Role of Toxin-Antitoxin Systems in Emergence of Persisters from Methicillin-Resistant *Staphylococcus aureus* Strain

Yehia A. Osman[*1], Hamdia Askr[2], Sally ElSheikh[1] and Attyia Mohamadeen[1]

[1] Btany Dept, Faculty of Science, and [2] Microbiology and Immunology Dept, Faculty of Medicine, Mansoura University, Mansoura, 35166, Egypt.

Abstract

Background: Persisters or metabolically dormant bacterial cells are much less sensitive to antibiotic and do not grow in their presence. Different explanations for this phenomena are widely circulated in the literature and in the heart of it is the role of toxin and antitoxin (TA) systems. Where, the more labile antitoxins are readily degraded under stress conditions, allowing the toxins to exert their effect and force some cells to be dormant.

Aim: The diversity of toxin and antitoxin system(s) in persisters derived from *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 43300-MINI-PACK™) was the major objective of this study.

Methods: MRSA-derived persisters were isolated under vancomycin stress. Classically they were characterized by persister's assay, catalase test, Baird-Parker assay and mannitol fermentation. Molecular characterization included: plasmid DNA profiles, protein banding patterns and toxin-antitoxin operons, comparing persisters to the parent strain ATCC43300 and its closely related stain ATCC6538 (FDA 209).

Results: Biochemically, persisters were similar to the ATCC43300 and ATCC6538 strains apart from the catalase reaction that was very weak in persisters. No significant differences were seen in the plasmid profile but distinctive differences were observable in the protein banding patterns and the toxin-antitoxin PCR products' profiles. Seven toxin-antitoxin operons namely, HipAB, RelEB, HigAB, MazFE, MqsRA, CcdAB, HicAB were detected in the standard MRSA strain (ATCC43300) and its laboratory derived persister.

Conclusion: This is to detect 7 toxin-antitoxin systems in the laboratory derived persisters from the MRSA stain ATCC43300. These systems appear to be the reason for remission of infection and further complicate the discovery of the real mechanism for persister's emergence.

Keywords: MRSA, Persisters, Toxin-antitoxin systems, antibiotic resistance, VRSA

Introduction

Antibiotic resistance constitutes a major challenge to modern life. The over use and misuse of antibiotics has increased the rate of emergence resistant bacteria. Physiologically, a few members of the bacterial population enter a state of dormancy to escape the danger imposed by antibiotics, i.e. become persistent. This bacterial population simply immunized itself against the harmful action of antibiotics and ensured its survival (Kussell et al., 2005). This phenomenon was first observed by Joseph Bigger (Bigger, 1944), who discovered failure of penicillin to eliminate exponentially growing *Staphylococcus aureus* cells. This constitutes major problems to eradicate infectious agents, especially in health care settings.

Numerous scientific reports and publications tried to unravel the exact mechanism or explain the real bacterial strategies for the emergence of persisters. However, the real answer is still hard to find. Although, toxin and antitoxin (TA) systems are well documented in bacteria and archaea, some reports implicated them as one of the contributors to emergence of persister cells (Lewis, 2008, Wang and Wood, 2011). (Moyed and Bertrand, 1983) were the first to link TA systems to microbial persistence. In the absence of antibiotics, toxins and antitoxins neutralize each other and hence their existence is not felt. Generally, toxins are more stable and target DNA replication, mRNA stability, protein synthesis, cell-wall biosynthesis, and ATP synthesis. In antibiotic-sensitive bacterial cells, antitoxins are readily degraded allowing the destruction of cells by the toxins.

A direct evidence for the involvement of TA systems in emergence of persister cell was presented by (Dörr et al., 2010). They deleted the HipA and MqsR components of the HipA/HipB and MqsR/MqsA TA systems, respectively from *E. coli* cells and found a huge reduction of persistence(Maisonneuve et al., 2011, Kim and Wood, 2010, Keren et al., 2004b). Moreover, the work of (Keren et al., 2004a, Korch et al., 2003) has strengthened the involvement of at least one toxin-antitoxin system in the generation of "persisters" to β-lactam antibiotics. Several reports stated that overproduction of type II toxins inhibited bacterial cell growth and induced non-growing cells that could not regain its vigorous growth even after the induction of cognate antitoxin genes (Christensen-Dalsgaard and Gerdes, 2006, Christensen-Dalsgaard et al., 2010, Pedersen et al., 2002). Therefore, in 2011, Hayes and Van Melderen tried to group the known five toxin-antitoxin systems according to their specific target inside the bacteria which were DNA, proteins or RNAs molecules. This was to facilitate the subsequent studies. Recently, it was discovered that *Escherichia coli* and *Mycobacterium tuberculosis* harbor more than 33 and 60 TA systems, respectively. The presence of such huge number of TA systems in these two highly pathogenic bacteria has complicated the relationship between the emergence of persisters and multidrug resistance(Yamaguchi et al., 2011) .

The objective of this study was to understand some of the genetic basis behind the emergence of persister cells from amongst multidrug resistant *S. aureus*. The genetic diversity between the multidrug resistant strain of *S. aureus* ATCC 443300 and its persister cells in comparison to a second *S. aureus* strain ATCC6538 was also a target for the current study.

Materials & Methods

1. Bacterial isolates and growth media:

Two standard isolates of *Staphyloccocus aureus* (ATCC 43300 a MRSA strain and the β-lactam sensitive strain; ATCC 6538) were obtained from Dr. Askar, Faculty of Medicine Mansoura University and from a local culture collection center (MIRCEN) at Ain Shams University, Cairo, Egypt, respectively. Mannitol-Salt agar (MSA), Brain-Heart infusion (BHI), Luria-Bertani (LB) and Baird-Parker (BP) media were used to grow bacterial strains. All were obtained from Sigma-Aldrich except the BP medium was from OXOID. All antibiotic discs and powders were obtained from Bioanalyse and Mylan companies through local suppliers. Extra tests were done such as Gram staining and catalase activity test to confirm the identity of the strains (Chelikani et al., 2004).

2. Antimicrobial susceptibility testing

A universal antibiogram was used to test the antibiotic sensitivity of the three bacterial strains involved in this study as originally described by Kirby Baure disc diffusion method (Sirrenberg et al., 1996). Antibiotics discs used were oxacilline (1µg), vancomycin (30µg), gentamycin (10µg) and ampicillin (10µg). After overnight incubation at 37°C the diameter of inhibition zone produced by each e antibiotic disc was measured along two axes at right angles in mm. The minimum inhibitory concentration MIC was determined only for vancomycin ((Rodríguez-Tudela et al., 2003).

3. Isolation of persister cells:

Persister cells were isolated from *S. aureus* (ATCC 43300) according to the protocol of (Keren et al., 2004b). Six cultures were exposed to different concentrations of Vancomycin (125, 150, 175, 200, 225, and 250 ug/ml, respectively) and left to grow at 37°C for an overnight. Once lysis was observed, persisters were collected by centrifugation at 12000 rpm for 20 min. Reactivation of persisters was done in liquid as well as on agar plates of Brain heart infusion medium. In broth, 0.1 ml of the persister's culture was used to inoculate a 5 ml fresh BHI broth, while, 100 µl were streaked onto BHI agar plates and incubated at 37°C.

4. Persisters assay:

The number of vancomycin-derived persisters was determined according to the method described by (Keren et al., 2004a). Briefly, a 5ml of an overnight grown culture of MRSA ATCC 43300 was used to inoculate 200 ml LB medium containing 250ug\ml final concentration of vancomycin. Cultures were incubated with shaking at 37^{o}C and 100µl were withdrawn after 3, 5, 7, 9 and 24 h ,and streaked on solid Brain heart media and measuring growth at OD_{600} (JENWAY 6305 spectrophotometer) and determining the colony forming units (CFU) on LB agar plates.

5. Molecular characterization of persisters:

Molecular characterization of living organisms depends not only on its own genome, but also upon its genome expression (chromosome and plasmid). Therefore, total cellular protein

banding pattern, plasmid profile and polymerase chain reactions (PCR) were used to differentiate between studied strains at the molecular level.

5.1. Plasmid profile:

The QIAprep Spin Miniprep Kit (cat.nos.27104 and 27106) was used for extraction of plasmid DNA. Studied strains were (MRSA ATCC, persister cells, reactivated persister cells and standard *S. aureus* ATCC 6538). Five ml overnight cultures were used for all studied strains except the persisters for which a 100ml volume was used to compensate for the low cell count. The extracted DNA was analyzed by agarose gel electrophoresis.

5.2. Protein banding pattern (SDS- PAGE analysis):

Sodium Dedocyle sulfate polyacrylamide gel electrophoresis (SDSP-PAGE) was used to examine the total cellular proteins of MRSA, persister cells, reactivated persister cells and standard *S. aureus* ATCC 6538 as described by Laemmli (1970). The fractionated proteins were photographed and analyzed by Quantity One software (BioRad, USA). Protein concentrations were determined using the Bradford method with a Bio-Rad dye reagent concentrate and bovine serum albumin (BSA) as a standard protein.

5.3. Detection of Toxin–Antitoxin genes:

PCR amplifications of TA system genes were performed using a single colony of each of *S. aureus* ATCC 6538, ATCC 43300 and its persister cells. Different sets of oligonucleotide gene-specific internal primers were used to amplify the MazEF, RelEB, HigAB, HicAB, MqsR, HipAB TA genes and separate intergenic primers were used to amplify the upstream and downstream flanking regions. The oligonucleotide sequences of the primers were listed in Table 1. The general reaction mixture consisted of 12.5 µl mastermix, 1 µl *Taq* polymerase enzyme, 2µl of each primer; forward or reverse (diluted 1/10) A single colony of each strain served as (DNA template) and steriled distilled H_2O completed the volume to a final 25 µl. PCR amplification was carried out in Proflex thermal cycle under the following reaction conditions: initial denaturation temperature 95^oC for 10 min. and denaturation at 94^oC for 1 min, extension at 72^oC for 1 min, and a final extension at 72^oC for 5 min. PCR amplified products were analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and photographed (Mullis et al., 1986, and (Saiki et al., 1986).

Toxin-Antitoxin systems of *Pseudomonas aeruginosa* : *HipA* (toxin T), *HipB* its (AT), *MazF* (T) and its AT *MazE, RelE* (T) and its AT *RelB, MqsR* (T), *CcdA* (T) and *CcdB* (AT), *HigBA*, *RelBE* ; *Acinetobacter baumannii HigBA, RelBE*; from *S. aureus MazEF* up and down*; E. coli HicA* and *B*, and *Burkholderia* sp. *HicAB* and *HigBA* were used in this study (Table 1).

Table 1: The nucleotide sequences of primers specific for the TA genes.

Gene	Sequence	Product	Annealing temp.	Reference
***HipA* Toxin**	F:5`- CTTGTCACTTGGATGAACAACCAG-3` R : 5`-TCACTTACTACCGTATTCTCGGC-3`	1314 bp	43^{o}C	(Hemati et al., 2014)
***HipB* Antitoxin**	F: 5`-AGCCCAACGCAATTGGCGAATGCA-3` R: 5`-CTGTTCTGTTGATTCTGGCGAGGC-3`	225 bp	43^{o}C	(Hemati et al., 2014)
***MazF* Toxin**	F: 5`-ATGGTAAGCCGATACGTACCC-3` R: 5`-TGGGGCAACTGTTCCTTT-3`	288 bp	43^{o}C	(Hemati et al., 2014)
***MazE* Antitoxin**	F: 5`-ATGATCCACAGTAGCGTAAAGCGT-3` R: 5`-TTACCAGACTTCCTTATCTTTCGG-3`	249 bp	43^{o}C	(Hemati et al., 2014)
***RelE* Toxin**	F: 5`-GACGAGCGGGCACTAAAGGAAT-3` R: 5`-TCAGAGAATGCGTTTGACCG-3`	267 bp	42^{o}C	(Hemati et al., 2014)
***RelB* Antitoxin**	F: 5`-ATGGGTAGCATTAACCTGCGT-3` R: 5`-TCAGAGTTCATCCAGCGT-3`	240 bp	42^{o}C	(Hemati et al., 2014)
***MqsR* Toxin A**	F: 5`-ACGCACACCACATACACGTT-3` R: 5`-GCCTGGGTCTGTAAACATCCT-3`	194 bp	42^{o}C	(Hemati et al., 2014)
***CcdA* Toxin**	F: 5`-GACAGTTGACAGCGACAGCT-3` R:5`- TCACCAGTCCCTGTTCTCGTC-3`	199 bp	42^{o}C	(Hemati et al., 2014)
***CcdB* Antitoxin**	F: 5`-GAGAGAGCCGTTATCGTCTGTT-3` R: 5`-TCCCCAGAACATCAGGTTAATG-3`	272 bp	42^{o}C	(Hemati et al., 2014)
***RelBE* Toxin**	F:5`-CAGGGGGTAATTTCGACTCTG-3` R:5`-ATGAGCACCGTAGTCTCGTTC-3`	505 bp	43^{o}C	(Williams et al., 2011)
***HigBA* Toxin**	F:5`-CTCATGTTCGATCTGCTTGC-3` R:5`-CAATGCTTCATGCGGCTAC-3`	469 bp	48^{o}C	Williams, et al. (2011)
MazEF	F:5`-GTCTTGAACACATCTTCACGCG-3` R:5`-GCGAAAATACCGACACATGTAGAG-3`	753 bp	45^{o}C	(Williams et al., 2011)
MazEF	(+)5`-GCTTCGTTCGCTAGGGAGAG-3` (-)5`-CTACAAGCGGGTGAGTCTGTAA-3`	556 bp	48^{o}C	(Williams et al., 2011)
***HicA* Toxin**	f:5`CCCTCTAGAGGATCCGTTTACTTTGTTGATATAC TCAG-3` R:5`_CCCAAGCTTGAATTCTTAACTCAAACCGAGTT GTTTC-3`	174 bp	50^{o}C	Jorgensen, et al (2008)
***HicB* Antitoxin**	F:5`CCCTCTAGAGGATCCCGCCAATTAAAAAGGTTA ATGAC-3` R:5`CCCAAGCTTGAATTCTTAAACCATCACCAGCGA TAAC3`	435 bp	50^{o}C	Jorgensen, et al (2008)
***HigBA* TA**	Forward: AGCACATCCGTACGATCTACTGC-3 Reverse: TGCACTCCTGCGATGCGGCGAA	440bp	45^{o}C	Ghafourian, et al, (2014)
***RelBE* TA**	Forward: ATGAAGTGAACGGTCAACAATA Reverse: ACAGACCTCGGAAAGTGGTCG	578bp	42^{o}C	Ghafourian, et al, (2014)

Results

1- Characterization of *S. aureus* persisters

Both ATCC *S. aureus* strains and the persiter cells derived from the ATCC 43300 were Gram positive -as expected- but the persister cells produced smaller colonies on LB agar plates. The three tested trains produced β-hemolysis on the Tryptone-Soy agar, grew on mannitol salt agar and produced characteristic black colonies on Baird-Parker medium. However, the catalase activity (1 min after H_2O_2 addition) of the persisters cells was highly reduced compared to the ATCC standard strains (Fig. 1).

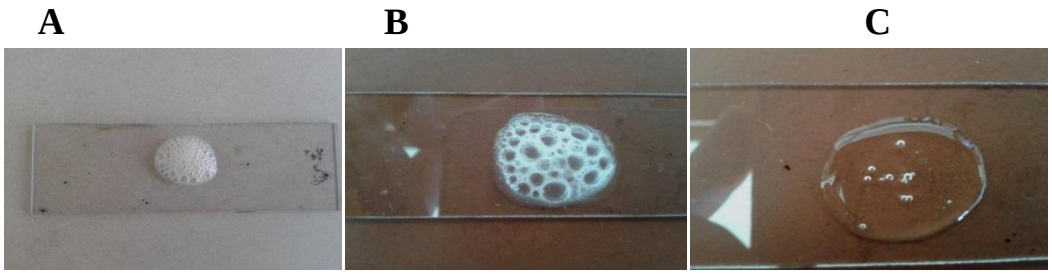

Figure (1): Catalase activity of the three *S. aureus* strain. Panel A: ATCC43300, panel B: ATCC6538 and panel C: vancomycin-derived persisters

2-Persister assay:

The 100 µl of bacterial(MRSA) culture with vancomycin were withdrawn at 0, 3, 5, 7, 9 and 24 hr and streaked onto plates labeled A, B, C, D, E and F, respectively. The number and size of the individual persister colonies was smaller and growth curves of the three strains showed distinctive differences. While the lag phase lasted for 2 hrs in ATCC strains, persister cells did not start the exponential growth phase before almost 38 hrs. It was also interesting to notice that the duration of the exponential phases for the ATCC43300, ATCC6538 and persister strains was 27, 40 and 22 hrs, respectively. Similar growth patterns in liquid media were monitored by measuring the OD at 600nm.

Figure (2): The number and intensities of the colonies appearing on Brain-heart infusion agar plates correlate with the surviving cells after Vancomycin treatment of MRSA (ATCC43300) cells.

Antibiogram

The data showed that MRSA strain (ATCC43300) and its persister cells were resistant to all tested β-lactam antibiotics such as oxacilline, ampicillin and penicillin. Both bacteria were sensitive to gentamycin (an aminoglycoside), vancomycin (a glycopeptide), Trimethoprim/sulphamethoxazole (a combination of sulfamethoxazole and trimethoprim). The antibiogram of the second standard *S. aureus* strain tested (ATCC6538) was similar to that of MRSA strain (ATCC43300) except for being sensitive to Oxicillin, ampicillin and penicillin. Moreover, the three bacteria showed an intermediate sensitivity to Ceftriaxone (a cephalosporin, β-lactam antibiotic). The vancomycin MIC was determined for the MRSA strain ATCC 43300 and found to be 1.5ug/ml.

Molecular Identification:

- **Plasmid profile:**

The four bacterial isolates showed a common plasmid profile with almost the same electrophoretic mobility. The only difference was the appearance of a plasmid band with electrophoretic mobility greater than 10 kbp in both persister cells and persister cells after regrowth on fresh media without antibiotic; lanes 2 and 3.

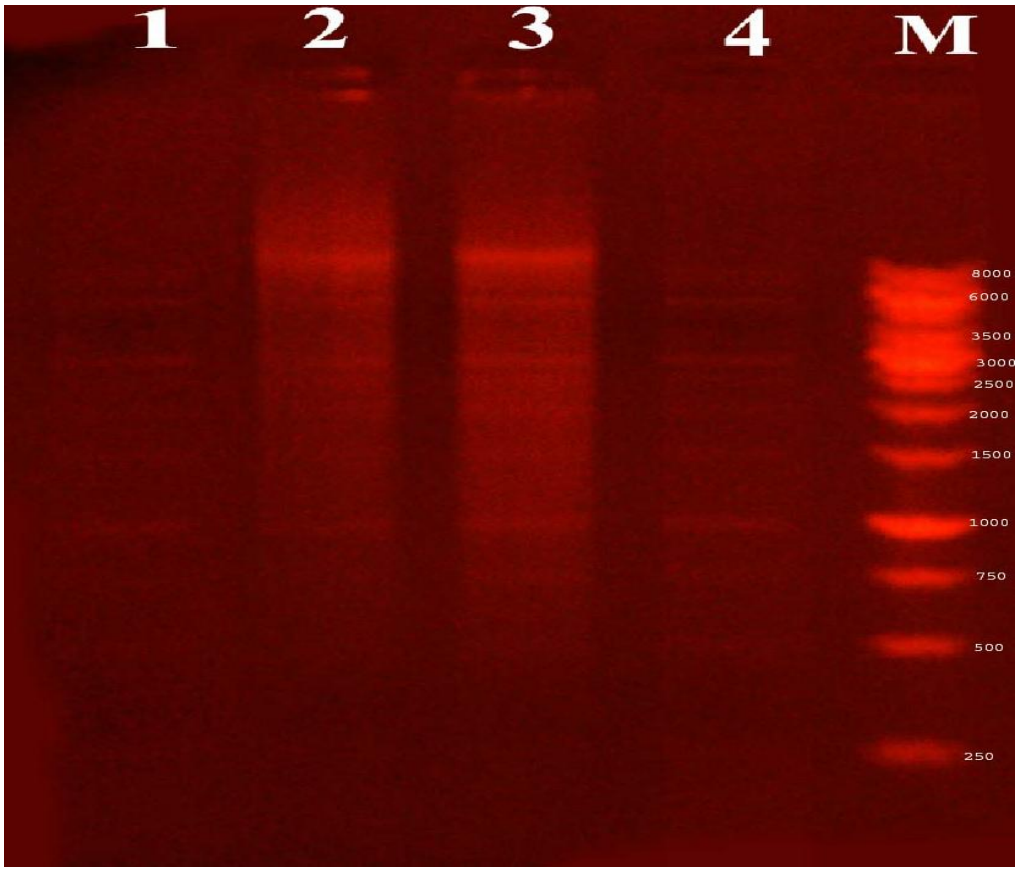

Figure (3): Plasmid profile of the three studied bacterial cells. Lane1: MRSA ATCC43300 strain, lane 2: Vancomycin-derived Persisters, lane3: activated persisters, lane 4: *S. aureus* ATCC 6538, lane M: GeneRuler 1Kb DNA ladder (10,000-8000-6000-3500-3000-2500-2000-1500-1000-750-500-250pb)

- **Protein SDS PAGE:**

Significant differences between the total cellular protein patterns of MRSA (ATCC443300) and its vancomycin-derived persisters (Fig. 4). Each cell type showed distinctive proteins which did not appear in the other strains. As shown in Fig.4 MRSA strain (ATCC443300) with no equivalence in the persister cells and/or the reactivated persister cells (14 days old). The following protein bands were expressed in MRSA cells with electrophoretic mobility corresponding to the molecular masses of 129.061, 112.918, 86.227, 72.741, 57.749, 47.664, 39.054, 33.43, 20.564, 19.876 kDa. Similarly, the protein banding pattern of the persister cells showed its own unique bands: 77.109, 56.089, 34.503, 21.852, 18.75, and 16.973 kDa.The protein banding pattern of the reactivated persister cells showed a greater diversity even more than the persister cells (19.876, 33.43, 43.04, 65.845, 72.741 and 133.203 kDa.). Three of the reactivated cells' proteins were shared with MRSA and the persister (43.04, 65.845, and 133.203 kDa.) while the other three were shared with MRSA only (72.741, 33.43, and 19. 876 kDa.) suggesting that the last ones may be crucial for cell survival.

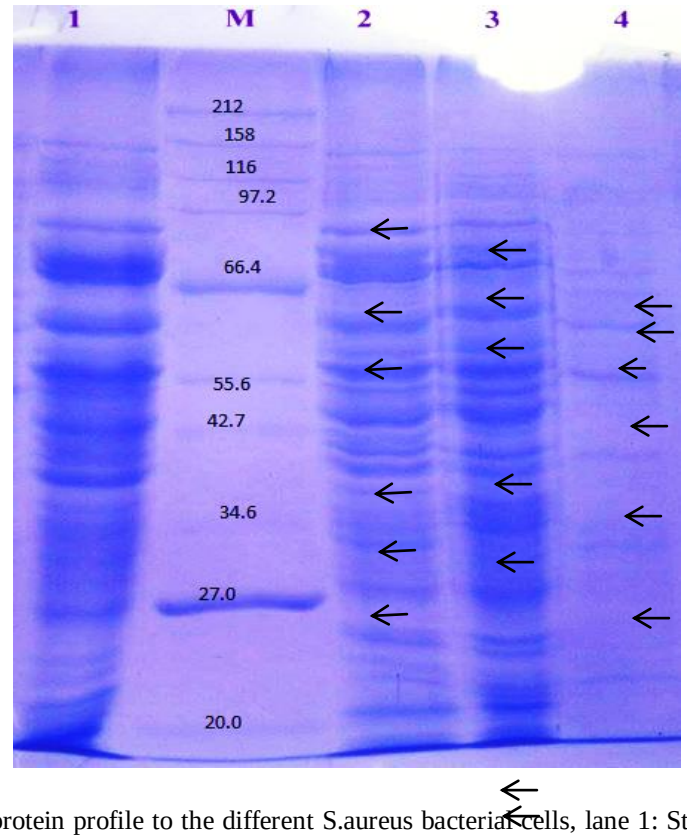

Figure (4): SDS -PAGE protein profile to the different S.aureus bacterial cells, lane 1: Standard *S. aureus*, lane M: protein molecular weight marker (212-6.5KDa (212-6.5KDa), lane 2: MRSA, lane 3: persister cells and lane 4: activated persister cells; 14 days old.

- **PCR product profile**

PCR product profile analysis of the different toxin-antitoxin (TA) bacterial systems scored as presence or absence of the expected size, in either the MRSA ATCC 43300 strain or its vancomycin-derived persisters are presented in the fig. 5 and table (2) below. Interestingly, almost all the PCRs produced DNA fragment with variable sizes than the expected ones for all tested toxin-antitoxin systems. Considering that MazEF toxin-antitoxin system is the only published one for *S. aureus*, and as expected, the exact amplicon size (556bp DNA fragment) did exist neither in MRSA nor its vancomycin-persisters. The PCR product profiles for MRSA (ATCC43300) and its persister strain were either specifically negative or non-specifically positive for the following toxin-antitoxin systems: *HipA/HipB, MazF/MazE, RelE/RelB, MqsR, CcdA/CcdB, HigAB, HicA/HicB,* and *RelEB.* A comprehensive list of all PCR products for the tested toxin-antitoxin systems are listed in the Table (2) below. The total experimental PCR products and those specific for the MRSA ATCC 43300 and its vancomycin persister were listed in separate columns.

Table (2): PCR-product profiles for all toxin/antitoxins systems in MRSA (ATCC43300) and its Vancomycin-derived persisters

Toxin/Antitoxin System	Expected PCR Product (bp)	Experimental PCR product (bp)	PCR product (bp)	
			MRSA	Persister
HipA (Toxin)	1314	679	679	679
HipB (antitoxin)	225	**235+271+300+400+962+1080**	235	rest
MazF (Toxin)	288	Nd*	Nd	Nd
MazE (Antitoxin)	249	314+Nd	314	314
RelE (Toxin)	267	Nd +556+124	556+124	556+124
RelB (Antitoxin)	240	124	124	124
MqsR(Toxin)	194	1437+1062+834+420+290+200	200	All
CcdA(Toxin)	199	Nd	280 124	280 124
CcdB(Antitoxin)	272	Nd +1226	No + 48	1226+48
HigAB(Toxin-Antitoxin)	440	Nd+1592+1091+910+702+220 +105+95	220+105+ 95	All
MazEFsa(Toxin-Antitoxin)	753	748	748	748
HicA(Toxin)	174	Nd+274+265	274+265	274+265
HicB(Antitoxin)	435	485+1010	485	485+101
RelEB(Toxin-Antitoxin)	578	1309+1120+1006+734+285+196 168+65+31+nd	Nd	All
HigAB(Toxin-Antitoxin)	440	1454+1060+734+352+285+197	Nd	All

Nd: the expected PCR product was not detected

The following are the agarose gel photos for all tested TA systems showing the experimental PCR-product profiles using MRSA ATCC strain 43300.

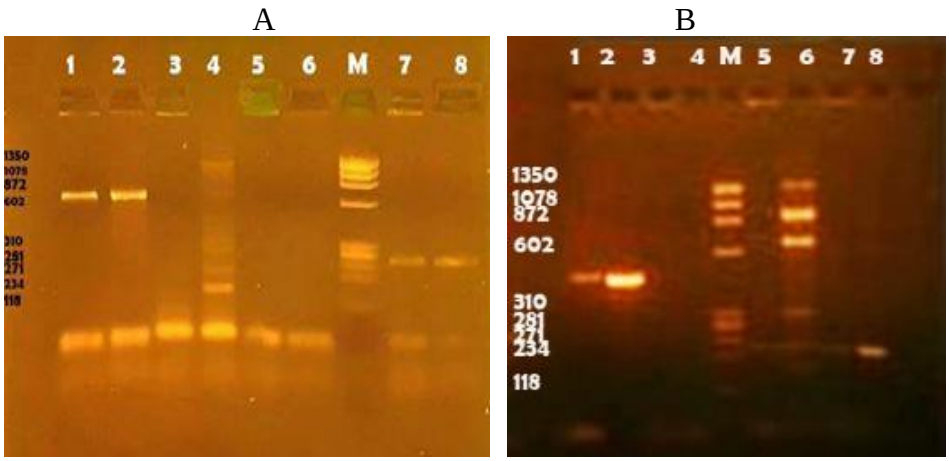

Figure (5A): PCR product profile for some TA systems. **Panel A:** lane: 1-*HipA* toxin gene in MRSA, lane 2-*HipA* toxin gene in persisters, lane 3-*HipB* antitoxin gene in MRSA, lane 4- *HipB* antitoxin gene in persisters, lane 5-*MazF* toxin gene in MRSA, lane 6-*MazF* toxin gene in persisters, lane M –DNA ladder (with molecular weight 1350 -194 bp) lane 7-*MazE* antitoxin gene in MRSA and lane 8-*MazE* antitoxin gene in persisters. **Panel B**: lane 1: *RelE* toxin gene in MRSA, lane 2: *RelE* toxin gene in persisters, lane 3: *RelB* antitoxin gene in MRSA, lane 4: *RelB* antitoxin gene in persisters lane M: DNA ladder (from 1350-194bp), lane 5: *MqsR* toxin gene in MRSA, lane 6: MqsR toxin gene in persisters, lane7*: CcdA* toxin gene in MRSA and lane 8: *CcdA* toxin gene in persisters.

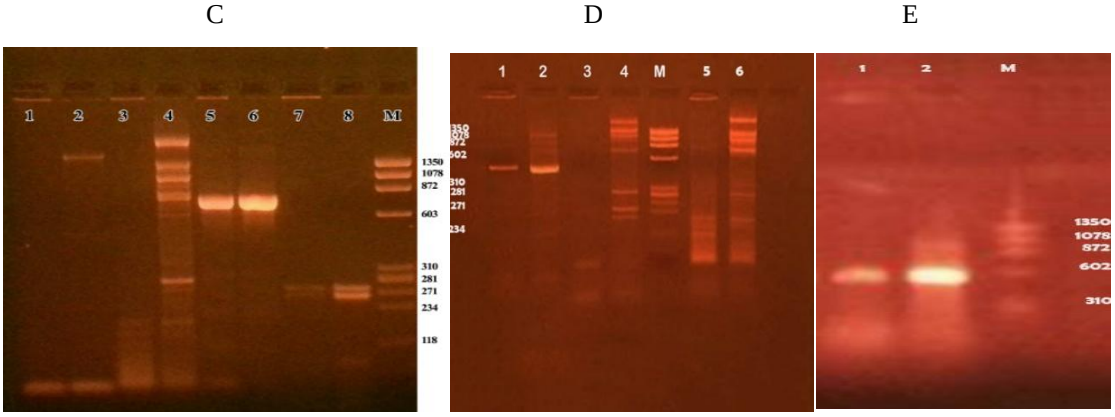

Fig. (5 B): Panel C: 1-ccdB toxin gene in MRSA, 2-*CcdB* toxin gene in persister cells, 3-*HigAB* toxin-antitoxin genes in MRSA. 4-*HigAB* toxin-antitoxin genes in persisters. 5-*MazEFsa* toxin-antitoxin genes in MRSA. 6-MazEFsa toxin antitoxin genes in persisters. M-DNA ladder (with molecular weight 1350-194 bp).7-*HicA* toxin gene in MRSA. 8-*HicA* toxin gene in persisters. **Panel D:** 1-*HicB* antitoxin gene in MRSA.2-*HicB* antitoxin gene in persisters .3-*RelEB* toxin-antitoxin genes in MRSA .4-*RelEB* toxin antitoxin genes in persisters .M-DNA ladder (with molecular weight 1350-194 bp). 5-*HigAB* toxin-antitoxin genes in MRSA. 6-*HigAB* toxin-antitoxin genes in persisters. **Panel E:** 1-*MazEFsa* toxin-antitoxin genes in MRSA showing a positive 556bp PCR product. 2-*MazEF*sa toxin-antitoxin genes in persister cells showing a positive 556bp PCR product. M-DNA ladder (with molecular weight 1350-194 bp)

Discussion

The recalcitrant nature of MRSA chronic infections is attributed to the emergence of persister cells. This state of antibiotic-tolerance and physiological dormancy affects a small subpopulation of MRSA cells in the culture due to the pressures exerted by antibiotics such as vancomycin. Electron micrographs showed extensive damage suffered by MRSA cells upon growth in presence of vancomycin and explicitly showed that some cells have escaped the killing action of vancomycin. These cells continue to grow and flourish even in the presence of this deadly antibiotic; VRSA cells. What contributed to its survival was the hypothesis of this study.

Historically, persisters were first reported by Bigger in 1944 (Bigger, 1944), but the exact mechanisms behind their development are still not fully understood. A major problem was obtaining enough cells to carry out reliable studies to uncover the real mechanism behind the emergence of this type of cells. This inability was overcome by using vancomycin which enhanced not only the conversion of the few survived MRSA cells (ATCC443300) into persisters, but also encouraged these persisters to divide and flourish in its presence. In addition to this, vancomycin overcame the number limitation for generation of persisters that was reported to be around 0.001 to 0.1% of the cells of a specific bacterial population. The availability of enough persister cells enabled us to establish some molecular evidences that might have contributed to the survival mechanism of these cells.

Although several mechanisms have been postulated and supported by experimentation (Keren et al., 2004b), most of them suggested the dependence of each mechanism on the type of the antibiotic used (Luidalepp et al., 2011). (Shan et al., 2015) have concluded that activation of motility and amino acid biosynthesis contributes to the formation of persisters tolerant to gentamicin. Recent studies have implicated several genes and their expression products in the formation of persisters (Kint et al., 2012, Lewis, 2010). In this study PCR and protein analysis of the vancomycin-generated persisters lend strong support to the involvement of some genes in this process. Seven TA systems were detected and seven different protein bands were present in MRSA with no resemblance in the persisters and seven proteins were detected in its vancomycine-emerged persister cells. Moreover, the protein banding patterns of MRSA and its persisters coincided with their PCR product profile for toxin-antitoxin systems. Importantly, none of the protein bands has the same electrophoretic mobility, meaning that all have distinctive molecular masses. This reflects the differential gene expression in both cell types. Most probably, the proteins detected in the vancomycin sensitive MRSA could represent the toxins, while those detected in its persisters could represent the corresponding antitoxins.

Considering the set of data obtained by us, we hypothesized a conservative mode for the emergence of non-spore forming persisters to survive in the presence of antibiotics. It does include the involvement of several genes encoding for TA systems. The complexity of the TA systems detected in the MRSA strain ATCC 43300 - at least 7 out of 9 tested TA systems (*HipAB, RelEB, HigAB, MazFE, MqsRA, CcdAB, HicAB*) - does support the genetic basis for the

emergence of persister; it is not just a physiological dormancy state. All of the detected 7 TA belong to type II and characterized by exerting different cellular roles.

The *HipA* gene encodes for a serine/threonine-protein (48.4kDa), specifically phosphorylates the Glu-tRNA-ligase, leading to inhibit replication, transcription, translation and cell wall synthesis.(Correia *et al*., 2006,Bokinsky *et al.*, 2013), While, *HipB* antitoxin (20.9kDa protein) binds to DNA and represses the *HipBA* operon promoter; which neutralizes the toxic effect of the toxin *HipA* (Black *et al*., 1994). *RelEB* interferes with translation process by cleaving of ribosome-bound mRNA specificity in the ribosomal A site between positions 2 and 3 (Christensen *et al*., Galvani *et al*., 2001, 2001, , Pedersen *et al*., 2003, Ryseck *et al*., 1992) *MazF*_Gene is a sequence-specific endoribonuclease it inhibits protein synthesis by cleaving mRNA and inducing bacterial stasis. Antitoxin *MazE* Gene antitoxin binds to the MazF endoribonuclease toxin and neutralizes its endoribonuclease activity. (Kolodkin-Gal et al.,Marianovsky *et al*., 2001, 2009, Zorzini *et al*., 2015). *HicAB* system. While *HicA* is translation-independent mRNA interferase prevents cell growth by cleavage of a number of mRNAs and tmRNA. However, expression of antitoxin HicB inhibits its action.(Jørgensen et al., 2009)

Then we have to ask, what specifically activates the dormancy state? and why in a small subpopulation only? This fits well with the paradox about the real mechanisms involved in the emergence of persisters (Lewis, 2010). They tried hard to explore all possibilities for existence of persisters, but found it multi-facets and the drug they developed to kill these cells affected more than 400 cellular proteins. In our study, the protein banding patterns analysis of vancomycin generated persisters showed numerous protein bands with no equivalents in the MRSA cells (ATCC43300) and vice versa. The molecular weights, the natures of these proteins and their functions should give a strong supporting evidence to our hypothesis. A correlation between the new proteins and the TA systems should explain the existing dilemma of dormancy and persister cells.

Kos, et al (2012) (Desjardins et al., 2012) have studied 12 VRSA strains, derived from the most common hospital lineage of MRSA causing infection. They concluded that clonal cluster 5 has acquired the vancomycin-resistant transposon Tn_{1546} from restricted enterococcal donors. This is totally different from our VRSA persister strain, since our strain was laboratory derived and not isolated from patients. This suggests the activation of internal genetic elements such as TA systems rather than externally acquired factors.

The arsenal of TA systems found the MRSA ATCC43300 strain and their variable and diverse functions significantly contributes to the multidrug resistance and emergence of the persisters. Moreover, they complicate the process of revealing the exact mechanism of persistence.

Our study appears to complement a tremendous amount of work that has been done to answer the mechanism behind the development of persisters in bacteria and specifically in *S. aureus.* Linking the TA systems to any of the mentioned antibiotic resistance mechanisms,

antitoxin proteins responsible for the active defense systems in the current persisters, control of ROS molecules, *etc*., all require more extensive studies to resolve this elusive problem.

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