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Investigation of Bacterial Persistence and Filaments Formation in Clinical *Klebsiella pneumoniae*: First Report from Iraq

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***Abstract*—Bacterial persistence is recognized as a major cause of antibiotic therapy failure, causing biofilms, and chronic intractable infections. The emergence of persisters in *Klebsiella pneumoniae* isolates has become a worldwide public health concern. The goal of the present study is to investigate the formation of persister cells beside filaments in Iraqi *K. pneumoniae* isolates. A total of fifty clinical *K. pneumoniae* isolates were collected from different clinical specimens and identified using the genotypic identification by using specific primer (rpoB gene) from housekeeping genes. Persister cells investigation is performed by exposure of stationary phase *K. pneumoniae* isolates to a high concentration of ciprofloxacin (×10 MIC) and counting the number of viable persister cells by CFU counts. Bacterial filament formation is detected and measured by light microscope scanning electron microscope. The results show the ability of these pathogenic bacteria to form persister cells to survive the bactericidal antibiotics and to cause chronic infection. Furthermore, persistent isolates have the ability to change in shape and size extensively, about 4 times increase in cell length than their normal length. These phenomena are possibly the initial stages of bacterial resistance prevalence.**

***Index Terms*—Filamentation, Klebsiella pneumoniae, Persistence, Scanning electron microscope, Survivor cells.**

I. Introduction

Antibiotic therapy failure is known as a global threat in modern medicine that is commonly attributed to resistance. Many studies have uncovered many genetic-molecular mechanisms of bacterial resistance that reduced the effective concentration of antibiotics (Blair, et al., 2015; Khazaal, et al., 2020). However, this is only part of the story. It has long been realized that the formation of persisters in the bacterial population allows a small subpopulation of bacterial cells to survive lethal doses of bactericidal antibiotics. In 1944, medical doctor Bigger noticed the capacity of a small number of bacterial cells in *Staphylococcus aureus* to tolerate and survive a high concentration of antibiotics (Podlesek, et al., 2016). Genetically, persister cells have the same ranges of minimal inhibitory concentration (MIC) as the susceptible cells, but they survive the lethal concentrations of bactericidal antibiotics (Brauner, et al., 2016). Persister cells are dormant, multidrug-tolerant, and non-heritable phenotypic variations of bacteria that differ from resistant populations (Helaine and Kugelberg, 2014). When the antibiotic stress is removed, persisters switch back to a growing state and generate a new population that is genetically identical to the wild-type cell (Aziz, et al., 2021). Persisters may be a middle stage for developing bacterial resistance due to their ability to undergo cellular division during persistence. In addition, persisters can accelerate mutagenesis and horizontal gene transfer due to their stress response mechanisms (Windels, et al., 2019). Therefore, persisters may play a role in the development of multi-drug resistant bacteria (Windels, et al., 2019). *Klebsiella pneumoniae*, belonging to the Enterobacteriaceae family, is an opportunistic bacterium with a capsule that can cause serious hospital-acquired infections and community-acquired infections, such as urinary tract infections, bloodstream infections, pneumonia, pyogenic liver abscesses, and endogenous endophthalmitis (Aziz, et al., 2019). At present, with the recent emergence and dissemination of antibiotic-resistant strains, *K. pneumoniae* have gained notoriety as an infectious agent due to a rise in the number of severe infections and the increasing lack of effective treatments so, it has become a major health concern (Da Silva, et al., 2019). The failure of antibiotics against *K. pneumoniae* has been widely studied, bacterial persistence may play a role in treatment failure (Navon-Venezia, et al., 2017). However, it still few studies about the persistence *K. pneumoniae* (Li, et al., 2018). Furthermore, there are no studies about persistence formation in Iraq. Therefore, to provide more information about *K. pneumoniae* persistence, the goal of the present study was to investigate the formation of persisters cells beside filaments formation in Iraqi clinical *K. pneumoniae* isolates.

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II. Materials and Methods

A. Bacterial Isolates, Identification, and Growth Conditions

A number of fifty clinical *K. pneumoniae* isolates were obtained from Teaching Laboratories in Medical city/Baghdad. The identification of the isolates was done using their growth characteristics in culture media and confirmed using and Vitek-2 system (BioMe´rieux, France). Conventional PCR was performed for the genotypic identification of *K. pneumoniae* isolates using specific primers for *rpoB* gene. The sequence of the primers and PCR cycling conditions are listed in Table I. The isolates were routinely grown in Luria Bertani (LB) broth at 37°C. for a 24 h.

B. The MIC Determination

The MIC values of ciprofloxacin antibiotic were investigated using E-test gradient strips (bioMérieux/France) on a Mueller-Hinton agar medium. The MIC was measured and the lowest concentration of ciprofloxacin antibiotic non-permissive for visible growth was reported to be the MIC. *Escherichia coli* ATCC 25922 was used as the negative control strain.

C. Phenotypic Detection of Persister Cells Formation

With few modifications, persister cells formation was carried out according to (Chung, Wi and Ko, 2017) by exposing exponential phase bacteria (optical density OD600 = 0.5) to a high concentration of ciprofloxacin (10 MIC), then incubated at 37°C for 16 h in LB media. The bacterial cells of *K. pneumoniae* were washed with phosphate-buffer saline and then spread on (LB) agar medium for 24 h. After the incubation, persister cells numbers in the agar medium were determined by colony-forming units. Furthermore, few colonies from each bacterial sample were re-inoculated on (LB) broth medium for 24 h and an antibacterial sensitivity test was performed, to confirm their identity as persister cells by non-changing of MIC values.

D. Molecular Detection of hipB and mazE Genes in Persister Cells

PCR reaction mixture for *hipB* and *mazE* genes, which are related to persistence state, was set up for each gene alone in a final volume of 25 µl. It was composed of 2.5 of Green master mix ×2, 5.5 µL of nuclease-free water (Promega, USA), 2 µL of primers (1 µL forward and 1 µL reverse), and 5 µL template DNA in PCR Eppendorf tubes. Negative control was used with all PCR experiments, which consisted of all the materials but without the template DNA. The mixture was vortexed and briefly centrifuged to move the contents to the bottom of the tubes before being placed in a thermocycler PCR. Table I shows the primer sequence and the size products of the PCR.

E. Detection of Filament Formation

Detection of filament formation was done using microtiter wells plate according to (Buijs, et al., 2008) with few modifications. Briefly, eight sub-MIC concentrations of ciprofloxacin antibiotic were prepared (5, 10, 20, 30, 40, 50, 100, and 120 mg/L). A negative control concentration that did not have any antibiotic also was used as a reference for *K. pneumoniae* isolates length. The ciprofloxacin antibiotic solution was diluted in distilled water then 20 µL of these dilutions were added microtiter wells plate followed by adding 180 µL of *K. pneumoniae* suspension with gentle mixing then incubated for 4 h at 37°C. After that, slide smear and Gram stain were done for each well to examine by light microscope. Following Gram's stain, determine the filament size which is the most characteristic often used to help identify filament formation bacteria, persistence *K. pneumoniae* isolate was detected. For determining the filament sizes, microscopy and scanning electron microscope were used.

F. Statistical Analysis

The data results of this study were analyzed using GraphPad Prism 8 software and Microsoft Excel 2013 for each biological replicate. The level of probability at $P \leq 0.05$, which used to identify a significant difference.

III. Results and Discussion

A. Bacterial Identification and Growth Conditions

The isolates of *K. pneumoniae* were collected from different clinical samples including 20 isolates from urine, 15 isolates from burns swab, 6 isolates from stool, 5 isolates from blood, and 4 isolates from sputum. The identification of *K. pneumoniae* isolate was confirmed by phenotypic identification using the Vitek 2 system (BioMe´rieux, France), then re-confirmed by gene sequence analysis of *rpoB* gene (β-subunit of RNA polymerase). Fig. 1 shows the amplification of *rpoB* gene in the gel electrophoresis for detecting *K. pneumoniae* isolates.

B. Phenotypic Detection of Persister Cells Formation

Out of 50 *K. pneumoniae* isolates, 2/50 isolates were persister cell formation. Ciprofloxacin dose-dependent killing

TABLE I
Primers Used in the Current Study for PCR Amplification

Activity	Name of gene	Primers 5 ---- 3	Size products	References
Housekeeping gene	*rpoB*	F: GGC GAA ATG GCW GAG AAC R: GAG TCT TCG AAG TTG TAA	1056 bp	(Kareem, et al., 2021)
Toxin-antitoxin system genes	*hipB*	F: AGCCCAACGCAATTGGCGAATGCA R: CTGTTCTGTTGATTCTGGCGAGGC	225 bp	(Hemati, et al., 2014)
	mazE	F: ATGATCCACAGTAGCGTAAAGCGT R: TTACCAGACTTCCTTATCTTTCGG	249 bp	(Hemati, et al., 2014)

(MIC = 0.031 µg/mL; sensitive) of *K. pneumoniae* test isolates. With 0.31 µg/mL ciprofloxacin (10-fold MIC), the majority of the population was efficiently killed, followed by a near plateau with minimal decrease in survival up to 40 g/mL ciprofloxacin concentration.

The percentage of survival at 30 µg/mL ciprofloxacin was 1.3% of the initial cell count, and it was about 1.5% at 40 µg/mL ciprofloxacin (1000-fold MIC).

Because they survived the ciprofloxacin treatment, the survivors along the plateau of the antibiotic dose-dependent killing curve could be considered persister cells. To ensure the persistence state, MIC of these survivor cells against ciprofloxacin remained unchanged when compared to the test organism's wild-type cell population. Furthermore, no colonies appeared after an incubation period of 24 h at 37°C on LB agar plates containing 30 µg/mL ciprofloxacin that were pre-inoculated with survival cells when compared to their growth on LB agar plates alone without added ciprofloxacin. Our study is resembling for many recent studies revealed that *K. pneumoniae* are the pioneer intricate bacteria that can persist and resist in the presence of distinct antibiotics, including fluoroquinolones (Lee, et al., 2019; Abokhalil, et al., 2020; Kareem, et al., 2021). However, in Iraq, there is a need to focus on bacterial persistence, especially with opportunistic bacteria like *K. pneumoniae*.

C. Molecular Detection of hipB and mazE Genes in Persister Cells

The results showed that the same two *K. pneumoniae* isolates which were able phenotypically to form persister cells were harboring both *hipB* and *mazE* genes as shown in Fig. 2. These results were supported by many other results such as a recent study by (Fraikin, Goormaghtigh and Van Melderen, 2020). Furthermore, in their report, (Wood and Songs, 2020) suggested that TA system can be a realistic choice for both inductions of persistence and their elimination. The current study supported by many international studies that indicated the wide distribution of TAs type II in *K. pneumoniae*, especially a high level of *hipAB*, *mazEF*, *ccdAB*, and *relEB* genes (Coskun, et al., 2018; Horesh, et al., 2020). When activated, TA stress-responsive elements cause reversible bacterial growth indictment, making them typical candidates for persistence drivers. The role of TA operons in persistence, on the other hand, is a hotly debated topic. When Moyed and Bertrand discovered a high-persistence mutation in HipAB, they established the first connection between persisters and TA modules (Gollan, et al., 2019). HipB encodes an auto repressor of *hipBA* transcription (Black, Irwin and Moyed, 1994; Harms, et al., 2018). HipA overproduction inhibited cell growth by reducing translation, DNA replication, and transcription, as well as significantly increasing tolerance to bactericidal antibiotics (Korch, Henderson and Hill, 2003).

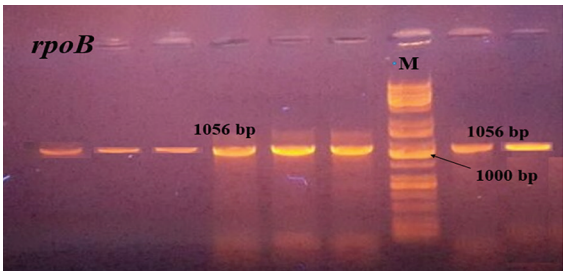

Fig. 1. Gel electrophoresis (1% agarose, 70 volt for 50 min) of *rpoB* gene (1056 bp). Lane M 1000bp DNA Ladder, the other lanes bands are that the other lines are the positive results of *rpoB*.

When exposed to bactericidal antibiotics, bacterial persisters form through stress responses that stimulate ppGpp synthesis, leading to MazE antitoxin degradation and MazF toxin activation. MazF inhibits RNA and protein synthesis effectively blocking cell elongation and protecting persister cells from death by lysis. Ciprofloxacin generates DNA breaks which, if not repaired through the SOS response path-way, quickly kill dividing bacteria that are actively synthesizing DNA. By inhibiting DNA synthesis, MazF blocks cell division and protects persisters from ciprofloxacin. Bactericidal antibiotics were reported to stimulate ROS production that could lead to mutant cells, but these conclusions were challenged by subsequent studies (Gollan, et al., 2019).

D. Detection of Filament Formation

Results of exposing persistent *K. pneumoniae* isolates to various concentrations from the ciprofloxacin sub-MIC showed that cell filament formed at 0.25, 0.19, and 0.125 µg/mL concentrations so, the bacterial isolate was shape shifter into a filamentous, Fig. 3. Our results showed that persistent *K. pneumoniae* was changed in

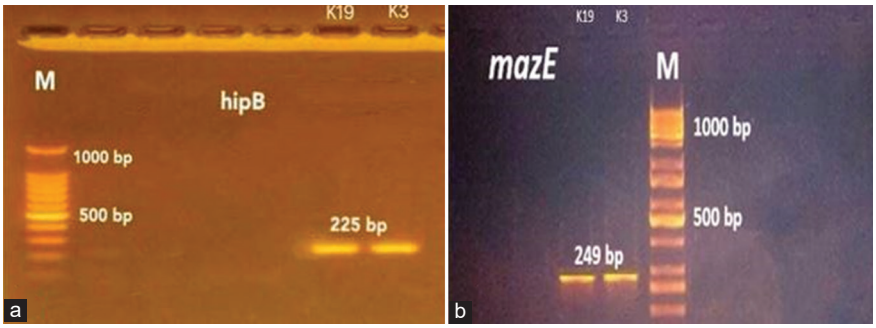

Fig. 2. Gel electrophoresis (1% agarose, 70 volt for 50 min) of (a) *hipB* (225 bp), (b) *mazE* (249 bp) genes.

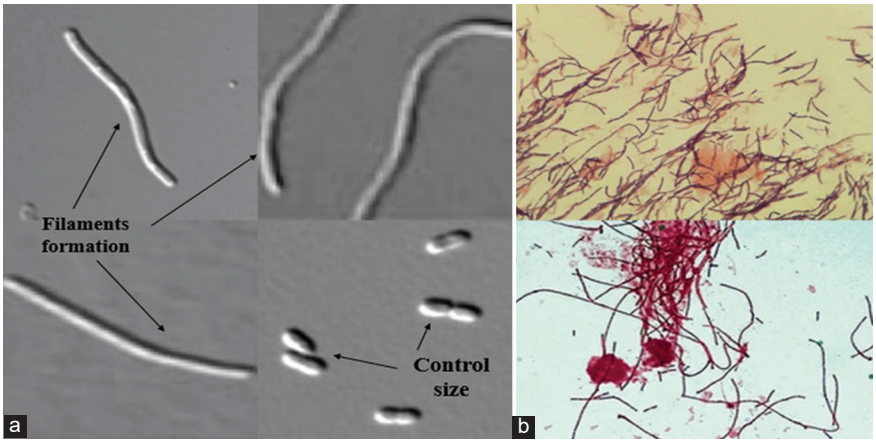

Fig. 3. (a) Scanning electron microscope, (b) light microscope, ×100 magnification, filament of *Klebsiella pneumoniae* isolate.

shape and size extensively (filamentation) associated with antibiotics therapy and persistence formation. Persistent *K. pneumoniae* isolates exhibited cell elongation, about 4 times increase in cell length when examined under a scanning electron microscope. A recent study suggested that Filaments Formation by persistent bacteria, during antibiotics exposure or under other stress conditions, is possibly the initial stage of emergence of the bacterial resistance (Joseph, et al., 2018).

IV. Conclusion

This study reports the formation of persistence *K. pneumoniae*. The isolation of persister cells can be done with antibiotic treatment (ciprofloxacin). The slow growth rate after resuscitation indicated that metabolisms of persistent bacteria had slowed. This emphasizes the significance of researching the underlying mechanisms contributory to the persistence state, which should be the focus of our future perspectives.

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