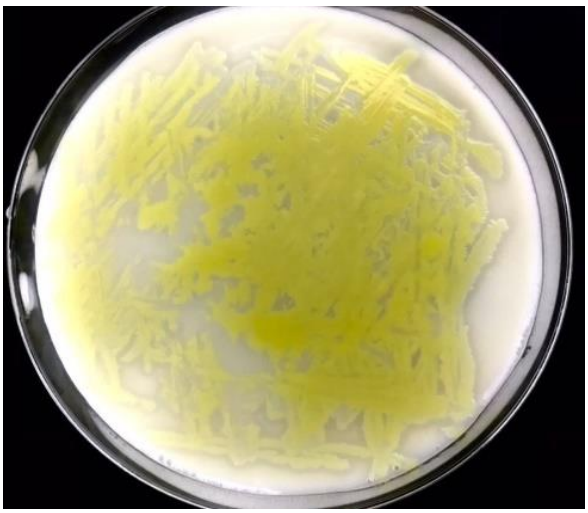

Figure (1): staphyloxanthin pigment on skim milk agar.

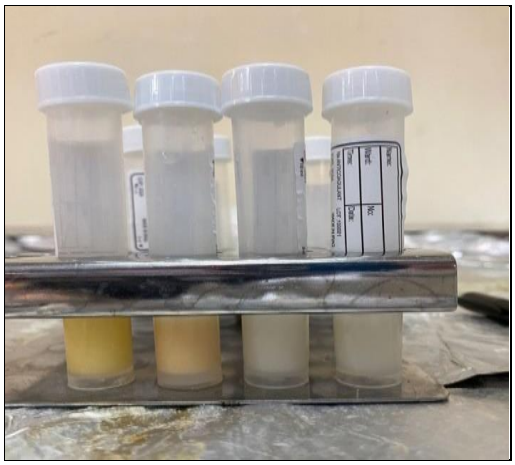

Figure (2): *S. aureus* isolates were subjected for screening of staphyloxanthin production.

Extraction of staphyloxanthin pigment

The sterile millipore filter was used to partially purify pigment extract, and then dried. Dark yellow powder of the extracted staphyloxanthin was gained which then either used immediately or stored in the fridge in a dark glass plate due to the light sensitivity of the pigments (20).

Estimation of Staphyloxanthin pigment by UV-Vis spectroscopy

The outcome indicated that the absorbance of staphyloxanthin pigment in UV visible was at about O.D460.The results were similar to Pelz, A., et al. (2005) stated that staphyloxanthin from *S. aureus* (20) had a maximum absorption spectrum at 462 nm.

Antibiotic susceptibility test of *Klebsiella pneumoniae*

-The Antibiotic susceptibility

The Antibiotic susceptibility test for eight different antibiotics was performed on thirty isolates. (meropenem, ciprofloxacin, amikacin, cefe-pime, tetracycline, augmentin, trimethoprim, carbencillin) by the disc diffusion method, recommended by the medical and laboratory standard association (CLSI, 2023) guide- lines, the results shown that (93%, 67%, 53%, 27%, 23%, 17% and 13%) of the isolates were sensitive toward (meropenem, amikacin, ciprofloxacin, trimethoprim, cefe- pime, tetracycline, augmentin) respectively, while 100% of isolates were resistant to carbencillin, in (Figure 3).

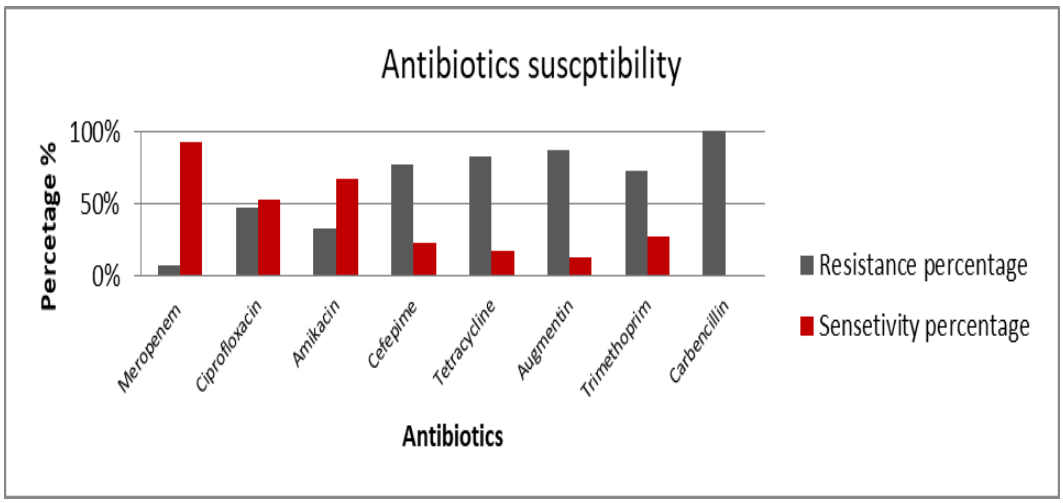

Figure (3): antibiotic susceptibility percentage of *K. pneumoniae* isolates to 8 antibiotics

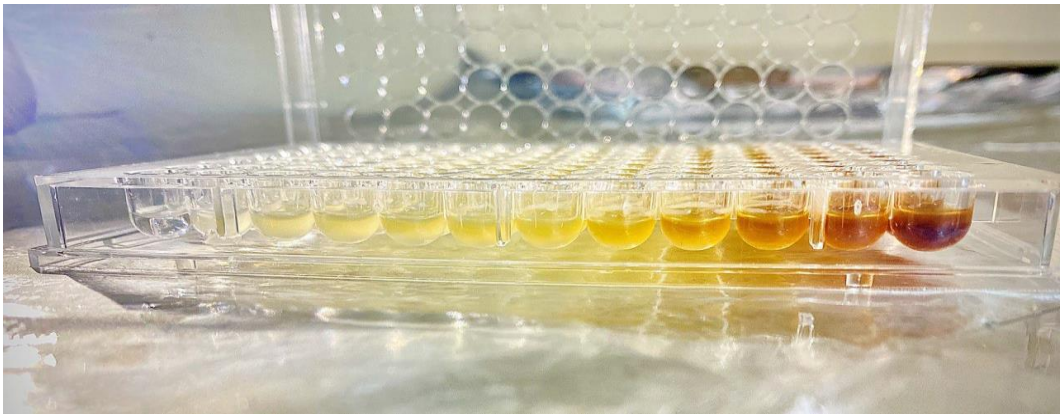

Figure (4): Microdilution plate to determine MIC of staphyloxanthin and antibiotics.

MIC of staphyloxanthin, meropenem and combination of them. And ciprofloxacin, combination of staphyloxanthin and ciprofloxacin against *K. pneumoniae*

The MIC for staphyloxanthin, meropenem and combinations were investi- gated using eight *Klebsiella pneumoniae* isolates. In addition, combination of staphyloxanthin and ciprofloxacin against *K. pneumoniae*, Using the microdilution technique, (Figure 4), was suggested by the clinical trial and laboratory values established (CLSI, 2023) guidelines.

The results presented that the MICs of Meropenem were (2µg/ml) and MIC of staphyloxanthin (250 mg/ml) and MIC of combination between staphyloxanthin and Meropenem distributed between 1.9 and 7.8 mg/ml for staphyloxanthin and between 0.25 and 1 µg/ml for meropenem) against eight isolates. Obviously it was clear that MIC of staphyloxanthin and meropenem decreased for both against the eight isolates of *K. pneumonia* by the synergistic effect, it decreased from 2 µg/ml to (1-0.25 µg/ml) for Meropenem and from 250 mg/ml to (1.9-7.8 mg/ml) for staphyloxanthin against the eight isolates as given in (Table 1).

One of the most often used antibiotic for treating *K. pneumoniae* infections, meropenem is a member of the carbapenem family of drugs and exhibits broad-spectrum in vitro resistance to both Gram-positive and Gram-negative bacteria (21). It easily passes through the cell walls of the majority of Gram-negative and -positive bacteria to reach the penicillin-binding protein (PBPS) that it is looking for and demonstrates stability against hydrolysis by the majority of -lactamases.

Table (1): MIC of (staphyloxanthin, meropenem, combination between staphyloxanthin and meropenem) for *K. pneumoniae*

Isolates	Meropenem	Staphyloxanthin	Synergistic between staphyloxanthin and Meropenem	
1	2 µg/ml	250 mg/ml	staphyloxanthin	3.9 mg/ml
			meropenem	0.5 µg/ml
2	2 µg/ml	250 mg/ml	staphyloxanthin	1.9 mg/ml
			meropenem	0.25 µg/ml
3	2 µg/ml	250 mg/ml	staphyloxanthin	1.9 mg/ml
			meropenem	0.25 µg/ml
4	2 µg/ml	250 mg/ml	staphyloxanthin	3.9 mg/ml
			meropenem	0.5 µg/ml
5	2 µg/ml	250 mg/ml	staphyloxanthin	3.9 mg/ml
			meropenem	0.5 µg/ml
6	2 µg/ml	250 mg/ml	staphyloxanthin	7.8 mg/ml
			meropenem	1 µg/ml
7	2 µg/ml	250 mg/ml	staphyloxanthin	7.8 mg/ml
			meropenem	1 µg/ml
8	2 µg/ml	250 mg/ml	staphyloxanthin	1.9 mg/ml
			meropenem	0.25 µg/ml

Bulik, *et al*.,sp(2010) found that The BMD MIC test range was 0.06 µg/ml to 64 µg/ml (22), while, Cojutti, *et al*.,(2018) stated that the meropenem (MIC≤2 µg /L) against *K. pneumonia*(23).

Abd-algabar, (2019) reported that extracted staphyloxanthin of *S.*

aureus strain and then studied its antibacterial and antioxidant properties, this pigment showed antibacterial action against drug resistant pathogens (MDR) such *as Pseudomonas aeruginosa, Serratia marcescens, Klebsiella pneumonia, and Enterobacter* species an inhibition zone of 8, 7, 7 and 7 mm at 2 mg/ml, respectively. And an inhibition zone of 15, 13, 12 and 10 mm at 20 mg/ml, respectively (24).

Eight *Klebsiella pneumoniae* isolates (isolates were resistance to ciprofloxacin) were tested to adjust the MIC for staphyloxanthin, ciprofloxacin and com- bination between staphyloxanthin and ciprofloxacin against *K. pneumonia,* by the microdilution method recommended by the clinical trial and laboratory values found (CLSI, 2023) guidelines, the results presented that the MICs of ciprofloxacin were (1000 µg/ml) and MIC of staphyloxanthin (250 mg/ml) and MIC of combination between staphyloxanthin and ciprofloxacin distributed between 125_250 mg/ml for staphyloxanthin and between 250-500 µg/ml for ciprofloxacin against eight isolates as given in (Table 2).

Table (2): MIC of (staphyloxanthin, ciprofloxacin, combination between staphyloxanthin and ciprofloxacin) for *K. pneumoniae*

Isolates	Ciprofloxacin	Staphyloxanthin	Synergistic between staphyloxanthin and ciprofloxacin	
1	1000 µg/ml	250 mg/ml	staphyloxanthin	250 mg/ml
			ciprofloxacin	500 µg/ml
2	1000 µg/ml	250 mg/ml	staphyloxanthin	250 mg/ml
			ciprofloxacin	500 µg/ml
3	1000 µg/ml	250 mg/ml	staphyloxanthin	250 mg/ml
			ciprofloxacin	500 µg/ml
4	1000 µg/ml	250 mg/ml	staphyloxanthin	125 mg/ml
			ciprofloxacin	250 µg/ml
5	1000 µg/ml	250 mg/ml	staphyloxanthin	125 mg/ml
			ciprofloxacin	250 µg/ml
6	1000 µg/ml	250 mg/ml	staphyloxanthin	250 mg/ml
			ciprofloxacin	500 µg/ml
7	1000 µg/ml	250 mg/ml	staphyloxanthin	250 mg/ml
			ciprofloxacin	500 µg/ml
8	1000 µg/ml	250 mg/ml	staphyloxanthin	125 mg/ml
			ciprofloxacin	250 µg/ml

In clinical therapy, ciprofloxacin is one of the antibiotics that are frequently used to treat infections brought on by *K. pneumoniae*. In *K. pneumoniae*, resistance to widely used quinolone antibiotics, such ciprofloxacin, has grown; increasing the risk of first antibiotic selection for *K. pneumoniae* therapy (25). The recent study also demonstrated that *K. pneumoniae* is resistant to ciprofloxacin (45%) (26,29). Our result demonstrate that combination between ciprofloxacin and staphyloxanthin revealed that their MIC values were decreased for both, have synergistic effect. So, we noted that such substances (pigments of bacteria) have the potential to improve the effect of antibiotics and provide a choice of alternative to antibiotics. Numerous studies comparable to this one have demonstrated the use of pigments such as staphyloxanthin, prodigiosin and pyocyanin against a wide range of microorganisms (19, 24, 30, 31).

Conclusion

It is better to use staphyloxanthin as alternative to meropenem or in combination with it to avoid bacterial resistance and side effect of this antibiotic. And in combination with ciprofloxacin which has been improved by using it with staphyloxanthin at a time when there were resistance towards this antibiotic by a not small number of *K.pneumoniae* isolates.

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