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Characterization of Persister Cells and Some Mutants from Clinical Isolate of a Multidrug-Resistant MRSA

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Abstract – Background: Some multidrug resistant bacteria such as MRSA, produce persister cells in the presence of bactericidal agents, which enables them to survive in the presence of the antibiotics. Evolution of persisters and the mechanisms of survival are still debatable and difficult to understand. Formation of persister cells establishes phenotypic heterogeneity within a bacterial population, causes relapses of bacterial infections and linked to failed treatments and the emergence of antibiotic resistance.
Method: MRSA-derived persisters were isolated under vancomycin stress and mutated by exposure to ultraviolet radiation; 254um. Persister cells and the mutants were classically and biochemically characterized. Persister cells were further subjected to persister's assay, grown on Baird-Parker medium, tested for biofilm formation, DNase production and mannitol fermentation.
Results: Bacteriologically and biochemically persisters were found to be similar to the clinical isolate and standard MRSA strains. But distinctive differences were observable in the DNase, coagulase and catalase activities.
Conclusion: A general method for isolating persisters was developed based on lysis of regular MRSA cells by vancomycin. These persisters retained all MRSA characteristics but shown down regulated enzyme activities.

Keywords – S. Aureus, MRSA, Persisters and Mutants.

I. Introduction

Many infectious bacterial diseases developed mechanisms to persist in their hosts and withstand antibiotic treatments. Many explanations have been circulated in the literature for this phenomenon such as development of antibiotic resistance by the infectious agents, immunosuppression of the host. A more recent interpretation was found to be associated with the physiological state of a fraction of the infectious agent cells called persister cells. Persister cells were documented among quite a significant number of bacterial infectious agents such as *Escherichia coli, Pseudomonas aeruginosa, Mycobacterium tuberculosis, Salmonella enterica subsp. enterica serovar Typhimurium and Staphylococcus aureus* [1] [2]. Failure of treatments of the numerous infectious agents was attributed to the existence of persister cells regardless of the antibiotic doses used [3]. A number of researchers have made a close link between these cells and the emergence of antibiotic resistance [4]. These dormant cells (the persisters) constitute huge challenge to the health industry and good deal of scientific efforts have been devoted to resolve this problem; which was first recognized by Joseph Bigger in 1944 [5, 6] A contradicting explanation was published by [7] challenging the metabolic inactivity of the persister cells.

In recent years, advances in the technologies have enabled scientists to study the mechanisms by which persister cells developed to escape killing by antibiotics without being genetically resistant [8]. This work is important for the

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development of effective drugs to treat the chronic and recalcitrant infectious bacterial pathogens exhibiting tolerance to different types of antibiotics. An in vitro study published by **Abokhalil *et al.*, (2020)** showed that different persisters subpopulations of different pathogenic bacteria respond differently to pretreatments before exposing them to antibiotics. For example, a 97% decreased in persister subpopulations of infectious *K. pneumonia* was recorded if pretreated by different pHs, while *Proteus mirabilis* showed a 25% decrease only [9].

In this study, we used recent advances in biological sciences to understand the formation and regrowth of persisters cells from a local isolate of methicillin-resistant *S. aureus* (MRSA). Moreover, we compared the bacteriological and biochemical behaviors of the laboratory developed persister cells to the clinical isolated of MRSA.

II. Methods:

Bacterial strain and growth media:

All bacterial isolates used in this study were local isolates from patients seeking treatments at Mansoura University Hospitals. All growth media were made up in one liter of distilled water and autoclaved at 121°C for 20 minutes. These were Mannitol-Salt agar (MSA), Brain-Heart infusion (BHI), Luria-Bertani (LB) and Baird-Parker (BP). All media and chemicals 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. The bacterial cultures were grown at 37°C for 24-48h, while the liquid cultures were kept in a shaking incubator at 180rpm for overnight [10]. The colonies were examined morphologically and biochemically for size, shape, edge, color and pigmentation, antibiotic sensitivity and hemolytic activity…etc.

Screening for MRSA:

The 24 isolates were screened for Methicillin-resistant *Staphylococcus aureus* (MRSA) as described by [11]. A 0.5 McFarland regular suspension was prepared in Mueller-Hinton Broth (MHB, Hi-Media, Mumbai, India) with each isolate plated on Mueller-Hinton agar (MHA, Hi-Media, Mumbai, India) plates containing 2 percent NaCl. Oxacillin (1µg) disc was used to select the *S. aureus* MRSA.

Minimum inhibitory concentration (MIC) of Vancomycin:

The lowest concentration of vancomycin that prevented growth of MRSA (MIC) was determined **[12, 13]**. In brief, each tube of MRSA cultures containing only one concentration of vancomycin as follow 0.0, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2 µg/ml were incubated for overnight shaking at 37°C at 200 rpm for overnight and bacterial growth was determined ($O.D_{600nm}$).

Amplification of persister cells from MRSA:

Vancomycin was used to select persister cells from clinical isolates of MRSA strains (**[14]**. Several concentrations of vancomycin (100 or 150 or 200, 250, 300, 350 and 400 µg/ml) were used to select for persisters from actively growing MRSA in 5 ml of LB broth. All cultures were incubated shaking (180 rpm) at 37°C for overnight. Persister cells were collected by centrifugation and used for subsequent analyses. The viability and identity of the persister cells of *S. aureus* were confirmed by microscopic examination and growth onto a variety of specific media. For example, the Baird-Parker selective medium contains glycine, lithium and tellurite encourages only the growth of *S. aureus*, MRSA and its persisters. These bacterial colonies should look grey-black shiny colonies with clear zone around them **[15].**

Persistence assay:

This is a time-dependent killing produce of MRSA cells or its emerged persister cells **[16]**. In this assay, samples were withdrawn at 0, 5, 7, 9 and 24h from an overnight MRSA culture growing shaking at 37°C for 24 h in presence of 500ug vancomycin. The withdrawn samples were streaked onto Brain-heart infusion agar plates to determine the colony forming units, which are proportional to the survived cells (CFU) and to confirm their identity **[17]**.

Biochemical identification of persisters:

Standard MRSA (ATCC 43300), locally-isolated MRSA and its persister cells were biochemically identified as recommended by [18]. The biochemical tests used to ensure the identities included fermentation of mannitol sugar, growth on Congo red agar, Baird-Parker medium, blood hemolysis, measuring catalase, oxidase, coagulase and DNase activities [19] [20] [21-24] [25] [26]; and [24, 27].

Antimicrobial Susceptibility Test:

The antimicrobial susceptibility of the selected MRSA and its persisters were done by using Kirby-Bauer disc diffusion technique **[28]** using commercially available antimicrobial discs (Bioanalyse/Ankara/Turkey). The antimicrobial discs and their concentrations were: methicillin (10µg), ampicillin (10µg), penicillin (10µg), oxacillin (1µg), gentamicin (10µg), ciprofloxacin (5µg), vancomycin (30µg), chloramphenicol (30 µg), sulfamethoxazole trimethoprim

(25µg). The diameter of the zones of inhibitions were recorded and graded as recommended by the Clinical and Laboratory Standards Institute (CLSI; 2011).

Screening for MRSA and resistance to Ciprofloxacin and Sensitive to Vancomycin:

A 0.5 McFarland standard suspension of Selected MRSA strains were prepared in Mueller-Hinton Broth (MHB, Hi-Media, Mumbai, India) and plated on Mueller-Hinton Agar (MHA, Hi-Media, Mumbai, India) plates containing 2% NaCl. A Ciprofloxacin (5 µg) and Vancomycin (30 µg) were placed on the surface of the plates and incubated at 37°C for 24 h.

III. Results

Screening for Staphylococcus aureus:

The 28 clinical isolates were screened for their ability to ferment mannitol sugar (Fig1). This differentiating test showed that 10 out of the 24 isolates fermented mannitol; as the pH indicator changed from red to yellow. This test suggested that the ten isolates belong to the *S. aureus* bacterium and they were subjected to further characterization using oxacillin.

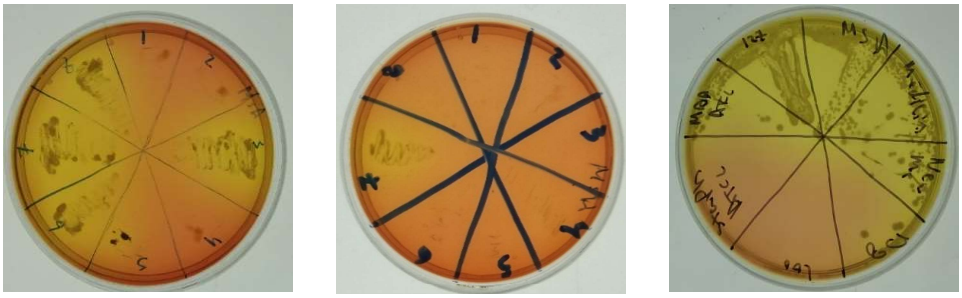

Fig (1): Screening the 24 *Staphylococcus* isolates by culturing on Mannitol salt agar medium yellow color is a fermentation of mannitol.

Oxacillin resistance:

The ten isolates of local clinical *Staphylococcus aureus* were further screened using oxacillin disk (1 µg/ml) to confirm their identities. All the ten isolates of local clinical *S. aureus* did show resistance to oxacillin and produced non-measurable zones of inhibition as recommended by the CLSI (Fig. 2). This data suggests to be members of MRSA.

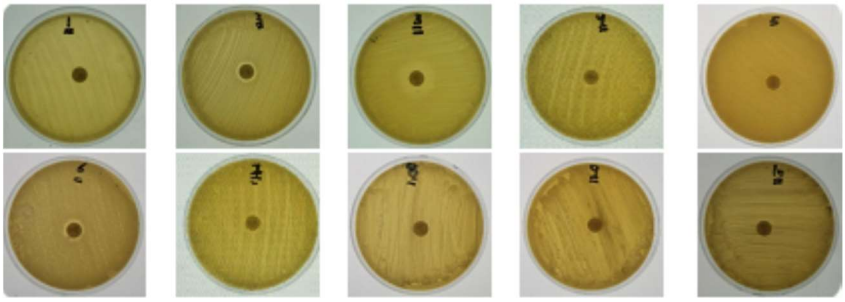

Fig (2): The oxacillin sensitivity test of the 10 selected MRSA local isolates; all were resistant to oxacillin.

Screening for MRSA and resistance to Ciprofloxacin and Sensitive to Vancomycin

The ten MRSA isolates were screened further using ciprofloxacin and vancomycin antibiotics. The results indicated that six of the local MRSA isolates were sensitive to vancomycin and only one MRSA (isolate number 10) was resistant to ciprofloxacin (Fig.3A). Isolate ten was also found to be resistant to ampicillin, chloramphenicol, methicillin, oxacillin, penicillin, sulfamethoxazole-trimethoprim and highly sensitive to gentamycin and moderately-sensitive to vancomycin (Fig.3B). Moreover, the MIC of MRSA isolates to vancomycin was found to be 1.5 µg/ml.

A B

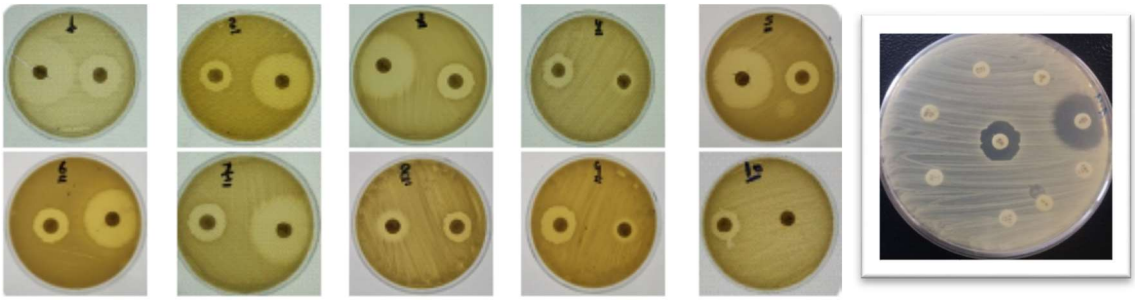

Fig (3): Double screening of the ten MRSA isolates using vancomycin and ciprofloxacin. Panel A showing the sensitivity to the two antibiotics and panel B shows sensitivity of isolate 10 to ampicillin, chloramphenicol, ciprofloxacin, methicillin, oxacillin, penicillin, sulfamethoxazole-trimethoprim and resistance to gentamycin and vancomycin.

Isolation and amplification of MRSA persisters:

Vancomycin treatments of locally-isolated MRSA cells did not kill all of cells when grown in brain heart infusion (BHI) broth. The cells that escaped the treatments were known to be persisters. The growth of MRSA in the presence of high concentrations of vancomycin was monitored by naked eyes, spectrophotometry and streaking on Baird-Parker agar plates. The few colonies resulted from streaking samples onto BP agar plates appeared to be proportional to the vancomycin concentration used in correlation with age of the culture (Fig.4). Moreover, the size of the colonies survived the vancomycin treatment were smaller than normal MRSA colonies.

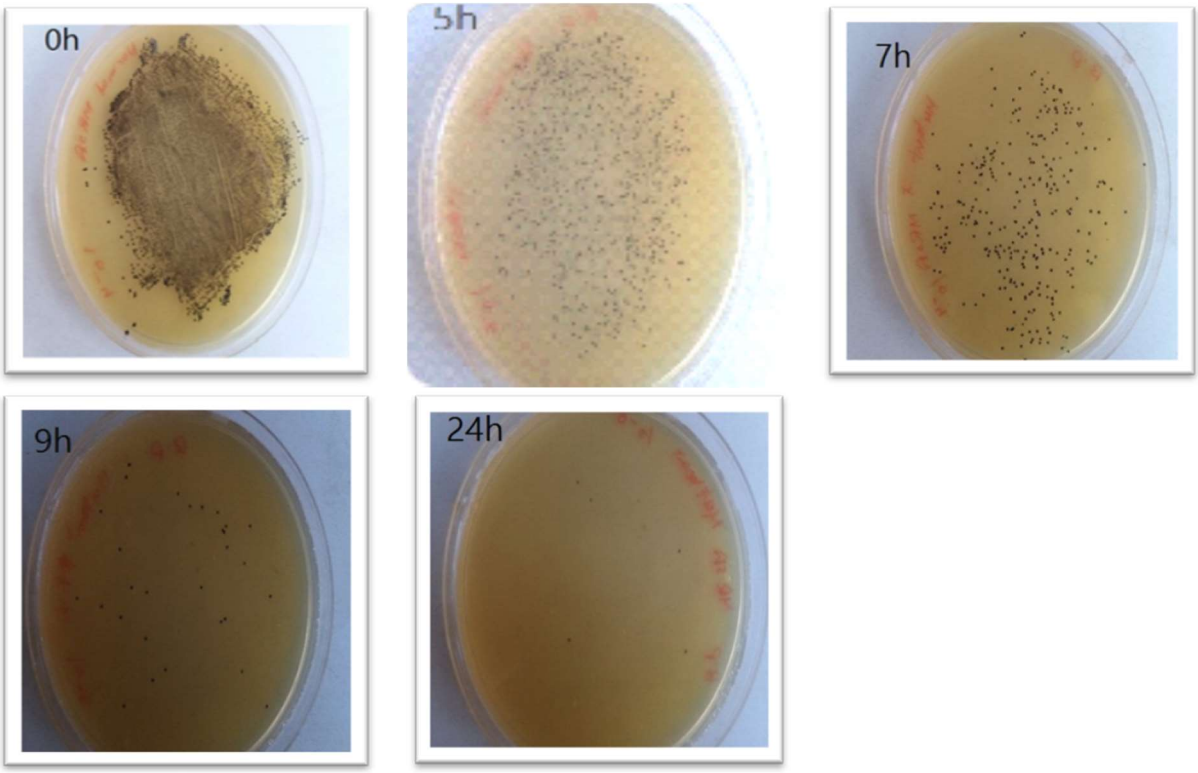

Fig (4): Persister assay show decreasing in persisters number with the increasing time exposure to antibiotic.

Biochemical characterization of MRSA persister cells:

Mannitol salt agar:

Three bacteria isolates belonging to *S. aureus* of MRSA, locally isolated MRSA- ATCC43300 standard MRSA, and vancomycin-derived persisters from locally isolated MRSA were capable of growing on mannitol salt agar (Fig. 5 (A, B, C).

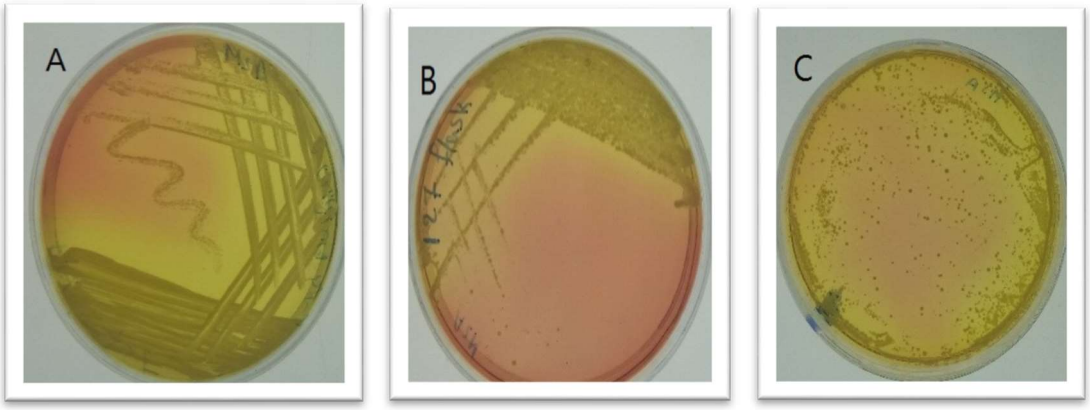

Fig (5): Fermenting Mannitol Salt Agar (selective and differential medium) by A: Standard MRSA ATCC 43300 standard MRSA, B: local clinical MRSA isolate, C: vancomycin-derived persister cells from local clinical MRSA.

The colonies of the three isolates appeared black when grown on Baird-Parker selective medium. Only one colony of seemingly persister cell survived the vancomycin treatment and regrown on Baird parker medium and produced black colonies (Fig. 6).

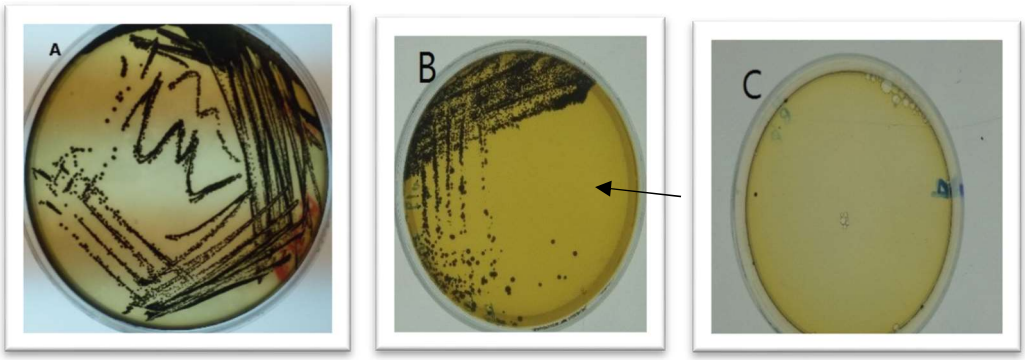

Fig (6): Black colonies of different S. aureus cells grown on Baird-Parker medium. Panel A: ATCC 43300 MRSA strain, panel B: locally-isolated MRSA, panel C: persister cells derived from local MRSA isolate (arrow referred to a single colony).

All three types of *S. aureus* isolates grown on blood-agar medium and hemolyzed blood. Again, a single colony of vancomycin persister cells hemolyzed blood medium (Fig.7); produced black colonies, on Congo red biofilm selective medium (Fig.8).

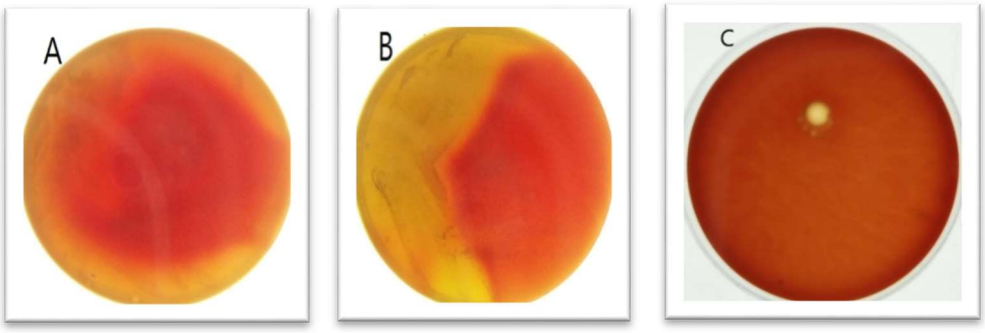

Fig (7): Blood medium showed complete hemolysis (Beta-hemolysis) of red blood cells in different S. aureus Panel A: Standard MRSA ATCC 43300, Panel B: MRSA ,Panel C: Persister cells.

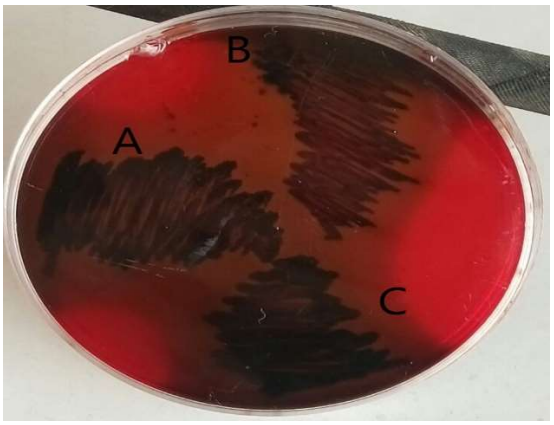

Fig (8): Congo red medium showed black growth due to formation of Biofilm in three types of S. aureus isolates; A: Standard MRSA ATCC 43300, B: MRSA isolate, C: Persister cells.

Moreover, the three studied *S. aureus* bacterial isolates reacted differently with catalase, coagulase and DNase tests. While locally isolated MRSA and standard MRSA (ATCC strain) produced positive activities with the three enzymes (Fig 9,10,11), the persisters cells showed negative activities with DNase and coagulase enzymes. On the other hand, it showed very weak catalase activity (Fig: 10C).

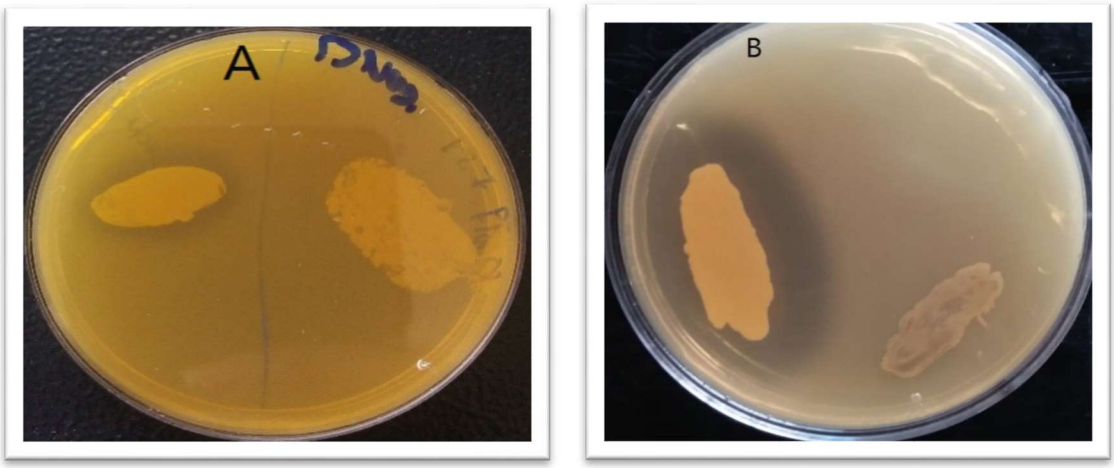

Fig (9): DNase activity test for MRSA and persister cells showed Clear zone around bacterial growth DNase positive in MRSA and DNase negative in persister cells . Panel A: Standard MRSA 43300 which contain DNase and produce clear zone after detected by HCl and negative control no clear zone around growth, Panel B: MRSA isolate have DNAse and its persisters with no clear zone.

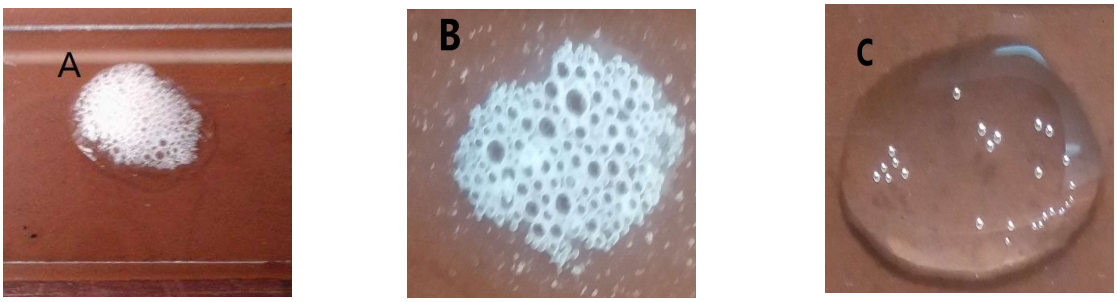

Fig (10): Catalase activity of three types of S. aureus isolates. Panel A: Standard MRSA ATCC43300. Panel B: MRSA isolate showing in the two panels air bubbles appear immediately after adding H2O2, panel C: Vancomycin derived persisters showing few air bubbles appear after about 60 seconds after adding H2O2.

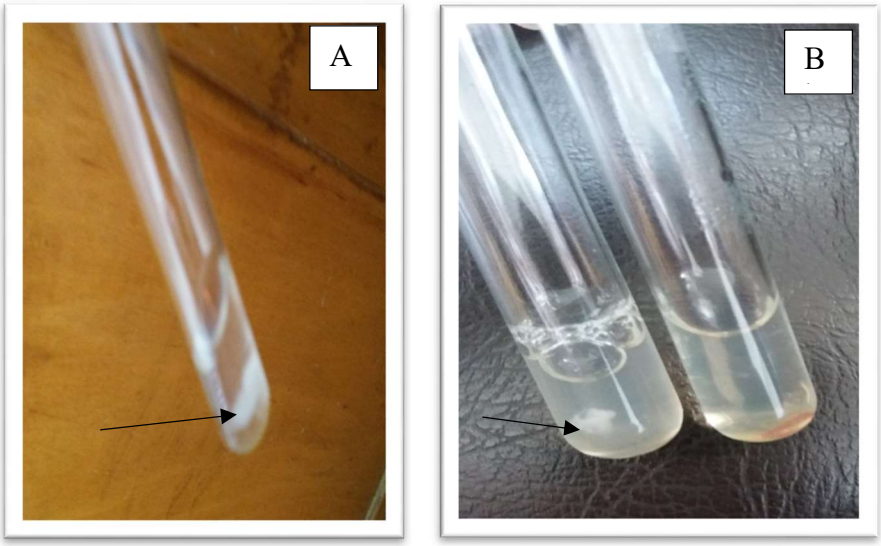

Fig (11): Coagulase test for three types of S. aureus isolates, panel A :MRSA ATCC 43300 , Panel B:MRSA isolate and its persister cells the arrows refer to the coagulation of plasma in Standard MRSA, MRSA local isolate and lack of coagulation of plasma in persister cells.

IV. Discussion:

Producing enough number of persister cells from any bacterium is of highest importance; so that we can study them at bacteriological, physiological and molecular levels. Persistence phenomenon was discovered in bacterial almost eighty years ago; but their implication in infectious diseases and multidrug resistance were sort of mystery [29]. The significance of the work presented here lies in the simplification of the laboratory process to get enough number of persister cells for studying and understanding the mechanisms behind their arousal. The identity of the MRSA, its persister and the mutants was confirmed biochemically; especially their abilities to ferment mannitol sugar, producing black colonies on Baird-Parker agar. Moreover, the abilities of the different isolates to produce biofilms on Congo red medium a prerequisite in virulence of these bacterial cells. The delayed hydrolysis of H_2O_2 and lack of coagulase activity were characteristics of persister cells which are in agreement of work published by [30].

A recent work by **Abokhalil *et al.*, (2020)** discussed the persistence phenomenon in MRSA isolate as a frequent cause of nosocomial infections and chronic recurrent infections. The use of vancomycin in persisters isolation from MRSA was of high importance since it is the last treatment resort of this dangerous infectious bacterial pathogen. And development of resistance to it will have serious health consequences on fighting such infectious pathogen and may be relative pathogens [9].

Raising persister from MRSA in the lab was so simple and according the protocols published by **Ren *et al.*, (2015)[31].** In this context we developed a dose-dependent killing biphasic curve; where rapid killing phase followed by a plateau of survivors. Persistence in MRSA was attributed to a triggered physiological dormancy state by the antibiotics; since this is not a spore forming bacterium. This is meaning a temporarily shutdown of most or all physiological function leading persister cells to be inactive and hence the antimicrobial agent cannot act. The team supporting this hypothesis has present convincing evidences in support of their theory and this study does agree with them. The depended on the same MIC values for persisters and MRSA cells and hence concluded that both are phenotypic variants and hence persisters are not resistant mutants [32]. They also concluded that the slow regrowth of persisters, was consistent with reactivation of the metabolic machinery [33]. The persister cell population showed doubling time in comparison to exponential cells. These observations indicate that persister cell waking is not synchronized. This result indicated that reversion of persisters to normal antibiotic-sensitive cells is very slow, which is consistent with previous results [29].

However, there must be a general signal(s) that transmits the orders for all cellular ports to shut and prevent the antibiotics from entering the cells of deactivate them rapidly. How could that happen without the knowledge of the control master of the cell; the genome? Therefore, the derivatization

of large number of persister cells from MRSA was excellent beginning to facilitate the deciphering of this phenomenon.

The ability of non-sporulating persister derived from MRSA to escape the toxic effect of antibiotics deserves extreme attention, despite the last reports of the drug discovered by Lewis et al [34]. The metabolic inactivity of these cells sounds good but the linkage with functionality of genes is lacking. More questions need answer what triggers the dormancy state of the persisters? How long it can last? What if the antibiotic persists for a week or more? These and similar question should be investigated al physiological, genetic and molecular levels.

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- **Fermentation of mannitol sugar:**

Selected MRSA isolate and persister *S. aureus* strains were inoculated onto mannitol-salt agar (MSA) plates, incubated at 37°C for 24-48 h and changes of pH indicator was observed [19] [25] [26].

- **Congo Red (Biofilm Formation Test):**

Standard MRSA ATCC (43300), MRSA isolate and its persister cells were streaked onto BHIA (Difco) containing 0.08% (w/v) Congo red (Sigma-Aldrich, Germany) and supplemented with 5% (w/v) sucrose. The cultures were incubated at 37°C under aerobic conditions for 24 h. The biofilm producers' staphylococci will form black colonies, while the non-biofilm producer strains formed red colonies [23].

- **Baird-Parker:**

S.aureus is determined by streaking bacterial colonies onto Baird Parker agar plate, incubated at 37°C for overnight. Strains belonging to *S.aureus* bacterium will produce black colonies surrounded by an opaque zone.

- **Blood Hemolysis:**

Standard MRSA ATCC (43300), MRSA isolate and its persister cells were streaked onto Tryptone Soya Agar plate containing blood, incubated at 37°C for overnight and the types of blood hemolysis was scored [22].

- **5- DNase test :**

Standard MRSA isolate and its persister cells were inoculated onto a DNase agar plates containing methyl green complex as an indicator for DNase production. Bacterial growth by observing changes in the indicator color [35].

- **6- Catalase Test :**

From growing colonies of MRSA or its persister cells mount part of the colonies on glass slide and add to each colony a drop of hydrogen peroxide, 3% (H_2O_2) and observe evolution of oxygen bubbles [24, 27].

- **7- Coagulase Test :**

A 1mL human plasma was added to 9mL of sterilized saline water inoculated with MRSA and persister cells. The tubes were then incubated at 37°C for 1.5 hours and watch for coagulation of plasma [36].

- **Oxidase Test**

A small portion of the bacterial colonies from MRSA and persister cells were placed onto moistened filter paper strip with 1% oxidase reagent; N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) and watch for color changes to either blue or purple within 30 seconds [22].