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Biofilm Formation And Quorum Sensing lasRGene Of Pseudomonas Aeruginosa Isolated From Patients With Post-Operative Wound Infections

Marwa Fotouh Mahmoud[1], Fayza Mahmoud Fathy[2],Maha Kamal Gohar[2], Wael Mahmoud Awad[4], Manar Hassan Soliman[2]

[1]Microbiology and Immunology Department, Al-Ahrar Teaching Hospital, Zagazig, Egypt.
[2,3,5]Medical Microbiologyand Immunology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt.
[4]General Surgery Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt

Corresponding Author:
Marwa Fotouh Mahmoud
M.B.B.CH., M.Sc., Faculty of Medicine- Zagazig University, Assistant Specialist of Microbiology& Immunology, Al-Ahrar Teaching Hospital, Zagazig, Egypt.
E-mail: marowa.fotouh@yahoo.com

ABSTRACT

Background: Pseudomonas aeruginosa (P.aeruginosa) is one of the most commonbacteria capable of forming biofilms which are important in the establishment of P. aeruginosa infections on different host tissues includingpostoperative wounds. P. aeruginosa possesses at least two well-defined, interrelated QS systems, las and rhl that control the production of different virulence factors including biofilm development.Objectives: To determine the antimicrobial susceptibility pattern, biofilm producing ability and the presence of QS lasR gene in isolated P. aeruginosa strains from patients withpost-operative wound infections.Methodology:The study was conducted on Fifty-four clinical isolates of P. aeruginosa from infected postoperative wounds in patients admitted in Zagazig university hospitals, Egypt. Isolates from all patients were tested for antimicrobial susceptibility using disk diffusion method, in vitro formation of biofilm in microtiter plates containing Tryptone Soya Broth (TSB) medium and detection of QS lasR gene using conventional PCR technique. Results: P. aeruginosa isolates showed high prevalence of resistance against ceftazidimeand aztreonam (74.1%), while they showed the lowest resistance to ceftolozan/tazobactam (7.4%). Biofilm formation was detected in 38 (70.4%) of P. aeruginosa isolates; 14.8%, 46.3% , 9.3% and 29.6% of isolates were strong, moderate, weak and non-biofilm producers, respectively. The lasR gene was detected in 42 (77.8%) of P. aeruginosa isolates. There was significant relation between biofilm formation and presence of lasR gene. Conclusion:Postoperative wound infection may serve as a reservoir for multidrug resistant biofilm forming P. aeruginosa. The QS lasR gene is strongly associated with biofilm formation,which can help in identifying lasR gene as a useful diagnostic marker for biofilm producing P. aeruginosa strains isolated from infected wounds.

Key words:Pseudomonas aeruginosa, biofilm, lasR gene,wound infections

1. INTRODUCTION

Post- operative wound infections by multi-drug resistant (MDR) microorganisms are a global threat among the nosocomial infections leading to higher treatment expenditure, longer hospital stay, morbidity and mortality [1,2]. As the skin constitute the first line of defense in human body, an injury to the skin can act as a portal of entry of pathogenic as well as opportunistic pathogens. The development of wound infection depends on the protective function of the skin which is a barrier of wound healing. Being most favorable site for biofilm formation, the wounds are considered as very high risk point for MDR microorganism infections. Post-opetative wound infection is universal and the bacterial types present vary with geographic location, bacteria residing on the skin, clothing at the site of wound and time between wound and examination [3]. Generally, the most commonly isolated MDR microorganisms from wounds are *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus*, *Escherichia coli*,*Acinetobacter spp.*and *Klebsiella pneumonia* [4].Though *P. aeruginosa* is an opportunistic pathogen, it is one of the most clinically significant organisms because of its multiple drug resistance properties, biofilm formation and production of several virulence factors such as exotoxin A, protease, leukocidin, lipopolysaccharide (LPS), phospholipase and other enzymes. The deadlines of *P. aeruginosa* is observed in post-operative wounds, burn wounds, chronic wounds and cystic fibrosis patients [5]. Biofilms are estimated to be responsible for over 65% of nosocomial infections[6], and 60% of all human bacterial infections[7]. Bacterial biofilms may impair cutaneous wound healing and reduce topical antibacterial efficiency in treating infected skin wounds[8].Biofilm formation occurs as a result of a sequence of events: microbial surface attachment, cell proliferation, matrix production and detachment[9].*P. aeruginosa* is one of the most commonly studied bacteria capable of forming biofilms [10]. Biofilm formation is important in the establishment of *P. aeruginosa* infections on different host tissues[11, 12]as well as different medical devices[13]. In these settings, the antibiotic resistance engendered by biofilms presents a serious challenge to the treatment of chronic *P. aeruginosa* infections[14].

Biofilm formation by *P. aeruginosa* involves the cell-to-cell communication quorum-sensing (QS) systems. QS is a cell-density-dependent mechanism through which bacteria coordinate different activities, including bioluminescence, plasmid conjugation and the production of different virulence factors [11,15,16.]*P. aeruginosa*possesses two well-defined, interrelated QS systems, las and rhl that control the production of different virulence factors. Each QS system consists of two components, the autoinducer synthases (lasI and rhlI, respectively) and their cognate transcriptional regulators (lasR and rhlR, respectively). LasI is the synthase for the autoinducer N-(3-oxododecanoyl) homoserine lactone (3OC12-HSL), while rhlI synthesizes the autoinducer N-butyryl homoserine lactone (C4-HSL) [15,16]. The two QS systems of *P. aeruginosa* are hierarchically linked. The las system positively regulates the expression of rhlR and rhlI[17,18,19]. *P. aeruginosa* also possesses an additional signaling molecule, 2-heptyl-3-hyroxy-4-quinolone (PQS). The production and activity of PQS is dependent on lasR and rhlR[20]. It has been suggested that in *P. aeruginosa*, QS is involved in both the initiation of biofilm formation and the maturation of the biofilm. The las QS system appears to be especially important during the late stages of biofilm development[21].This study aimed to determine the antimicrobial susceptibility pattern, biofilm producing ability, and the presence of Quorum sensing lasRgene in

isolated *Pseudomonas aeruginosa* strains from patients with post-operative wound infections at Zagazig University Hospitals, Egypt.

2. METHODOLOGY

Clinical isolates:

Fifty-four non repeat clinical isolates of *P. aeruginosa* were obtained from patients with post-operative infected wounds at Zagazig University Hospitals, Egypt. The isolates were collected within the period from December 2017 tillMarch 2020. *P. aeruginosa* strains were isolated and identified based on standard microbiological techniques[22].

Antimicrobial susceptibility testing:

Antibiotic susceptibility of all isolated strains was done by disc diffusion method (discs supplied by Oxoid, UK and Liofilchem, Italy), using Muller-Hinton agar plates (Oxoid, UK). After overnight incubation, results were reported and interpretation was done according to CLSI[23]. ceftazidime,cefepime, aztreonam, levofloxacin, amikacin,imipenem, meropenem, ceftazidime/avibactam and ceftolozan/tazobactam were used for the antibiotic susceptibility testing.

Biofilm formation and quantification:

The tissue culture plate assay (TCP) is the most widely used and is considered as a standard test for detection of biofilm formation. The assay is based on the colorimetric measurements of the crystal violet incorporated by sessile cells[24].Overnight cultures of tested strains of *P. aeruginosa* were inoculated into 5 ml of trypticase soya broth (TSB) and incubated at 37°C for 24 hours. The turbidity was then adjusted to that of 0.5 McFarland standard.200 µl of previously prepared suspensions were added to the wells of sterile 96-well flat bottomed polystyrene microtiter plates. Each strain was tested in triplicate (three wells per strain) and three wells in each plate were used as negative control (the negative control wells contain broth only: 200 µl of TSB), then the plates were covered with lids and incubated aerobically at 37°C for 24 hours.After incubation, the bacterial suspension of each well was gently removed. The wells were washed three times with 200 µl of phosphate buffered saline (PBS) to remove free-floating planktonic bacteria, and then the wells were air dried for 45 min.Adherence of bacteria to the culture plate was detected by crystal violet, in which 200 µl of (0.1%) crystal violet was added to each well and the plates were incubated at room temperature for 10 min. Excess stain was rinsed off by washing with deionized water (washed 3 times with 300 µl of water) and plates were kept for drying 20 min. After drying, 200 µl of 95% ethanol was added to the wells to solubilize the incorporated dye, the plate was covered with the lid (to minimize evaporation) and left at room temperature for 30 min. The optical densities (ODs) of the stained adherent bacteria were determined with a microplate reader at 600 nm.The average OD values were calculated for all tested isolates & negative control, the cut-off value (ODc) was detected. It is defined as a 3 standard deviations (SD) above the mean OD of the negative control: ODc = average OD of negative control + (3xSD of negative control). Final OD value of a tested strain was expressed as average OD value of the strain reduced by ODc value (OD= average OD of a strain-ODc); ODc value was calculated for each plate separately. When a negative value was obtained, it was presented as zero, while any positive value indicated biofilm formation. Strains were divided into the following categories;non biofilm producers = OD ≤ODc,weak biofilm producers = ODc <OD

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≤2×ODc, moderate biofilm producers = 2×ODc <OD≤4×ODc and strong biofilm producers = 4×ODc <OD[25](figure 1).

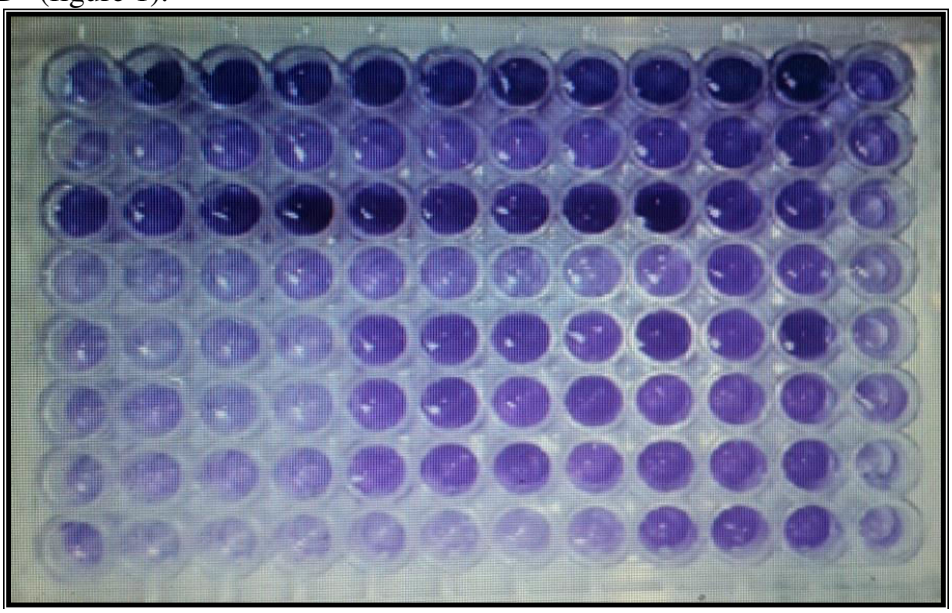

Fig. 1:Microtiter plate showing different grades of biofilm formation.

PCR for detection of the quorumsensing lasR gene:

DNA was extracted from tested *P.aeruginosa* isolates, one colony of each strain cultured on solid medium was inoculated into 5 ml of TSB medium.After overnight incubation at 37°C; DNA was extracted from these cultures utilizing DNA extraction kit (Thermo FisherScientific, USA) according to manufacture instructions.PCR amplification was carried out using thermal cycler (Biometra, Germany) with specific primers for lasR gene[26](table 1).PCR was performed in 25 µl of reaction mixture containing 2 µlof bacterial DNA template, 12.5 µlDream Taq PCR Master Mix (Thermo Fisher Scientific, USA), 100 pmol (1µl) of each primer and 8.5 µL distilled water was added to bring the final volume to 25 µl. PCR conditions were as follows: initial denaturation at 94°C for 1 min followed by 34 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 2 minutes, and a final extension at 72°C for 10 minutes [27]. Agarose gel electrophoresis was used for examining the amplified products; PCR products were run on 2% agarose gel, stained with ethidium bromide visualized under UV transilluminator (Cole-Parmer, USA) and photographed [28].

Table 1: Primer sequence used in the study.

Gene	Primer direction	Primer sequence	Amplicon size (bp)
LasR	Forward Reverse	5′ aagtggaaaattggagtggag3′ 5′gtagttgccgacgacgatgaag 3′	130

Statistical analysis:

Data were analysed using SPSS, version 25 (SPSS Inc., Chicago, IL) using Descriptive statistics and Chi-square test. Descriptive statistics including number and percentage for qualitative variables and mean and standard deviation for quantitative one. Chi-square test was used for analysis of qualitative variables and P-values were calculated. A P-value <0.05 was considered as statistically significant.

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3. RESULTS

This study was conducted on fifty four clinical isolates of *P.aeruginosa* from post-operative wound infected patients admitted in Zagazig University Hospitals, Egypt in the period from December 2017 till March 2020.

Antibiotic susceptibility of P. aeruginosa isolates:
P. aeruginosa isolates showed high prevalence of resistance against ceftazidimeand aztreonam 40 strains (74.1%) and31 (57.4%) strains were resistant to cefepime,while 25 (46.3%) strains were resistant to levofloxacin, 17 (31.5%) strains were resistant to amikacin, 16 (29.6%) strains were resistant to imipenem, 12 (22.2%) strains were resistant to meropenem,5 (9.3%) strains were resistant to ceftazidime/avibactam and 4 (7.4%) strains were resistant to ceftolozan/tazobactam (table 2). 41 (76 %) of *P. aeruginosa* isolates were MDR(figure 2).

Table 2: Antibiotic susceptibility results of P. aeruginosa isolates.

Antimicrobial agent	Concentration (µg)	Rssistant No. (%)	Intermediate No. (%)	Sensitive No. (%)
Cephems				
Ceftazidime	30 µg	40 (74.1%)	3 (5.6%)	11 (20.4%)
Cefepime	30 µg	31 (57.4%)	9 (16.7%)	14 (25.9%)
Monobactams				
Aztreonam	30 µg	40 (74.1%)	0 (0)	14 (25.9%)
Fluoroquinolones				
Levofloxacin	5 µg	25 (46.3%)	3 (5.6%)	26(48.1%)
Aminoglycosides				
Amikacin	30 µg	17 (31.5%)	0 (0)	37(68.5%)
Carbapenems				
Imipenem	10 µg	16 (29.6%)	0 (0)	38(70.4%)
Meropenem	10 µg	12 (22.2%)	0 (0)	42 (77.8%)
B-Lactam combination agents				
Ceftazidime/avibactam	30/20 µg	5(9.3%)	0 (0)	49(90.7%)
Ceftolozan/tazobactam	30/10 µg	4(7.4%)	0(0)	50(92.6%)

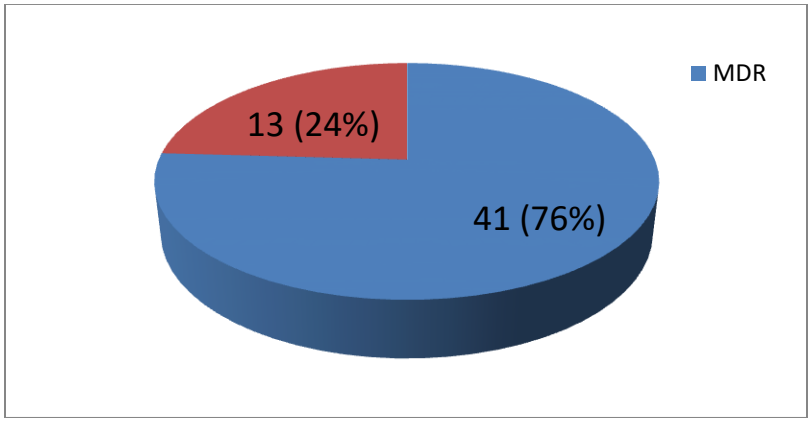

Fig.2: Multidrug-resistant isolates among *P. aeruginosa* (No. =54).

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Biofilm formation:

Out of 54 *P. aeruginosa* isolates; 38 (70.4%) isolateswere biofilm producers; 8 isolates (14.8%) were strong biofilm producers, 25 (46.3%) were moderate biofilm producers and 5 (9.3%) were weak biofilm producers, whereas 16 (29.6%) isolates were non biofilm producers(figure 3).The association between the MDR and biofilm formation is statistically highly significant. Out of 38 biofilm forming isolates, 34 (89.5%) were MDR positive while 4 (10.5%) were MDR negative. Regarding 16non biofilm forming isolates, 7 (43.8%) were MDR positive while 9 (56.2%) were MDR negative (figure 4).

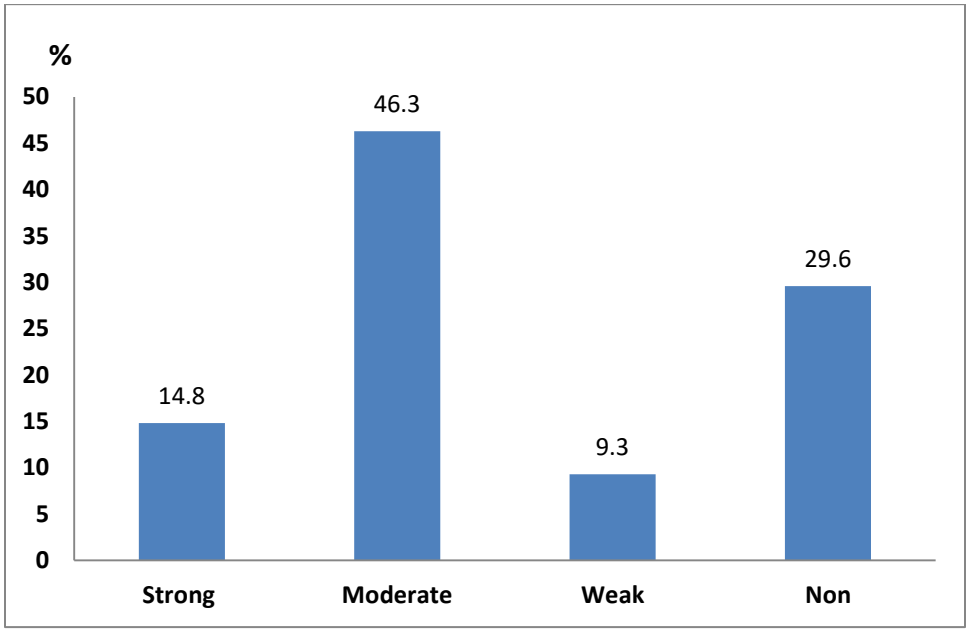

Fig.3: Degrees of biofilm formation of *pseudomonas aeruginosa* isolates (No. =54).

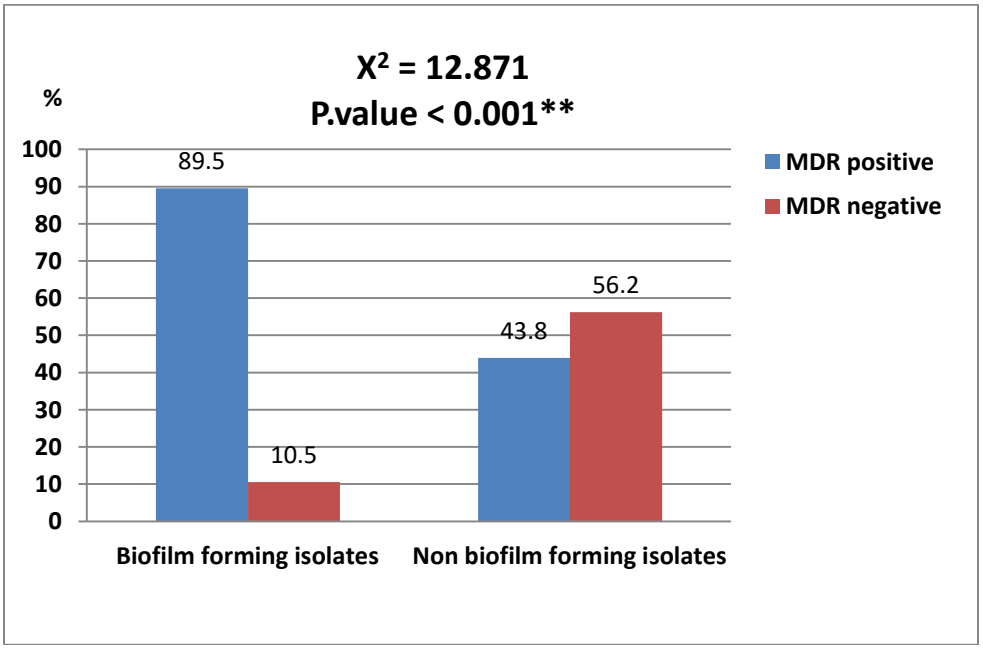

Fig. 4: MDR detection among biofilm forming and non-biofilm formingisolates.

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PCR results:

The lasRgene was detected in 42 (77.8%) out of 54*P. aeruginosa* isolates collected, while 12 (22.2%) isolates did not harbor the gene (figure 5&6). LasR gene was detected in all biofilm forming isolates (100%) and in only four (25%) of non-biofilmformingisolates. There was highly significant relation between biofilm formation and detection of lasR gene (figure 7).

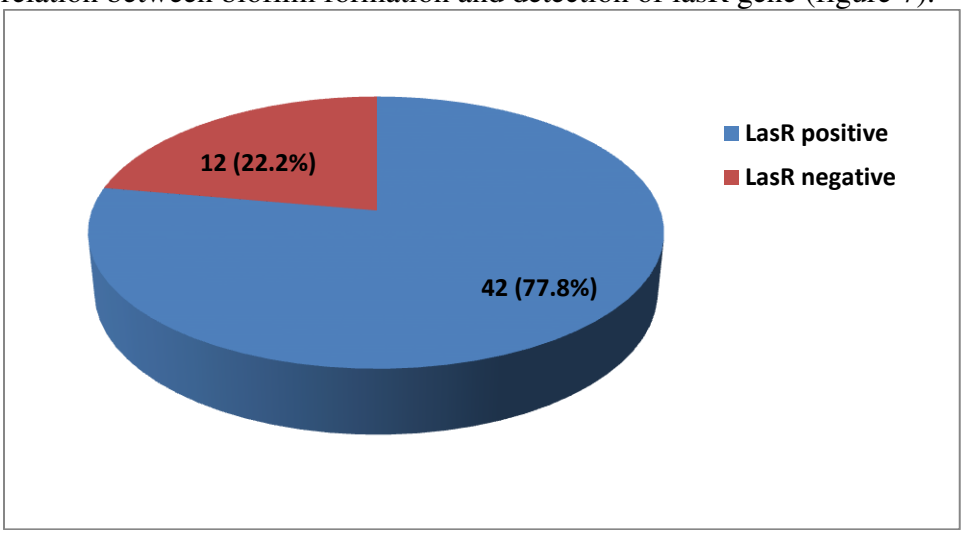

Fig.5: LasR gene detection among the 54 studied *P. aeruginosa*isolates.

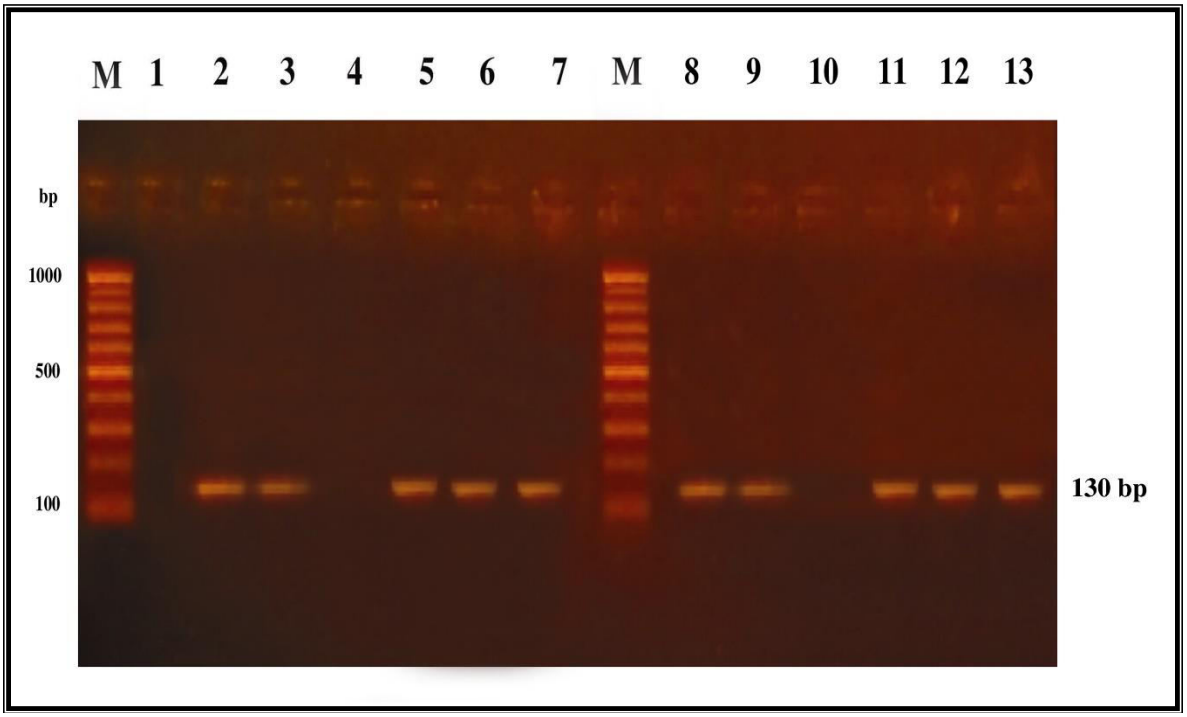

Fig.6: Agrose gel electrophoresis for lasR gene in *P. aeruginosa*isolates.

Lane M: Molecular size marker which gave 10 bands ranging from 100-1000 bp.
Lane 1: Negative control (no bands).

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Different strains of *P. aeruginosa* with lasR gene products detected at 130 bp, strains 4 and 10 are negative for lasR gene

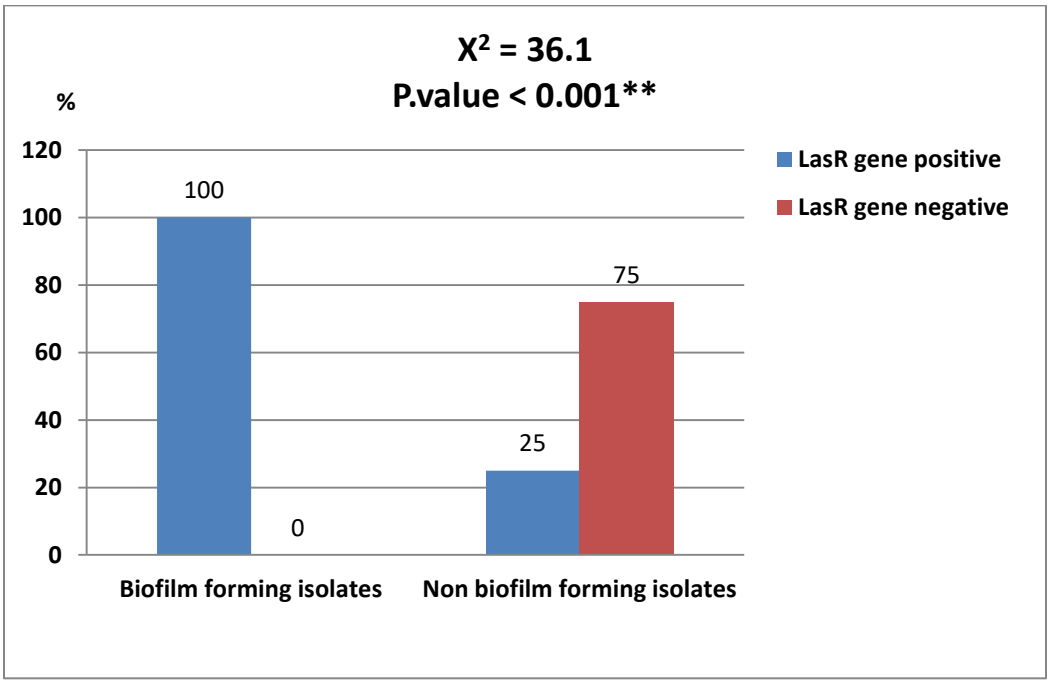

Fig.7: LasR gene detection among biofilm forming and non-biofilm formingisolates.

4. DISCUSSION

Bacterial communication via quorum sensing (QS) has been reported to be important in the production of virulence factors, antibiotic sensitivity, and biofilm development. Two QS systems, known as the las and rhl systems have been identified previously in the opportunistic pathogen *P. aeruginosa*[29,30].

This study included 54 isolates of *P. aeruginosa* from post-operative wound infected patients admitted in Zagazig University Hospitals, Egypt.In the period from December 2017 tillMarch 2020.

As regards antibiotic susceptibility results in this study, *P. aeruginosa* isolates showed high prevalence of resistance against ceftazidime (74.1%),aztreonam (74.1%) and cefepime (57.4%). The obtained findings are probably due to the wide use of these antibiotics in the hospitals. Hashem et al.[31]in Egypt reported similar results where 75.5% and 66% of isolates were resistant to ceftazidime and cefepime respectively. Abaza et al. [32] in Egypt recorded a high level of resistance to ceftazidime 80%, aztreonam 85.7% and cefepime 79.4%. Another study in Egypt also showed a resistance rate of 68.1% to each of ceftazidime and cefepime and 57.4% to azetronam [33].In contrast, El-Ageery and Al Otibi [34]in Saudi Arabia, reported that 33.3% of *P. aeruginosa* isolates were resistant to ceftazidime and cefepime and 29.6% were resistant to aztreonam. Regarding levofloxacin and amikacin in this study; 46.3% and 31.5% of *P. aeruginosa* isolates were resistant respectively. Elmaraghy et al. in Egypt reported similar results where 46.8% and 36.2% of isolates were resistant to levofloxacin and amikacin respectively [33]. Saleem and Bokhari [35]in Pakistan also showed similar results, as they reported that 37.2% of *P. aeruginosa* isolates were resistant were resistantto amikacin.A higher incidence was detected by

Yekani et al. [36]In Iran who reported that 68.02% and 55.48% of their *P. aeruginosa* isolates were resistant to levofloxacin and amikacin respectively.One of the alarming results is the resistance against carbapenems; where 29.6% of isolates were resistant to imipenem and 22.2% were resistant to meropenem. In agreement with our results, Elmaraghy et al. [33]revealed that 14.9% and38.3 % of *P. aeruginosa* isolates were resistant to imipenem and meropenem respectively. A higher incidence was detected by Abaza et al.[32] in Egypt who reported that 78.3% and 73.7% of their *P. aeruginosa* isolates were resistant to imipenem and meropenem respectively.The lowest rate of resistance in the current study was to ceftazidime/avibactam andceftolozane/tazobactam; where only 9.3% and 7.4%of *P. aeruginosa* isolates were resistantrespectively. Similarly, Liao et al. [37]in Taiwan found 9% and 7% resistance rate to ceftazidime/avibactam and ceftolozane/tazobactam. A lower incidence was detected by Alatoom et al. [38]in United Arab Emirates who reported resistance rate of 6% and 3% to ceftazidime/avibactam and ceftolozane/tazobactamrespectively. This was an expected finding with ceftazidime/avibactam and ceftolozane/ tazobactam, owing to their unavailability in the Egyptian market.

In the present study, the prevalence of MDR isolates were 76% which is slightly higher than the result of Helmy and Kashef [39]which were 65.4%. Lower results were recorded by Talaat et al. [40]and Yekani et al. [36]who stated that (59.8%) and (65%) *P. aeruginosa*, respectively, were MDR. Undoubtedly, the rate of MDR *P. aeruginosa* at the Egyptian hospitals is alarming, probably due to the excessive use of a wide range of antibiotics to treat nosocomial infections in hospitals [41]. Added to that, the ability of this bacteria to survive for months in hospital environment increases the likelihood of its passage to other patients. Thus awareness, continuous surveillance and antibiotic stewardship to control and prevent the spread of resistant strains have become an indispensable necessity [32].

Regarding biofilm results in this study, 38(70.4%) of *P. aeruginosa* isolates were biofilm producers, 14.8%, 46.3% and 9.3% of isolates were strong, moderate and weak biofilm producers, respectively, whereas, 16 (29.6%) of isolates were non biofilm producers. El-Khashaab et al. [42]found that among 96 burn and surgical isolates of*P. aeruginosa*, 91.4% showed biofilm formation, among which 25.7%, 40% and 25.7% were strong, moderate and weak biofilm producers respectively. In study done by Dumaru et al. [43] biofilm formation was found in73.6% out of 38 isolates of *P. aeruginosa* of different sources.In contrast, Saha et al. [14]showed biofilm formation in only 29.1% out of 134 *P. aeruginosa* isolates of different sources; 11.9% were strong biofilm producers and 17.2% were moderate biofilm producers.

Yekani et al. [36] found that among 50 clinical isolates of *P. aeruginosa* from endotracheal aspirate, 28 of 50 (56%) and 19 of 50 (38%) isolates were biofilm- forming by microtitre and tube methods, respectively.The discrepancy in results between different studies may be attributed to many factors such as the different countries from which the samples were collected, the number and the type of clinical specimens from which the isolates were obtained and also the differences in isolates capability to form biofilm. The primary number of cells that succeeded in adherence and the differences of quality and quantity of autoinducers (quorum sensing signaling molecules) that were produced from each isolate may also play an essential and an important role[44].

In the present study we found a significant correlation between MDR and biofilm formation,where 89.5% of biofilm forming isolates wereMDR. So the strains capable of forming biofilms were more frequently observed to be an MDR phenotype. These findings come in accordance with other studies which reported the same results[36, 14, 45]. This proposes that physiological features particular to biofilm formation; efflux pumps expression, pharmacologic

characteristics, B- lactamase and amino-transferase production might play a role in improve biofilm antimicrobial resistance. However, biofilm-producing bacteria are 10 to 1000 times more resistant to antimicrobial agents than the planktonic cell [46]. This can be one explanation as to why there is a higher failure rate in the eradication of biofilm- related infections.

The lasRgene was detected in 42 (77.8%) out of 54 *P. aeruginosa* isolates collected, while only 12 (22.2%) isolates did not harbor the gene. LasR gene was detected in all biofilm forming isolates (100%) and in only four (25%) of non biofilm forming isolates. There was highly significant relation between biofilm formation and detection of lasR gene.Our results showed that 4 biofilm deficient isolates were lasR QS gene proficient. This finding is in concordance with Schaber et al.[26], who stated that three factors (flagellar-mediated swimming motility, pilus-mediated twitching motility and QS) are considered important for biofilm formation in *P. aeruginosa*. Thereby, in the present study, it could be related to our isolates might deficient in either or both remaining factors. Moreover, the isolates may be deficient in other genes rather lasR gene, such as the lasI, rhlR and rhlI genes.There is proportion of isolates (12/54) that were deficient in both lasR and biofilm, whereas, these isolates causing clinical wound infection.This observation was explained by several other clinical studies which showed that the loss of any single virulence factor appeared to be compensated by other virulence factors during infection[47].Further, Dénervaud et al. [48]concluded that there were *P. aeruginosa* strains defected in the production of both signaling molecules and extracellular virulence factors and inducing infection because of uncharacterized virulence factors other than known ones.

In conclusion, our results indicate that Postoperative wound infection may serve as a reservoir for multidrug resistant biofilm forming *P. aeruginosa* and that the QS lasR gene is strongly associated with biofilm formation and could therefore be used as a as a useful diagnostic marker for biofilm producing *P. aeruginosa* strains isolated from infected wounds. Further studies regarding the molecular mechanisms involved in biofilm formation including studying expression and sequencing of QS genes will give a better understanding of the pathogenesis of biofilm formation which will ultimately lead to novel strategies for controlling recalcitrant biofilms.

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