The Correlation Between the Persistence of Methicillin Resistant Staphylococcus Aureus Isolates to Mupirocin and Toxin-Antitoxin Type II Genes

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Abstract
Under high concentrations of antibiotics, a fraction of the bacterial population exhibits a phenomenon known as persistence. Toxin- system (TA system) has been reported to be involved in the formation of E. coli, Mycobacterium, and S. aureus persisters. In this study, the ability of thirty Iraqi isolates of MRSA to form in vitro persister cells after exposure to three different antibiotics (Ceftriaxone 30 µg, Mecillinam 10 µg, and Mupirocin 20 µg) was examined by TD test. Additionally, efflux pump inhibitor [Fluphenazine 0.25 mg/ml] was combined with the antibiotic that triggered persister formation. The distribution of mazEF and yefM-yoeB (Type II TA system) in the tested isolates was detected by PCR. 91% of Mupirocin susceptible isolates formed persister cells. 42% of the persistent level was reduced when Mupirocin was combined with the Fluphenazine. Genes for homologs of the yefM-yoeB and mazEF TA system were present in 100% of the tested isolates. The prevalence of these genes in the tested isolates suggested a link between persistence and the TA system. Further investigation is required to study the expression of these genes under stress conditions.

Keywords: Persistence, MRSA, Toxin- system, mazEF, yeEF-yoeB

العلاقة بين ثباتية عزلات المكورات العنقودية المقاومة للميثيديلين إلى المضاد الحيائي الميبروسين و جينات النوع الثاني من النظام السمسي و ضد السمسي

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الخلاصة
تحت التراكيز العالية من المضادات الحيوية، يظهر جزء من النمو البكتيري لظاهرة الإصرار (المثابرة).

يعتبر نظام السم و ضد السم أحد الأنظمة التي تم توثيق دورها في تكون الخلايا المثابرة في الأشكال القولونية والمعطرات والمكورات العنقودية. في هذه الدراسة قابلية ثلاثون عزلة عراقية من المكورات العنقودية المقاومة للميثيديلين على تكون الخلايا المثابرة في المختبر بعد التعرض للثانيان من المضادات الحيوية

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Introduction

The most common untreated surgical infections in hospitals are those caused by S. aureus. This bacterium possesses different defense strategies that provide the ability to survive various stress conditions [1]. In a high concentration of antibiotics, some bacterial populations would use one of the resistance mechanisms, such as mutations, and become resistant [2]. While some subpopulation would alter their metabolic process and enter the dormancy state and become persistent [3,4]. After stress factors removal, these persistent cells will resume normal growth and generate viable cells that are just like the original cell’s phenotypes [5,6]. Studies performed on persistent E. coli cells revealed an increase in toxin-antitoxin transcripts levels and suggested the implication of the TA system in the formation of persistent cells [7, 8, 9]. The formation of persistent cells was decreased upon toxin coding genes disruption or deletion, but resulted in more acute pathogenic infections [9,10,11]. These observations confirmed the notion that the TA system is implicated in persistent cells formation. Many other studies performed on E. coli showed that antibiotics would induce the formation of persistence in a toxin-antitoxin dependent manner [7]. Toxin-antitoxin system is prevalent in various types of bacteria due to their movement among bacterial genome by horizontal gene transfer [12]. This system was first identified in plasmid as an element that enhances plasmid’s stability. But then, by homology searches, it was detected in bacterial chromosomes with an ambiguous function [13,14,15]. Later on, many studies have conducted to understand the structure, function, and role of the chromosomal TA system to answer questions regarding the different cellular processes, like stress response, gene regulation, and persistence. At a molecular level, this system consists of two genes: toxin coding gene and antitoxin coding gene. The toxins are stable proteins of various activities (kinase, RNase, DNase, and acetyltransferase), while the antitoxins are unstable and can be either proteins or small RNAs [16,17,18,19]. The reason that the antitoxin proteins are less stable than the toxin proteins is that they are intrinsically disordered and very vulnerable to degradation by ATP-dependent protease or ATP-independent protease [20,21,22,23]. The toxins have certain cellular targets to attack that lead to cell growth inhibition. In normal growth conditions (absence of stress) the neutralize the effect of toxin to make sure that cells are in save of toxins effect [24,25]. Antitoxins neutralize the toxins either by direct interaction or by coupling with other elements of toxins expression regulation [21]. According to how antitoxin neutralizes the activity of toxins, the TA system has been classified into six types (I-VI). Type one and type three TA modules, the antitoxins are small RNA, while the rest of TA Types the antitoxins are proteins (type II, IV, V, and VI) [26]. The TA systems regulation and activation are triggered by stress response. Stress response elements increase the expression of toxin coding genes and increase the activity of toxins by decreasing antitoxin stability [19,20,27,28]. Among the six different types of TA systems, mazEF and yefM-yoeB type II TA systems are the well-studied modules, especially in E. coli and Staphylococcus aureus [29, 30]. The mazEF operon
consists of mazF gene locates downstream of mazE gene [31]. mazF is a stable ribosome-independent mRNA cleavage (activity is independent on coupling with ribosome) [32]. mazE is an unstable protein that is vulnerable to proteases. YoeB toxin is a ribosome-dependent mRNA sequence specific interferes that inhibits the initiation of the translation process. The action of yoeB toxin is counteracted by yefM anti-toxin which binds to the yefM-yoeB operon´s promoter and represses the transcription process [33]. The TA system, as mentioned above, has been associated with the formation of persister cells in gram-negative bacteria, but its role in Staphylococcus is controversial. So, this study aimed to investigate the ability of methicillin resistance S. aureus isolated from Iraqi patients to form persister cells using TD test (Tolerance and persistence Disk Test) which promotes the growth of persistent cells after removing the inducer agent (antibiotic disks), in addition, the association with the presence mazEF and yefM-yoeB chromosomal loci genes.

2. Materials and Methods

2.1. Bacterial isolates

Thirty isolates of Staphylococcus aureus were isolated from the noise, blood, and wounds of Iraqi patients. Staphylococcus aureus isolates were identified by biochemical tests oxidase, coagulase, and catalase [34]. The isolates were confirmed by PCR for the presence of nuc and mecA genes.

2.2. Persister cells detection

2.2.1. Antibiotic sensitivity test

With a sterile loop, four morphologically similar colonies were transferred to Mueller Hinton broth. Inoculated media was incubated at (35-37 °C) until the visible turbidity is equal to 0.5 McFarland. The inoculum was spread evenly over the entire surface of the Muller Hinton agar. Discs with defined concentrations CRO, Ceftriaxone (30 µg), Mupirocin, MUP (20 µg), and Mecillinam, MEC (10 µg) were applied to the surface of an agar plate. Plates were incubated at 37 °C for 18hr.

2.2.2. Persistence disk test

After incubation at 37 °C for 18hr, the inhibition zones were measured and determined for each isolate and antibiotic, the antibiotic disks were carefully removed with sterile forceps, and disks containing a sufficient amount of nutrient broth were substituted. The substituted disks were prepared by pipetting 100 µl of nutrient broth onto the blank disks. These disks were used immediately after their preparation. Then the plates were incubated at 37 °C for 18hr.

2.2.3. Spot plating Assay

Exponential growing cells were exposed to Mupirocin (20 µg/ml) and Ceftriaxone (30µg/ml) for four hours at 37 ºC. 2 µl of each isolate was plated to determine the number of survived cells (level of persistence).

2.2.4 Efflux pumps inhibitor

Exponential growing cells were grown in nutrient broth containing Mupirocin (20 µg/ml) only and in nutrient broth containing Mupirocin (20 µg/ml) and Fluphenazine with final concentration (0.25 mg/ml). the cultures were incubated for 4hr at 37°C. Cultures were centrifuged and re-suspended in nutrient broth and 2µl of suspended cells was spotted in nutrient agar media and incubated at 37°C for 18hr.

2.3. DNA Isolation.

Extraction of DNA from 1 ml of overnight culture using Promega DNA extraction kit supplemented with (30 µg/ml) lysozyme enzyme.

2.4. PCR Detection of mazEF and yefM-yoeB loci.

1µl of 100ng DNA was used as a template for PCR. mazEF loci presence among the extracted DNA of isolates was determined by polymerase chain reaction by using forward primer (5’-AGCTACTGCATTCAGCCCTA-3’) and reverse primer (5’-
AGGGATCTGAACAAGGGGA-3'), which will yield 300 bp fragment. yefM-yoeB loci were detected by using forward primer (5'-CGACCTGTGCTGATGTTA-3') and reverse primer (5'-CACTAGCCCTACAGAAGCGAG-3'), which will yield 451 bp fragments. 25 µl of PCR reaction contains 1x master mix buffer, 10 pmol/µl forward and reverse primers, 200 ng/µl DNA, and water. The PCR reaction was carried out with the following parameters: initial denaturation at 94 °C for 5min, second denaturation at 94 °C for 1 min, 1 min of annealing at 56 °C and 55 °C for mazEF and yefM-yoeB, respectively, extension at 72 °C for 50 sec. 30 cycles of amplification were applied. To analyze the PCR products, 10 µl of PCR mixture was loaded to 2% agarose in the presence of 100 bp DNA ladder. After performing gel electrophoresis, the gel was exposed to UV by using UV Transilluminator.

3. Results and Discussion

3.1. Cells with distinct phenotypes were detected in the inhibition zone of Mupirocin-susceptible Isolates after the Substitution of the antibiotic disk with nutrient broth disk.

Bacterial populations are genetically and phenotypically heterogeneous. The phenotypic heterogeneity is due to the presence of normal growing cells and slow growing cells in a dormant state (persistor cells). Persistent cells can survive stress conditions like antibiotics due to their low metabolic activity [35, 36]. To analyze the failure of antibiotic treatments, susceptibility testing usually focuses only on identifying resistance levels neglecting the possibility of persistor cells presence. Studying the persistence in *Pseudomonas* and *E. coli* concluded that there is a positive correlation between persistence and resistance. Once the bacteria have the ability to evolve persistor cells, it will increase the chance to evolve resistant either by mutation or horizontal gene transfer [37]. The minimum inhibitory concentration (MIC) of persistor cells is unchangeable, therefore, they will be neglected if the MIC assay is used only to detect isolates with higher values. Standard Disk diffusion assay is not efficient in distinguishing persistent from susceptible. Therefore, Persistence in isolates of methicillin resistant *S. aureus* was detected by TD test. In this study, to detect the presence of cells with distinct phenotype among the population of tested Iraqi isolates of MRSA (Methicillin Resistant *S. aureus*), the isolates were exposed to three different antibiotics. Among the three tested antibiotics, Mupirocin was more effective in inhibiting the growth of isolates. 73% of isolates were susceptible to Mupirocin Whereas, 60% and 100% of isolates were resistant to Ceftriaxone and Mecillinam, respectively [Figure 1] and [Figure 2]. To detect the presence of survival persisters inside the inhibition zone of Mupirocin and Ceftriaxone susceptible isolates, the antibiotic disk was substituted with nutrient broth saturated disk. After 18hr of incubation at 37C, detectible colonies were obtained [Figure 4]. Of the 22 Mupirocin-susceptible isolates, 91% (20 isolates) were triggered to form persisters, while no persistence was observed in the inhibition zone of 9% (2 isolates) (non-persistent susceptible isolate) [Figure 3]. All Ceftriaxone susceptible isolates formed persister cells [Figure 3]. The number of persister cells generated in the inhibition zones after disk substitution (persistence level) was ranged from medium (tens to hundreds of colonies in the inhibition zone) to high (Bacterial lawn in the inhibition zone) [Figure 4]. 70% (14 isolates) of the Mupirocin susceptible isolates formed lawn in the inhibition zone, while 30% (6 isolates) formed hundreds of colonies. Exponential growing cells exposed to Ceftriaxone had lesser persister frequency compared to when exposed to Mupirocin 20 µl [Figure 5].
Figure - 1 Standard Disk diffusion assay of three different antibiotics Ceftriaxone (CRO) 30 mcg, Mecillinam (MEC) 10 mcg, and Mupirocin (MUP) 20 mcg. A. The isolate was susceptible to MUP, B. Intermediate to CRO, and C. Resistant to MEC.

Figure - 2 Antibiotic susceptibility disk diffusion assay of methicillin resistant *Staphylococcus aureus*. S: Susceptible, I: Intermediate, R: Resistant.

Figure – 3 TD test for persistence detection in Mupirocin and Ceftriaxone susceptible isolates. The number of isolates detected in this test is indicated.
Mupirocin has been used as an excellent agent to treat *Staphylococcus* infections [38]. Based on the outcome of this study’s TD test, Mupirocin is not free of persistent cells. The substitution of the antibiotic disk with nutrient broth saturated disks allowed the detection of late growing cells that managed to survive the effect of antibiotic in the inhibition zone of Mupirocin susceptible isolates and Mupirocin and Ceftriaxone intermediate isolates. To make sure that these cells are persisters, colonies inside the inhibition zones were picked and the TD test was repeated, same susceptibility results were obtained. The level of persistence, represented by the number of colonies inside the inhibition zones and the number of colonies obtained after exposing exponential growing cells to antibiotics, was varying between isolates and antibiotics from medium to high. Isolates treated with the same antibiotic showed different persistence levels which can be attributed to cells growth rates. Since antibiotics target active mechanisms within bacterial cells, fast growing cells would be attacked by antibiotics faster than slow growing cells.

Therefore, the low growing isolates will form a higher level of persister cells. The persistence level is affected by the type of antibiotics. Exposing isolates to Mupirocin resulted in a higher level of persistence compared to Ceftriaxone. Mupirocin triggers the formation of persisters through inhibiting the translation process. Mupirocin mimics Isoleucine and binds to the active sites of Isoleucyl–tRNA synthetase resulting in the latter inactivation. The accumulation of Isoleucyl-tRNA synthetase molecules within cells activates the ppGpp alarm one synthesis which in turn activates regulatory pathways and mechanisms implicated in stress-related phenomena like persistence [39].

![Figure 4](image)

**Figure 4**-Different levels of persistence detected by TD test. Regrowth of colonies of Mupirocin and Ceftriaxone Persistent Methicillin resistant staphylococcus aureus. Colonies in the inhibition zone after the substitution of Mupirocin and Ceftriaxone disks with nutrient broth disk. The result was obtained after the 18hr of incubation at 37 °C. (a, c, and d) High-persistence. (b) Medium persistence
Exponential growing cells were exposed to two different antibiotics (Mupirocin and Ceftriaxone) for 4 hr at 37 ºC. 2 µl of treated bacteria was plated on antibiotic free nutrient agar for 12 hr at 37 Cº. next day, the number of viable persister cells was counted.

**3.2. The effect of Fluphenazine [Efflux Pumps Inhibitor] on the persistence of bacteria to Mupirocin**

Toxin - antitoxin system is not the only strategy for persistence, efflux pumps are another strategy. Studying persistence in E. coli and Mycobacterium tuberculosis concluded that toxin - antitoxins system and efflux pumps are implicated in persister cells formation [40]. Relying on our previous work about the impact of efflux pumps inhibitor Fluphenazine on the ability of MRSA isolates to form a biofilm, Fluphenazine (0.25 mg/ml) was used to investigate whether the ability of the tested isolates to form persister cells is conferred by TA system or efflux pumps. The persistence of isolates to Mupirocin in the presence of efflux pumps inhibitor was tested. Combining Mupirocin with efflux pumps inhibitor resulted in a 42% reduction in the number of persister colonies compared to number of survived persister colonies when Mupirocin was applied alone.

**Figure- 6 Efflux pumps inhibitor and persistence.** Exponential growing cells were exposed to Mupirocin (a) and Mupirocin + Fluphenazine (b) for 4 hr at 37 ºC. 2 µl of treated cells was plated in nutrient agar and incubated for 18 hr at 37 ºC. The number of viable persister colonies of the two treatments was counted and compared (c).
3.3. The loci of mazEF and yoeB/yefM in the genome of tested isolates.
Although the efflux pump inhibitor enhanced the effect of Mupirocin, the isolate formed persister cells, confirming that tested isolates depend on different mechanisms to promote dormancy state to survive the effect of antibiotics. Additionally, many reliable studies attribute the formation of persister cells to TA system that usually is triggered by stress responses. PCR was used to detect the prevalence of Two type II Toxin - antitoxin systems (mazEF and yefm-yoeB) among the tested isolates. Results consider positive if a distinct band at 300 bp and 451 bp for mazEF and yoeB/ yefM chromosomal loci, respectively, is observed on 1.5 % agarose gel. Results showed the presence of these two loci in 100% of the investigated isolates [Figure 7]. The prevalence of these genes among the Iraqi MRSA isolates and the ability of these isolates to evolve persister cells urged the need for new antibiotics that target the TA system.

![Figure 7 Polymerase chain reaction screen for mazEF (A and B) and yeEF-yoeB loci (C). Lane L: 100 bp DNA ladder. Lane N negative control. The number above the lanes represented the screened isolates. mazEF loci and yeEF-yoeB loci are present in all the screened isolates.](image)

4. Conclusion
The incorrect use of antibiotics resulted in the development of bacterial resistance that led to an increase in the number of cases of failed antibiotic treatments. Methicillin resistant Staphylococcus aureus emerged in society as a result of the unnecessary or an inappropriate use of antibiotics. Persistence is another cause of treatment failure. Detecting the ability of bacteria to evolve persister cells is very important to choose the right antibiotic or anti-persistent therapies to treat infectious diseases. Different strategies have been made to prevent the formation of persister cells and to eradicate their presence. Exposing tested isolates to Mupirocin resulted in a high level of persistence. The lethal effect of Mupirocin was enhanced by combining efflux pumps inhibitor. This urged the need to discover agents targeting genes code for efflux pumps and suitable inhibitors to be combined with an
antibiotic. Due to its Prevalence distribution among pathogenic bacteria, the TA system has been considered as a promising therapeutic target.

5. Ethical statement
All experiments followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000.

References


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