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Characterization of Biofilm production in XDR *Proteus mirabilis* isolated from Baghdad city hospitals

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Abstract

Objective(s): *P. mirabilis* is considered as extensive drug resistant pathogen in many studies as it can resist complex antibiotic regimes, such pathogen can be threat to public health, especially when it has the ability to produce biofilm. Therefore, biofilm production was characterized among XDR *P. mirabilis* local isolates in this research.

Material and Methods: 100 *P. mirabilis* isolated from wound infections from patients admitted to Baghdad hospitals. They were identified using biochemical test and Vitek2 system. The MIC test for antibiotic sensitivity was done by Vitek 2 automated system. Biofilm production was identified phenotypically by twitching motility test, scanning electron microscope, and microtiter plate assay.

Results: It could be revealed that 8/100 isolates were MDR, 90/100 isolates were XDR and pan drug resistance level was shown in only two isolates. 80% of isolates had motile ability through twitching assay, and scan electron microscope study revealed that 76% of XDR isolates could produce different stages of biofilm on coverslip placed in MacConkey broth. Microtiter plate assay revealed 81% of XDR isolates were biofilm producers.

Conclusion: it could be concluded that extensive drug resistant *P. mirabilis* can produce biofilm hence resist several important antibiotics; making treatment of infection among wounded patients is such a challenge in many hospitals in Baghdad.

Key words: XDR, wounds, *P. mirabilis*, and microtiter plate assay, SEM

تشخيص انتاج الغشاء الحيوي في بكتريا *Proteus mirabilis* المعزولة من مستشفيات محافظة بغداد

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الخلاصة:

الهدف من البحث: تعتبر بكتريا *Proteus mirabilis* من مسببات الأمراض المقاومة للأدوية على نطاق واسع في العديد من الدراسات لأنها يمكن ان تقاوم أنظمة المضادات الحيوية المعقدة و يمكن ان يشكل هذا العامل الممرض تهديدا للصحة العامة خاصة عندما يكون لديه القدرة على أنتاج الأغشية الحيوية. لذلك شخص أنتاج الغشاء الحيوي بين العزلات المحلية من النوع XDR *Proteus mirabilis* في هذا البحث.

المواد وطرق العمل: تم عزل 100 عزلة من بكتريا *P. mirabilis* من التهابات الجروح من المرضى الذين أدخلوا الى مستشفيات محافظة بغداد. تم التعرف على هذه العزلات بأستخدام الأختبارات البايوكيميائية و جهاز Vitek2. تم أيضا أجراء اختبار MIC لحساسية المضادات الحيوية بواسطة جهاز Vitek2 . أيضا تم التعرف على قابلية البكتريا لأنتاج الغشاء الحيوي من خلال أختبار الحركة بشكل الوخز Twitching motility assay و المجهر الألكتروني الماسح و كذلك بواسطة فحص microtiter plate.

النتائج: وجد بأنه 100/8 عزلة كانت متعددة المقاومة للمضادات الحياتية, 100/90 كانت شديدة المقاومة للمضادات الحياتية, بينما المقاومة القصوى وجدت فقط في عزلتين. أيضا وجد بأن 80% من العزلات كان لها القابلية على الحركه بشكل الوخز و 76% من العزلات الشديدة المقاومة للمضادات الحياتية استطاعت تكوين مراحل مختلفة من أنتاج الغشاء الحيوي على الشريحة المقطعية

الموضوعه في مرق MacConkey. فحص microtiter plate أظهر 81% من العزلات هي منتجة للغشاء الحيوي.
الأستنتاج: يمكن أستنتاج ان هذه البكتريا المقاومة للمضادات الحياتية على نطاق واسع يمكن أن تنتج غشاء حيوي و بالتالي ستؤدي الى مقاومة العديد من الأدوية المهمة و بالتالي جعل علاج العدوى بين الجرحى تحديا كبيرا في العديد من مستشفيات العاصمة بغداد.
الكلمات المفتاحية: XDR ,الجروح, *P. mirabilis* ,microtiter, المجهر الالكتروني الماسح.

1. Introduction:

Wounds are defined as the disruption of any soft region of a body, and could be infected depending on different factors (1). Microorganisms like fungi, parasite, and bacteria usually invade wounds and cause infections due to proliferation and colonization in any tissue generating damage and pus (2). Gram negative bacteria are such examples of invading microorganisms (3). *P. mirabilis* is one of *Enterobacteriaceae* family that can cause different diseases, and it is usually recovered from urinary and wound infections (4). It could be shown that this bacterium resist several antibiotics like tigecycline, colistin, aminoglycoside, carbapenems, fosfomycin and naturally resist polymyxin B (5) ending in extensively drug resistant conclusion which make it hard to treat infections caused by such a pathogen (6 and 7). This bacterium shows swarming motility that aid in moving across the medium; therefore, when noticing swarming in wound or urinary infections, *P. mirabilis* is suspected (8). It is reported that *P. mirabilis* can produce biofilm in many studies, especially when colonize in catheter surfaces and wounds (9). Biofilm production usually needs many steps that start with attaching single bacteria to substratum until making the mushroom like architecture. Bacteria when making biofilm produce different extracellular substances such as quorum sensing molecules (AHL) (10), polysaccharides, proteins, and extracellular DNA, etc. (11). To have a big picture of normal flora *P. mirabilis* pathogenicity, it would be crucial to study the virulence factors of this bacterium and how it could initiate infection (12). XDR *P. mirabilis* obtained locally from Baghdad hospitals were tested to reveal their ability to form biofilm, initiating and proceeding infections.

2. Material and Methods:

2.1 Specimens: 100 *P. mirabilis* isolates were collected from May 2019 to January 2020 from different wound infections such as burns, trauma, and post-surgery of patients admitted to hospitals in Baghdad city. Isolates were identified using biochemical tests and Vitek 2 system. All isolates were 99% *P. mirabilis*.

2.2. Antibiotic sensitivity test

MIC of antibiotic sensitivity was performed using Vitek 2 system for the following antibiotics (break point of 2µg/ml): Ampicillin, Piperacillin, Cefixime, Cefotaxime, Cefepime, Amoxicillin/clavulanic acid, Ticarcillin/ clavulanic acid, Aztreonam, Imipenem, Meropenem, Kanamycin, Gentamycin, Levofloxacin, Gatifloxacin, Trimethoprim and Trimethoprime Sulphamethoxazole, Tetracyclines, Tigecycline, colistin sulphate and Polymyxin B.

2.3 Biofilm production assays

2.3.1 Twitching motility assay: the medium included M63 medium, CAA and glucose (20% each), $MgSO_4$ then solidified with 1.5% agar- agar. Each isolate was stabbed in the center of plates containing 3mm media [LB+M63 media] (13). Plates then incubated at 37ºC/ 24 hr. Twitching motility zones were noticed by visualizing using crystal violet stain prepared in lab and comparing with control: *Pseudomonas* PA14 as positive and *Pseudomonas* ΔPel A mutant as negative. Isolates were put into three groups as revealed in literature (13 and 14) [negative non-motile (<5 mm), intermediate (5-20mm), and positive motile (>20 mm)].

2.3.2 Scanning Electron microscopy SEM: MacConkey broth tubes were used with sterile cover clip embedded inside. Isolates were cultured into these tubes and labeled with the date of starting procedure and then incubated for 37˚C/24 hr. After twenty four hours, cultures were sat horizontally for 15 days at 25˚C. Then samples of SEM were prepared as described by Aziz and Al-Jubori (15) and as follows:

1- Cover clips were removed carefully from MacConkey broth keeping their horizontal level.
2- Fixed with glutaraldehyde 2.5% for 2 hours
3- Washed with D.W
4- Samples then coated with Au (80): Pd (20). Diameter was 57mm and thickness was 0.1mm
5- Images were taken by scanning electron microscope [5µm to 2mm]

2.3.3 Microtiter Dish assay: Process was done as described by Aziz and Al-Jubori (15) and the same standard strains were used. Standard stains of *Pseudomonas* (P14) (OD 0.35) was used as positive control, while negative control was *Pseudomonas* ΔPel A mutant (OD 0.051). Isolates having optical density more than 0.35 were considered as strong biofilm producers, and when they had less than 0.051, they were considered as non-producers, but if they had in between these two values, they were classified as weak biofilm producers. Absorbance was tested for each triplicate isolates using ELISA system at 550nm with adding acetic acid concentrated at 30% in distilled water that used as blank control.

3. Results:

3.1 Antibiotic sensitivity tests: Most of isolates were extensive drug resistant except of eight isolates were MDR and two isolates were PDR, consequently they were excluded from this study ending in 90 XDR *P. mirabilis* isolates to be tested in consequent assays (figure1)

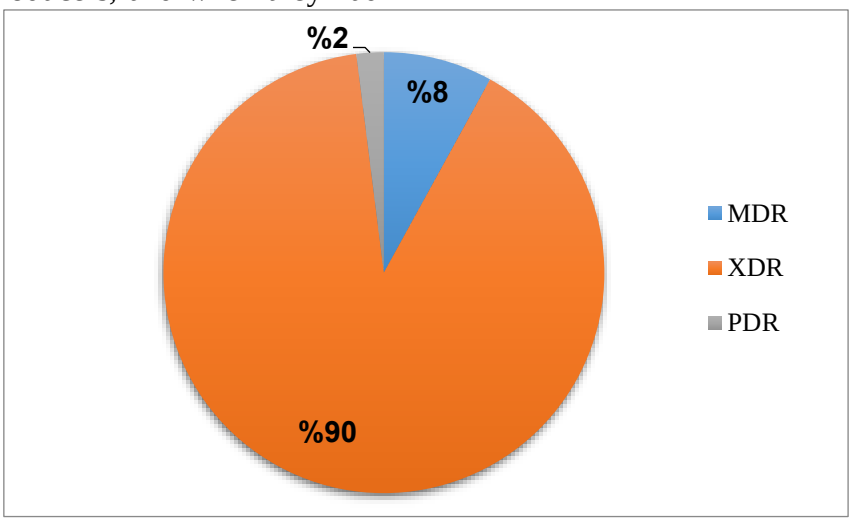

Figure 1: Antibiotic resistance levels among 100 P. mirabilis isolates.

3.2 Twitching motility assay: Results (figure 2) showed 80% of *P. mirabilis* had high ability to motile and the migration zone was more than twenty millimeters (figure 3). Five percent of isolates exhibited migration zone at range of 5-20mm revealing intermediate ability to motile. None motile isolates which showed less than 5 mm of migration zone were 15%. It is interesting to note that five isolate *P. mirabilis* W47, W37, W73, W82, and W21 showed range of 28-30mm migration zone, indicating highly biofilm production (figure 4).

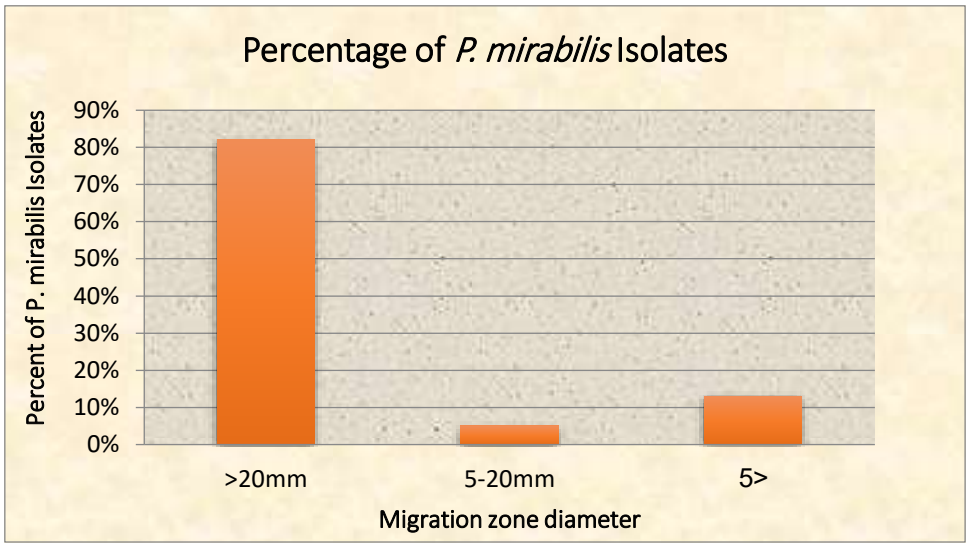

Figure 2, show the percentage of *P. mirabilis* isolates that could motile compared with non-motile isolates

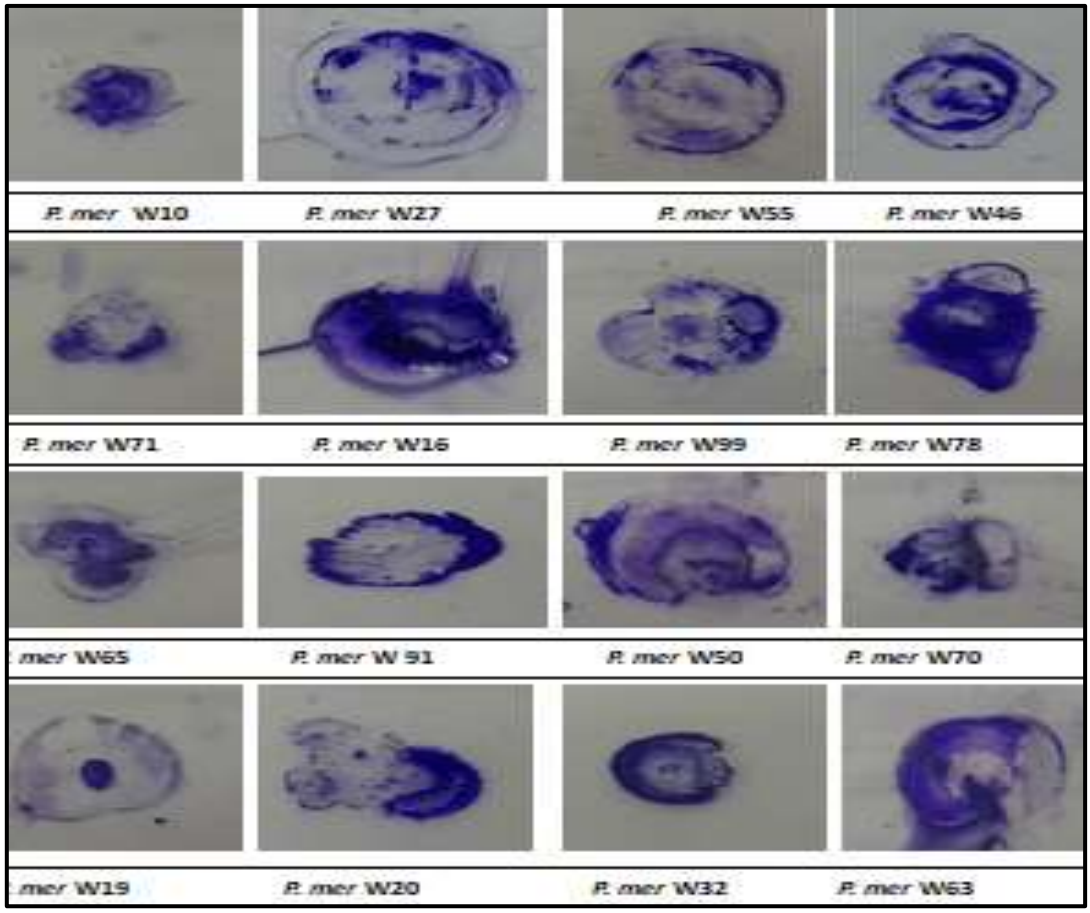

Figure 3: *P. mirabilis* isolates had migration zone >20mm in twitching motility assay.

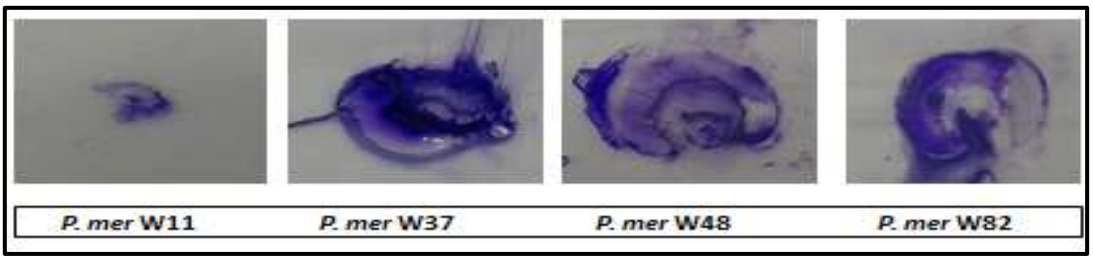

Figure 4: *P. mirabilis* isolates W37, W48, and W82 had migration zone of (28-30mm) compared with isolate W11 that was negative (<5mm).

3.3 Scanning Electron microscopy SEM: studies showed around 76% of isolates obtained from wound infections could produce different steps of biofilm on glass cover clip liquid interface after 15 days. It could be visualized planktonic cells, mat dense of aggregate cells of *P. mirabilis* embedded in extracellular substances, and even final architecture (figure 5). Highly magnification images revealed condense monolayer formed by XDR *P. mirabilis* W82 that produce a large amount glycocalyx that was generated by the interaction between MacConkey medium components and extracellular substances produced by bacteria (figure 5 A). Microcolonies of isolates were also noticed under SEM revealing the second stage of biofilm production (figure 5B). It was interesting that final stage of biofilm formation was abundant for XDR *P. mirabilis* W82 and 48 (figure 5C and D, respectively). These results proved that isolates which were motile in the previous assay could produce different biofilm stages.

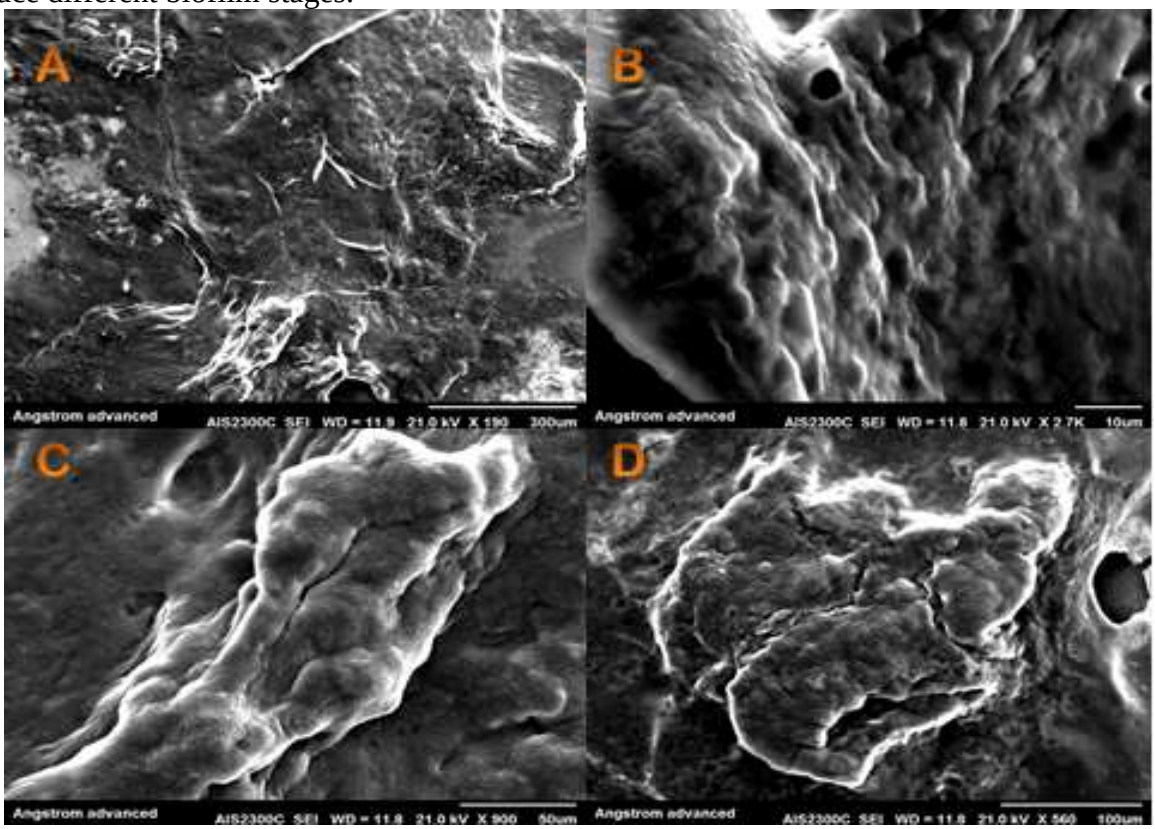

Figure 5: Biofilm of XDR *P. mirabilis* W82 and W48 on glass cover slip obtained by scanning electron microscope; A) large amount of extracellular substances produced by bacteria, (x300µm), B) Microcolonies performed by XDR *P. mirabilis* W82 of isolates (x10µm) C) Final stage of biofilm for XDR *P. mirabilis* W82 (x50 µm), and D) for W48 (100 µm).

3.4 Microtiter plate assay: 81% of *P. mirabilis* isolates (figure 6) showed to be biofilm producers, comparing with control strains; however, some were either weak or strong producers. It was interesting that W82, W48, W21, W73, and W37 had high range of optical density of OD≥0.80-0.89 compared with other positive isolates (figure 7).

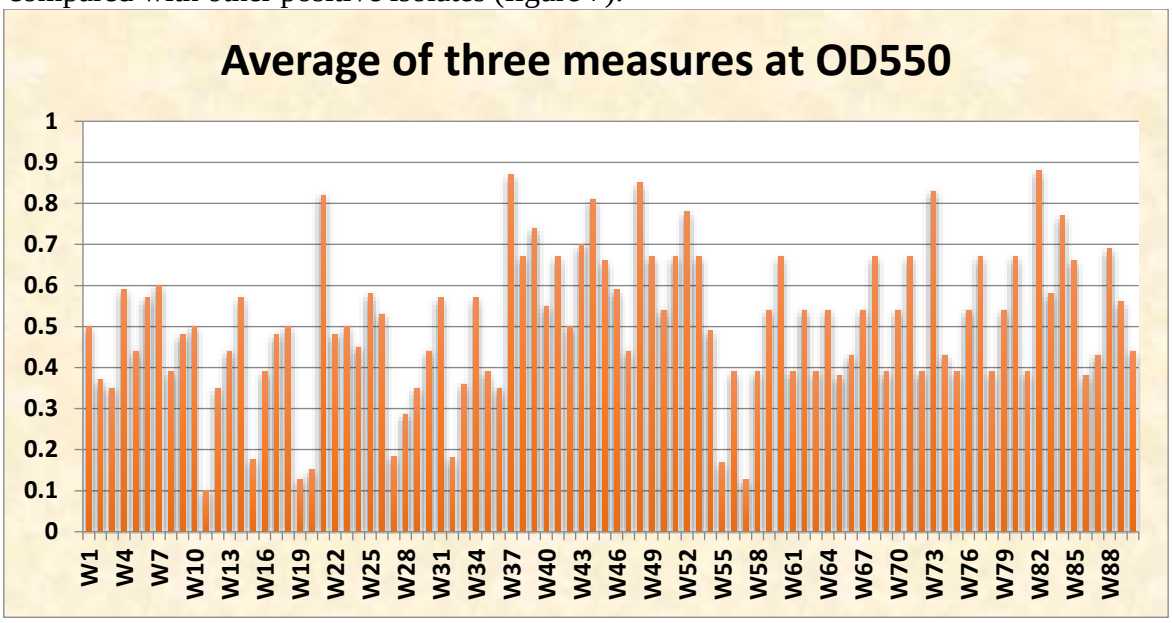

Figure 6: 90 XDR *P. mirabilis* isolated from wound infections; biofilm producers' readings at OD550nm by ELISA.

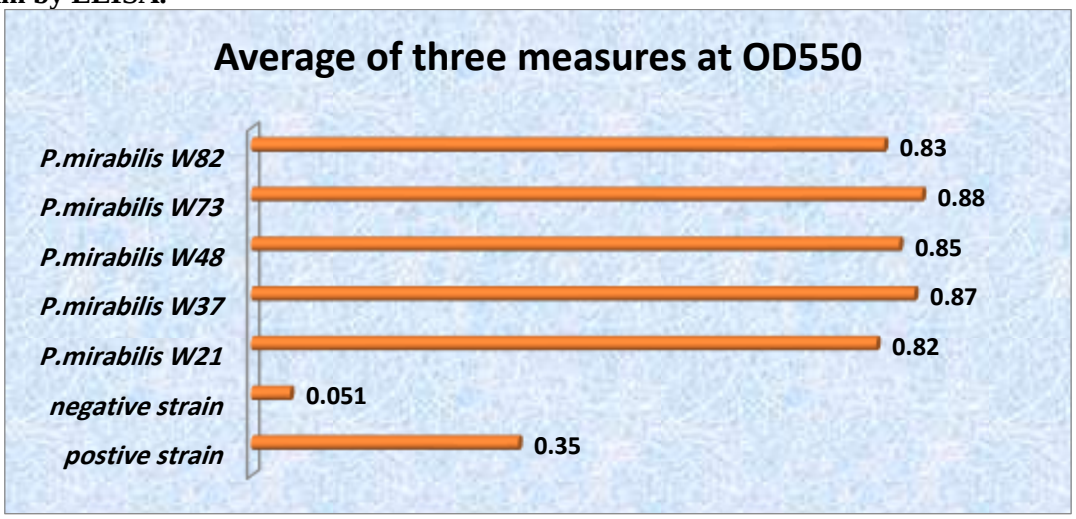

Figure 7: XDR *P. mirabilis* W21.W37, W48, W73, and W82 readings at OD550nm compared with standard strains.

4. Discussions: A Hundred *P. mirabilis* isolates were collected and tested for antibiotic sensitivity pattern using Vitek2 automated system as it is evaluated by many studies in literature (16; 17, and 18). Results obtained showed that 90 isolates were extensive drug resistant, while MDR and PDR were appeared among the last ten. This indicating that local *P. mirabilis* isolated from wounds resemble many studies showed increasing antibiotic resistance levels among such bacteria (19). Gabr *et al.* (12) reported that high percentage of extensive and multidrug resistant *P. mirabilis* were isolated from UTI patients in Egypt, and concluded that it must use new antibiotic regime to treat this opportunistic bacterium. Similar study was also done by Philips *et al.* (20) who showed public threat against MDR *P. mirabilis* infections found in wounds.

Results of twitching motility assays showed that 80% of *P. mirabilis* had high

ability to motile and migrate for more than twenty millimeters, especially W48, W37, W73, W82, and W21 isolates which migrated for 28-30mm. These results indicating high production level of biofilm. Gabr *et al.* (12) showed similar results where they found that 100% of isolates they tested were motile on semisolid twitching motility media assay. However, other study reported 33% of *P. mirabilis* isolates were non motile and exhibited only two to three millimeters traveling on media and the rest of their isolates had intermediate twitch moving, which resemble our 15% and 5% *P. mirabilis* isolates moving pattern (21). Fusco *et al.,* (22) showed that P. mirabilis isolated from UTI patients could associate with anti-inflammatory response from host cells and could have high level of moving around the medium. Study revealed that *P. mirabilis* could highly attached to biotic and non-biotic surface and produce crystalline when initiating biofilm formation, resulting in initiating colonization in urinary tract promoting infection (22, 23, and 24).

Scanning electron microscopy showed that most *P. mirabilis* isolates tested in this study were strong biofilm producers after 15 days. The isolates *P. mirabilis* W82 and W48 showed different stages on glass coverslip proving their results from twitching motility assays and microtiter dish assay that showed these isolates as highly twitched and biofilm producers. Many studies have relied on EM to identify biofilm formation stages for this bacterium (25). *P. mirabilis* was showed to perform adhesion process after only twenty four hours and after four days; all scaffold layer was covered with this bacterium surrounded by large amounts of additional material (25). Research reported that after 24 hours bacterial cell started to attach to the surface, initiating biofilm production (26). It was also found that this bacterium could produce large cluster in UTI patients leading to kidney stone (27). It was shown that *P. mirabilis* could perform biofilm in burn wounds when mixing with other microorganisms (28). Sabbuba *et al.,* (29) reported that *P. mirabilis* can perform biofilm easily in catheterized patients' bladder and can block it.

Microtiter plate assays revealed that 81% of our local isolates were highly biofilm producers in the range of 0.35-0.88 OD at 550nm. It was interesting that W82, W48, W21, W73, and W37 had high range of optical density of OD≥0.80- 0.89 compared with other positive isolates. Results resemble what was found by Czerwonka *et al.,* (30). They found that *P. mirabilis* isolated from UTI had high optical density at 600nm using microtiter dish assay, and they could adhere on glass and polypropylene initiating biofilm. Many studies were done using this assay to screen for biofilm production among this bacterium (31, 32, 33, and 34). It is studied that 100% of P. mirabilis isolated from catheterized patient in Alanbar province in Iraq were biofilm producers when using microtiter dish assay [brain heart infusion broth, 0.2% glucose, and 37°C/48hr] at OD 550= 0.7 as a maximum biofilm formation ratio (33). In addition, Sahal and Bilkay (34) revealed that 13% of 15 *P. mirabilis* isolates from different clinical samples were found to be strong biofilm formers using 1% crystal violet in 96- wells of microtiter dish assay. They also reported that multidrug resistant isolates were stronger in biofilm production than non-resistant bacterium (34). Rajivgandhi *et al.,* (35) also tested P. mirabilis isolates ability to form biofilm at OD 540nm and reported 16.6 % isolates were strong biofilm producers, 33.3% were intermediate, and 46.06% isolates were weak biofilm formers.

5. Conclusion

As revealed in this study, XDR *P. mirabilis* isolated locally from wound infections from different Iraqi hospitals could resist wide range of antibiotic categories used in market besides their ability to be stronger biofilm producers as tested by different phenotypic methods, leading to be public threat when infected with such pathogen. It has to think how to eradicate this pathogen before developing biofilm and become resistant.

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