Eradication of *Klebsiella pneumoniae* biofilms and persister cells using silver nitrate

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Abstract

To perceive the relationship between the emergences of antibiotics persistence, biofilm formation, and to study the role of silver nitrate in fighting these microbes, a total 50 clinical *Klebsiella pneumonae* isolates were collected from clinical sources and identified using genotypic identification by using specific primer (*rpoB* gene) from housekeeping genes. Detection of their biofilm-forming abilities were carried out. The eradication effect of silver nitrate to control bacterial persistence and reducing the biofilm-forming abilities were studied. We found that 40 isolate (80%) were biofilm-formers. From bacterial persisters, (75%) were biofilm-formers. Silver nitrate showed a great role to eradicate persister cells and inhibition of bacterial biofilm formation of clinical isolates of *K. pneumoniae.*

Keywords: Biofilms; *Klebsiella pneumonia*; persister cells; *rpoB*; silver nitrate.

1. Introduction

Klebsiella pneumoniae (*K. pneumoniae*), is a gram-negative, opportunistic pathogen, which belong to the "ESKAPE" group bacteria These bacteria are often inhabiting the skin, mouth, respiratory and gastrointestinal tracts (Li, *et al.,* 2014). Many infections and diseases caused by *K. pneumoniae* for examples, pneumonia, urinary tract infection, pyogenic liver abscesses, endogenous endophthalmitis and septicemia (Navon-Venezia *et al.,* 2017). Recently, *K. pneumoniae* being a universal concern in health threat due to prevailing capacity of acquiring antibiotic resistance. (Qin, *et al.,* 2020). Furthermore, persistence of bacteria due to antibiotic or other stress-response phenomenon, is also one of the most reasons for antibiotic therapy failure (Jung, *et.al.* 2019).

Persister cells formation is a phenomenon in which subpopulation inside a susceptible bacterial population can survive treatment of lethal doses of antibiotics. The persister cells show some characteristics such as being about only 0.0001% to a maximum 10% (Spoering & Lewis, 2001; Lewis, 2005), unlike resistance, persister cells cannot grow within antibiotic presence, and they barely survive (Lewis, 2010). Persister cells are not dependent on MIC and in contradiction to the resistance in which result in variations (a rise) in antibiotic MIC levels based on the bacterial capabilities to counterbalance the antibiotic's efficiency. Furthermore, they inheritable and cannot pass their factures to their new generations when stresses are removed.

Bacterial persistence is the main reason of the recalcitrance of chronic bacterial infections (Abokhalil, *et al.,* 2020). Antibiotic therapy failure is known as a serious threat which is typically related with the antibiotic resistance. But it is not the only issue. In fact, bacterial persister cells

which is antibiotic stress-response phenomenon, is also one of the most reasons for antibiotic therapy failure (Jung, *et.al.* 2019).

The ability of *K. pneumoniae* to produce biofilm encourages its survival and persistence in the hospital environments (Donlan & Costerton, 2002), which in turn participates in the *K. pneumoniae* extreme prevalence across many environments. Silver nitrate has typically been applied to newborn infants' eyes to combat their eye infections. Silver nitrate are is frequently employed in the clinic and for public health management as potent antimicrobial agents (Milionis *et al*., 2018). Besides being a strong topical broad-spectrum antimicrobial used in wound burns, as coatings for catheters and endotracheal tubes, and as disinfectant (Phan, *et al*., 2019). However, this compound has a toxic effect (especially if administered with high concentrations. This minimizes the use of silver nitrate in clinical practice and gave the light for the combination usage of silver compounds and antibiotics to promise the path for both of antibiotics activates restoring and the chemicals' toxicity precluding (Mohamed, 2019). A recent study by (Abokhalil, *et al*., 2020) have reported the complete eradication of persisters by using both of silver nitrate and ciprofloxacin antibiotic. In Iraq, there are few studies on silver nitrate role to control bacterial persistence. Therefore, the current study amid to investigate the activity of silver nitrate in eradicating bacterial persistence and biofilm formation of clinical isolates of *K. pneumoniae.*

2. Materials and methods

2.1 Bacterial isolation, identification, growth conditions and chemicals

A total of 50 *Klebsiella* species were collected from Teaching Laboratories in Medical city/ Baghdad from September 2020 to November 2020. These isolates were identified as *K. pneumonae* by Conventional PCR assay using a primer of *rpoB* gene from housekeeping genes with the following sequence (F - GGC GAA ATG GCW GAG AAC) (R- GAG TCT TCG AAG TTG TAA). The PCR reaction mixture consisted of 12.5 µl of 2X GoTaq®Green Master Mix (KAPA, 166 South Afriqa), 2 µl template DNA, 1.5 µl primers for each forward and reverse primers with final concentration (0.6 pmol/ µl), and nuclease free water up to 25 µl (6.5 µl). The amplified PCR product was run in agarose gel electrophoresis, the amplification size 1056 bp as compared with 1000 bp DNA ladder (KAPA, south Afriqa). The *rpoB* gene was visualized under UV trans-illuminator.

The bacterial isolates were routinely cultured in LB broth or agar at 37 °C. Silver nitrate powder ($AgNO_3$) was purchased from (Sigma/Aldrich/U.S) and 0.5 gm were dissolved in 50 ml dimethyl sulfoxide (DMSO), mixed and filtered by using sterile filtration (0.22 µm) membrane filter to produce 10 µg/ml of stock solution then stored at room temperature.

2.2. Detection of persister cells.

Persister cells assay was determined by measuring the ability of persister cells to survive the bactericidal doses of ciprofloxacin. An overnight culture was exposed to centrifugation at 12,000 rpm for a few minutes at 37°C and washed twice with normal saline then re-suspended and exposed to different ciprofloxacin concentrations (100–1000 ug/ml) and shacked overnight at 37 °C and 200 rpm. In the next day, the cells were washed twice with normal saline to remove ciprofloxacin.

Finally, 10 µl of the culture spread onto LB agar plates for measuring the frequencies of persister cells.

2.3. Antibacterial activity and MIC of silver nitrate

Antibacterial activity of silver nitrate toward persistence *K. pneumoniae* isolates was tested using the agar well diffusion method (Yaseen, *et al.,* 2019). The minimum inhibitory concentration (MIC) was carried out using the 96-well microtiter plate. Briefly, 100 µl of LB broth was added to flat well-microtiter plate wells, then wells of the first vertical row were filled with 100 µl of silver nitrate (10 mg/ml) followed by mixing 1:2 serial dilutions. 100µl from the last well was discarded. Then all wells of the microtiter plate were filled with 2 µl of bacterial suspension (1.5×10^8 CFU/ml) from fresh growth of an overnight (except negative control wells were filled with LB broth only) and incubated overnight at 37 °C. The next day, the MIC of silver nitrate was registered by using the optical density (OD) of ELISA reader. The test was carried out in triplicate.

2.4. Biofilm formation assay

For evaluating the biofilm-form capacity, the total 50 *K. pneumoniae* isolate were cultured in brain heart broth (BHB) overnight at 37 °C. Then, bacterial cultures were diluted to make a bacterial suspension equivalent to 0.5 McFarland solution. 20 µl of bacterial suspension were pipetted inside wells of flat microtiter plates with 180 µl of BHB added up to 2% sucrose and incubated overnight at 37 °C. After the incubation period, the plates were washed three times with sterile normal saline for removing unattached bacterial cells. After drying at room temperature, wells were stained with 200 µl of crystal violet solution for 15 min. Therefore, the unstained dye was discarded by washing with normal saline. Finally, 200 µl of 95% ethanol was added to each well. The OD was determined at 630 nm using ELISA reader (BioTek). Each isolate was tested in triplicate. The adherence capacities of the assay isolates were classified into four categories; the mean optical density of the negative control was counted as:

$OD \leq ODc$, the bacteria were non-adherent;
If $ODc < OD \leq 2 \times ODc$, the bacteria were weakly adherent;
If $2 \times ODc < OD \leq 4 \times ODc$, the bacteria were moderately adherent;
If $4 \times ODc < OD$, the bacteria were strongly adherent

2.5. Inhibition of biofilm-forming assay

The activity of silver nitrate on biofilm formation of persistence *K. pneumoniae* isolates was achieved according to the previous study (Theodora, *et al.,* 2019). Briefly, *K. pneumonia* isolates were inoculated in BHI broth overnight at 37 ˚C. The growing cultures were suspended to the (1.5×10^8 CFU/ml).Then, 20 µl of bacterial suspension was inoculated in 96 well flat microtiter plate containing 80 µl of BHI broth with 2% sucrose, and 100 µl from sub-MIC of silver nitrate (2.5mg/ ml) were added and mixed by pipette sucking up and down then incubated for 24 h at 37 ˚C. After two washes with normal saline (pH: 7.2). For draining the wells, the microtiter plate allowed to be dried at room temperature. Unattached bacterial cells were removed. 200 µl of crystal violet solution was used for biofilm staining. The stain was eluted with 200 µl of 95% ethanol, and

the optical density was measured at 630 nm using an ELISA reader. The negative control was filled with BHI broth only, while the biofilm production with bacterial suspension without silver nitrate were positive controls. The assay was carried out in triplicate. To calculate the rate of inhibition caused by silver nitrate, the following formula was applied:

Inhibition rate (%) = (Control OD − Treated OD) / (Control OD) × 100

2.6. Statistical analysis

The data results of this study were analyzed by using Graph Pad Prism 8 software and Microsoft Excel 2013 for each biological replicate. The level of probability at P values below of ≤ 0.05 that used to identify a significant difference.

3. Results and discussion

3.1 Bacterial isolates, identification and growth conditions

The isolates were collected from different clinical sources and the identification of *K. pneumoniae* isolates were carried out by gene sequence analysis of *rpoB gene*. Figure (1), shows the amplification of *rpoB* gene in the gel electrophoresis for detecting *K. pneumoniae* isolates. The advancement of *rpoB* gene analysis has given rise to rapid diagnostic techniques for the identification of *K. pneumoniae* (Kareem *et al.,* 2021).

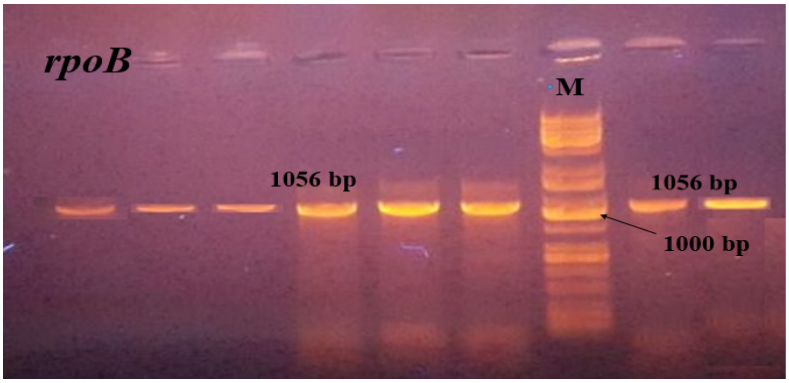

Fig. 1. Gel electrophoresis (1% agarose, 70 volt for 50 min) of 16S *rpoB* gene amplification for detecting *K. pneumoniae* isolates. Line M 1000 bp is the ladder, other lines are the positive results of *rpoB* gene of Isolates.

3.2 Detection of persister cells formation

Persister cells of *K. pneumoniae* were generated by phenotypic detection using different doses of sub-MIC for the ciprofloxacin (up to 1000 µg/ ml). Results exposed that eight isolates had persistence ability which were formed during the stationary growth phase (Song & Wood, 2021). Bactericidal antibiotics such as ciprofloxacin killed the normal growing cells, while the dormant cells during antibiotic stress, remained survived. Clinically, these persister cells are the start of the chronic infections, which results in the overuse of antibiotics and reduced antibiotic activity. *S. aurous* for an example, engages in persistent, relapsing infections that are often difficult to eliminate with antibiotics, such as osteomyelitis (Bakkeren *et al*., 2020; Ali *et al*., 2021).

3.3 Detection of persister cell inhibition by silver nitrate

To study the silver nitrate antibacterial activity against eight persistent *K. pneumoniae* isolates, agar-well diffusion assay were carried out. Results showed a potential bactericidal activity of silver nitrate toward persister cells of the *K. pneumoniae* isolates with inhibition zones between (24 mm) and (35 mm) Moreover, the MIC of silver nitrate realized the 0.31 µg/ml in the eight isolates, while the potential anti-persistence forming ability of the sub-MIC was about 0.5mg/ml. Our results confirmed the resent result of (Öztürk, *et al.,* 2020) when they reported both of the anti-biofilm and anti-microbial activities of green synthesized silver nanoparticles. Therefore, our results represent the ability of silver nitrate to eradicate the persister cells of a very urgent pathogen such as of *K. pneumoniae* which emerged as one of the most antibiotic resistant microbes responsible for outbreaks in both community and healthcare settings (Narimisa, *et al.,* 2020). Furthermore, to investigate the elimination of persister cells by silver nitrate, the method used by Aziz *et al*,2022. were done by treating of persister cells with silver nitrate for (6 h, 12h, and to 234h) and results showed that there is any observing for the persister cells in the media, which confirmed the total eradication of persister cells by silver nitrate at the concentration of 1024 mg/ml (Aziz *et al*, 2022).

3.4 Detection of biofilm and Its inhibition assay

Forty *K. pneumoniae* isoltes were biofilm producers, while only ten isolates were non producers. Fourthermore, all persistence isolates (8/50) were with biofilm forming ability. The biofilm may serve to induce resistance, persistence, and also act as a shield of persister cells from elimination by bactericidal agents. This supported by (Baek *et al.,* 2020; RAY *et al.,* 2021). Many publications have indicated that persister cells of various pathogenic bacteria were generated by lowering metabolic activity in biofilm after triggering some severe stringent responses such as the toxin-antitoxin system (Amato *et al.,* 2014; Harms *et al.,* 2016; Carvalho *et al.,* 2018; Soares *et al.,* 2019; Karimaei *et al.,* 2021; Uruén *et al.,* 2021). The biofilm inhibitory effect of silver nitrate against persister cells of *K. pneumoniae* isolates were significant due to the converting of the strong and moderate forming isolates to weak and non-biofilm producers after exposed to 2.5mg/ ml concentration dose of silver nitrate. Silver ions known as antimicrobial and so can change the biofilm profile of persister cells by many mechanisms as disrupting the multiple bacterial cellular networks and processes, resulting in the destabilization of the cellular envelope and the production of ROS in Gram-negative bacteria (Morones-Ramirez *et al*., 2013).

The frequency of persistence *K. pneumoniae* prevalence in Iraqi hospitals and laboratories has been increased, to control bacterial persistence, our study showed the effectiveness of silver nitrate in eradicating *K. pneumoniae* persister cells , and their derived biofilm formation. To our knowledge, this is the first study from Iraq to show the activity of silver nitrate compounds in inhibiting persister cells and biofilm formation of *K. pneumonia* isolates in vitro. Importantly, our findings suggest silver nitrate compounds may facilitate eradicating bacterial persistence which ssociated with chronic and periodic infections, and antibiotic therapy failure. We recommend further studies to investigate the relationship between the presences of some persisters genes and silver nitrate role of silver nitrate in persistence.

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