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# Detecting of Biofilm Formation and Associated Genes, and Assessing PilA Gene Expression in Different Pseudomonas aeruginosa Wounds and Burns Isolated in Baghdad Hospitals

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الكشف عن تكوين الأغشية الحيوية والجينات المرتبطة بها، وتقييم التعبير الجيني في الجروح والحروق المختلفة الناتجة عن الزائفة الزنجارية المعزولة من مستشفيات بغداد

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## ABSTRACT

A total of 103 clinical specimens were obtained from patients have burns, and wounds at ages between 10 - 60 years from both genders in three hospitals in Baghdad – Iraq: Al Yarmouk Teaching Hospital, Baghdad Teaching Hospital, and Al-Karkh General Hospital and at the period from (23/9/2022 to 29/12/2022). *Pseudomonas aeruginosa* was isolated and identified, which showed that from a total of 130 specimens, only 103 isolates (71.87%) belonged to *P. aeruginosa*. The isolation rate was higher from burn infections followed by wound infections, at ratios 40.62%, and 23.75%, respectively, the rest 27 isolates (16.87%) were related to other bacterial spp. like *Escherichia coli, Klebsiella pneumonia, Proteus mirabilis, Staphylococcus aureus* and *Enterobacter cloacae*. The results showed that all *P. aeruginosa* isolates were a multidrug resistance to many antibiotics. Estimating the growth for isolates at different time (6, 12, 18, 24, and 36) hrs. revealed that a high OD value was detected between 18-24 hrs.

The results of the molecular study to detect biofilm associated genes *pil A*, *Psl A, Psl B*, *RHII* and *rhIAB* revealed that these genes were detected in *P. aeruginosa*isolates at ratio (93%, 80%, 90%, 90%, and 86.6%) respectively. Also, the detection of gene expression for *PilA* gene in three *P. aeruginosa* isolates at different time 6, 12, 18, 24, and 36 hrs. by RT-PCR revealed that a high gene expression of *pilA* gene in *P. aeruginosa* isolates detected between 18-24 hrs.

**Keywords:** *Biofilm, Pseudomonas aeruginosa, genes pil A, Psl A, Psl B, RHII and rhIAB, gene expression*.

## 1. INTRODUCTION

Biofilms are complex structures and communities of microorganisms embedded in an extracellular polymeric substance EPS matrix. EPSs mainly is composed of polysaccharides, proteins, DNA, lipids (Costa *et al*., 2018, Di Martino 2018). Bacterial biofilm typically involves several stages, starting from attachment to living or a non-living surface leading to microcolony formation, proliferation, and maturation, finally detachment, its formation and infections are the most frequent wound complication (Shineh *et al*., 2023). The development of biofilm classified into five stages (Parasuraman *et al*., 2020). Figure 1 depicts biofilm development (Rendueles and Ghigo 2012). The biofilm is regarded as one of the strategies for survival against an expected changes in the surrounding environment like temperature and viability of nutrients, drugs and host immune responses (Skariyachan *et al*., 2018; Riquelme *et al*., 2020).

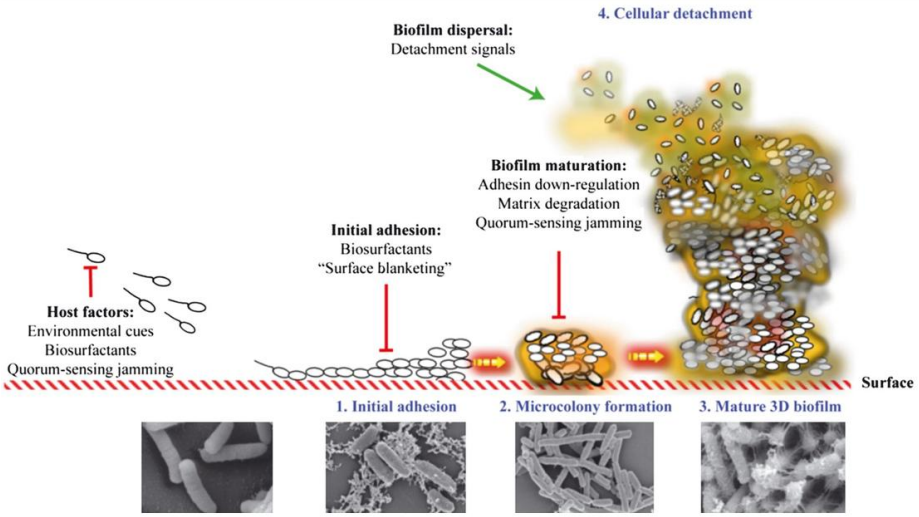


**Figure 1. Stages in the biofilm formation process, including scanning electron microscopy imaging of each stage. Reproduced from Rendueles and Ghigo (2012), with the permission of Oxford University Press and FEMS.**

*Pseudomonas aeruginosa* found occasionally as a member of the normal group at a rate of 1 - 2% on skin, 0 - 3.4% on nasal mucosa, 0.5 - 6% on oral cavity, also it may present in the intestine, urine and fecal specimens of non-hospitalized individuals (Stone and Krachler 2015), may be attach to surfaces and equipment like respiratory instrument, catheters, dialysis tube and anesthetic mask, or colonize on equipment that used water such as bathroom, sink drains, and showers (Ghafoor et.al., 2022).

*P. aeruginosa* is a widespread, Gr- opportunistic pathogen, regarded as one of the top three most frequently bacteria linked with several human diseases leading to high morbidity and mortality (Organization 2020), it can cause acquired infections such as burn and wound infections, skin and soft tissue infections (Breijyeh *et al*., 2020, Impey *et al.,* 2020). Pathogenicity depends on a wide range of cell associated virulence factors like pili, flagella, protein F, Lipopolysaccharide LPS, rhamnolipid, quorum sensing mechanism QS, and biofilm, in addition to many extracellular products like pigments, toxins, alginate, enzymes (Caschera *et al*., 2021, Webster *et al*., 2021). Exopolysaccharide is required for adhesion of sessile cell to the surface and in cell-cell communication during biofilm initiation (Bilici *et al*., 2020). Psl polysaccharide is a signaling molecule to lead to formation a thicker biofilm (Yang *et al*., 2021). Biofilm possesses a distinct physiology and represents the conversion from chronic to acute infections (Zadeh *et al*., 2022).

*P. aeruginosa* genome is a quite large contains 5.4–7 million base pairs with GC content 65-67% that made up of a single circular supercoiled chromosome and a variable number of plasmids (Hesse *et al*., 2019). Its strains are genetically diverse, and the core genome is generally large collinearly arranged that present within a respective DNA (Nimri *et al*., 2017, Yi and Dalpke 2020). It is highly conserved among clonal complexes but may showed a low level of sequence diversities about 0.5–0.7% such as the presence of a few diversity loci for flagellar regulon, Pil A and O-antigen biosynthesis locus (Liao *et al*., 2022).

The accessory genome of *P. aeruginosa* encodes a lot of extrachromosomal elements like plasmids, and many genes involved in the catabolism of organic chemicals (Paker *et al*., 2017), and their elements appeared to be acquired by horizontal gene transfer from a variable source included other species or genera (Zowawi and Bail 2021). The more important pigment pyocyanin and pyoverdine are toxic to the host cells (Invernizzi, 2020). Pyoverdines can mediate biofilm formation (Tacconelli *et al*., 2018). Protein F contains two fibronectin binding domains (Shaaban *et al*., 2017). Rhamnolipid has a role in biofilm development (Lee and Yoon, 2017). Las, Rhl, IQS and pqs of *P. aeruginosa* are utilized respectively for 4 autoinducers like OdHL, C4-HSL, IQS and PQS (El-Sayed, *et al*., 2020); that allowed bacterial cell itself communication, to sense their own density and together with their transcriptional activate or allowed to express specific genes as a population instead of individual cells (Karballaei *et al*., 2020). Most of the environmental and clinical *P. aeruginosa* strain have been shown to have a proteolytic activity, LasA, LasB protease degrades the hosts tissue proteins, and elastase may assist in escaping from phagocytosis and antibody- mediated cytotoxicity at the burn or wound site (Leiris 2021, Oluwabusola *et al*., 2022). Both alkaline protease and protease IV can damage the host tissues protein and enhance bacterial infection through degradation of fibrinogen, lactoferrin, transferrin, andelastin (O'Callaghan *et al*., 2019). Toxic Exoenzyme S encoded by exoS gene, has a role in phagocytosis (Cigana 2021), also it is disrupting actin cytoskeletal rearrangement, signal transduction cascades (Kamalanathan 2020). ExoT is acted by disrupting the host actin cytoskeleton,

and inducing apoptosis of host cells (Abbasi et al., 2017). Toxin A (PEA) is one of the more toxic extracellular factors (Liao *et al.,* 2022), exotoxin A is a significant virulence factor in a burn unit, and intensive Care Unit ICUs (Hossein *et al*., 2015). PEA inhibits host protein synthesis, and induces programmed cell death (Michalska and Wolf 2015). Leukocidin is a pore-forming toxin (Dai *et al*., 2021), targets phagocytes, natural killer cells, dendritic cells and T lymphocytes, therefore, targets both innate and adaptive immune responses (Lu *et al.*, 2021). There are many forms of burn according to their sources that are either caused by direct exposure to heat like flames, boiling water leads to skin layers destructions (Jeschke *et al*., 2020). The severity of burn injury is varied depend on several factors such as size of burned area, the site of injury if it's surface or deep burns (Durant et al., 2017). Other factors like exposure time, contamination of burn site may also contrast with burn healing (Phan *et al*., 2020). The acute type of wounds is characterized by healing through a routine inflammation process, formation of tissue and remodeling that take about 4-6 weeks, chronic wounds are more serious because the healing process takes longer than six weeks without expressing any signs of resolution (Brandenburg 2019). Research aims to detect biofilm formation of *P. aeruginosa* isolated from Baghdad Hospitals wounds and burns, assess genes expression in different isolates, detect of three biofilm associated genes by PCR, and the gene expression for one biofilm associated gene by RT-PCR.

## 2 Methods

Tables 1 and *2* show the types of primers used in the research.

### 2.1 Primers

**Table 1. Primers of biofilm associated genes.**

| Gene name | Primer sequence (5´- 3´) | Product size (bp) | Reference |
|---|---|---|---|
| *pilA* | F: ACTGTTGGTCGTCGTCTTCC | 160 | Abd ElGalil *et al*., 2013 |
| | R: CCGTCCTACCAGGGTTACCT | | |
| *pslA* | F: GTTCTGCCTGCTGTTGTTCA | 230 | Divyashree *et al*., 2021 |
| | R: GGTTGCGTACCAGGTATTCG | | |
| *pslB* | F: GCTTCAAGATCAAGCGCATC | 220 | Divyashree *et al*., 2021 |
| | R: ACCTCGATCATCACCAGGTC | | |
| *Rhll* | F: CTCTCTGAATCGCTGGAAGG | 245 | Abd ElGalil *et al*., 2013 |
| | R: GCGAAGACTTCCTTGAGCAG | | |
| *rhlAB* | F: TCATGGAATTGTCACAACCGC | 151 | Neha Campus *et al*., 2014 |
| | R: ATACGGCAAAATCATGGCAAC | | |

**Table 2. Primers used in RT-PCR.**

| Primer name | Sequence 5`-3` | Reference |
|---|---|---|
| *pilA* | F: ACTGTTGGTCGTCGTCTTCC | Abd ElGalil *et al*., 2013 |
| | R: CCGTCCTACCAGGGTTACCT | |
| *16s rRNA* | F: CCGTGTCTCAGTTCCAGT | Shriparna 2013 |
| | R: TGAGCCTAGGTCGGATTA | |

### 2.2 Samples collection

A total of 103 specimens were collected from various clinical cases including burns and wounds in three hospitals in Baghdad city: Al-Yarmouk Teaching Hospital, Baghdad Teaching Hospital, and AlKarkh General Hospital at the period from 23/ 9/ 2022 to 29/ 12/ 2022. A sterile swap in a transport media was used for burns and wounds specimens. These specimens were collected from both genders at ages (10-60) years, then immediately streaked on nutrient agar and MacConkey agar plates and incubated overnight at 37ºC.

### 2.3 Isolation and Identification of *Pseudomonas aeruginosa*

A loopful of non-lactose fermented MacConkey agar colonies were subcultured on cetrimide agar plates and incubated at

37°C overnight. After incubation, selected pure isolated colonies were streaked on blood agar plates and incubated overnight at 37 ºC to identify type of bacterial hemolysis (Algammal-*et al*., 2020). A pure isolated colonies were stained by gram stain for microscopic observation (Cappuccino and Welsh, 2018), and idented by biochemical tests: Catalase test, Oxidase test (Zina et al., 2020), Indole test, Citrate utilization test (Saleem and Bokhari 2020), Methyl red test (Cappuccino and Welsh, 2018), Voges-Proskauer test (Al-Mayyahi, 2018), Urease test (Welde *et al*., 2020), Motility test (Forbes *et al*., 2007), Triple Sugar Iron (TSI) test, Oxidative – fermentation test (Algammal *et al*., 2020). Growth of bacteria at 42°C (Forbes *et al*., 2007), Screening for protease production (Chongbing *et al*., 2022), Identification of isolates by VITEK 2 system (Minh *et al*., 2020).

**2.4 Enumeration of the growth curve of *Pseudomonas aeruginosa***

For enumeration of the growth curve of bacteria, isolate was inoculated in 5 mL of (De Conti et al., 2021), of brain heart infusion broth BIB and incubated at 37ºC for 24 hours, then centrifuged at 5000 rpm for 5 m, supernatant was discarded and the deposits' was diluted to a concentration 1.5×108 CFU/ml in compared with MacFarland turbidity standard tube No. (0.5), 0.5 ml from it was inoculated into 50 ml of BIB prewarmed at 37ºC, immediately after mixing , 5 ml portion of the inoculated broth culture was transferred into a sterile test tubes (1-6), than inocubated at 37ºC in a shaker condition. A 5 mL portion of not inoculated BIB was transferred into a sterile test tube as a blank. Optical density of the broth culture was estimated at 600 nm by spectrophotometers at different time (6, 12, 18, 24 and 36 hrs), then 0.1 mL portion of P. aeruginosa broth culture at each time was streaked on a nutrient agar plate to detect the viability of bacteria.

**2.5 Detection of Biofilm Formation** (Minh *et al.,* 2020)

The quantitative microtiter plate method was employed for testing biofilm formation by *P. aeruginosa*. 40 tested bacterial isolates were cultivated in 5 mL of nutrient broth while shaking overnight at 37 ºC, centrifuged at 5000 rpm for 10 m to precipitate it, the deposit was diluted to nearly concentration of 1.5 $\times 10^8$ CFU/ml in compared with MacFarland standard tube (0.5).

In Eppendorf tubes, putting 980 µl of BIB containing 2% glucose, 20 µl of the tested isolates were added, 200µl of each bacterial suspension was transferred to a well in a microtiter plate (96 walls) as triplicate, while 200 µl of non-inoculated BIB was transferred as a negative control, then incubated at 37 ºC for 24 hrs. After incubation, the excess culture was decanted of, then washed the wells carefully with PBS three times to remove unattached bacterial cells. For fixation of the adherent cells, 200 µl of absolute ethanol was added to each well for 10 min, excess ethanol was removed, the plate left to dry at room temperature, later stained the wells with 200 µl of 0.5% crystal violet for 15 min, washed three times with PBS and left to air – dried. The adherent cell in each stained well was resolubilized in 200 µl of 33% glacial acitic acid and incubated at room temperature for 15 minutes. Optical density (OD) of the stained bacteria was measured by ELISA reader at a wavelength of 630 nm. OD values were considered as an index of adherent bacteria that form a biofilm. The data calculation and the biofilm forming capacity for all tested isolates were classified into different categories (Table 3).

**Table 3. Classification of bacterial strength for biofilm formation.**

| **Biofilm producer ranking** | Non | Weak | Moderate | Strong |
|---|---|---|---|---|
| | 0 | + | ++ | +++ |
| **OD values** | OD≤ODc | ODc<OD≤2×ODc | 2ODc<OD≤4×ODc | 4×ODc<OD |

**ODc = mean OD of the negative control+(3×SD of the negative control)**

**2.6 Molecular analysis**

The **technique** amplification is based on RNA/miRNA concentration that conversion into cDNA. All steps a total RNA purification, qPCR amplification and later data analysis. Colony PCR technique based on using a sterile wooden stick for transferring selected *P. aeruginosa* colonies from nutrient agar plate and suspended in 100 µl of TE buffer (Jabar 2020), the tubes were tightly mixed by vortex, then incubated at 90 ºC for 20 m. Samples centrifuge at 12000 rpm for 5 min, the supernatant contained DNA is added to the PCR mixture in place of purified DNA.

Stock solution of **primers** was prepared by dissolving the lyophilized primers in nuclease-free water until reaching 100 p mol/ml final concentration. Primers working solution was prepared by adding 10 µl of the primers stock solution to 90 µl of nuclease-free water to obtain a working primer solution at a concentration 10 p mol/µl. **PCR mixture**: In Eppendorf tube (0.2 ml), putting the primers, nuclease-free water and master mix, final volume of PCR mixture was 20 µl as shown in table 4.

**Table 4. The reaction mixture for PCR working solution.**

| **Component** | Master Mix (2X) | Forward primer | Reverse primer | Nuclease Free Water | DNA template | Total volume |
|---|---|---|---|---|---|---|
| **Reaction volume, µl** | 10 | 1 | 1 | 4 | 4 | 20 |

**Reaction Setup**, the PCR tubes were carried to the thermal cycler to initiate the amplification reaction based on using a specific program for each pair of primers (table 5).

**Table 5. PCR amplification program for detection gene in isolates.**

| **pilA gene *P. aeruginosa* isolates** | | | | | |
|---|---|---|---|---|---|
| **Step** | initial den. | denaturation | annealing | extension | final extension |
| **No. of cycle** | 1 | 30 | | | 1 |
| **Time** | 5 min | 30 sec | 30 sec | 30 sec | 7 min |
| **Temperature, ºC** | 95 | 95 | 55 | 72 | 72 |
| **pslA gene *P. aeruginosa* isolates** | | | | | |
| **No. of cycle** | 1 | 30 | | | 1 |
| **Time** | 5 min | 30 sec | 30 sec | 30 sec | 7 min |
| **Temperature, ºC** | 95 | 95 | 52 | 72 | 72 |
| ***pslB* gene *P. aeruginosa* isolates** | | | | | |
| **No. of cycle** | 1 | 30 | | | 1 |
| **Time** | 5 min | 30 sec | 30 sec | 30 sec | 7 min |
| **Temperature, ºC** | 95 | 95 | 52 | 72 | 72 |
| ***RhII* gene *P. aeruginosa* isolates** | | | | | |
| **No. of cycle** | 1 | 30 | | | 1 |
| **Time** | 5 min | 30 sec | 30 sec | 30 sec | 7 min |
| **Temperature, ºC** | 95 | 95 | 55 | 72 | 72 |
| ***RhIAB* gene *P. aeruginosa* isolates** | | | | | |
| **No. of cycle** | 1 | 30 | | | 1 |
| **Time** | 5 min | 30 sec | 30 sec | 30 sec | 7 min |
| **Temperature, ºC** | 95 | 95 | 55 | 72 | 72 |

**Agarose gel electrophoresis** was used to confirm the presence of amplified band. PCR technique was completely dependent on DNA extracted criteria. For preparation of agarose at concentration 1%, 1gm of agarose were suspended in 100 ml of TAE 1X buffer that's freshly prepared, then heated the mixture in a microwave to dissolve completely. The agarose was left until it cooled to 50 ºC, then adding Ethidium promide 2 µl (0.5 mg /ml) concentration to it, mixing well by vortex. The comb was fixed in the taped tray about 1cm from the edge, then pre-warmed agarose solution was poured in the tray, left at room temperature to solidify. The comp is raised and removed the tapes from both sides of the tray, then place gel in gel tank that's filled with TAE 1X buffer until it reaches 3-5 mm above the surface of the gel. Five µl volume of PCR product was loaded slowly in the wells of the gel by using a micropipette. Five µl of the molecular DNA ladder 100pb was loaded in a single line of the gel as a marker. the tank is closed, then run the electrical power at 70 v/cm for 1 hr. DNA of the isolate was moved from cathode to anode poles. At the end of the electrophoresis, put the gel in UV transilluminator for isolation DNA bands, and took a photograph.

**RNA extraction** from three bacterial samples was done at time (6,12, 18, 24 and 36 hrs) according to the protocol of TRIzol TM Reagent as follows:

**Bacterial Cells lysis**: Bacterial isolates were inoculated in 4 ml of BIB, incubated at 37C for 24 hrs., then centrifugation at 13000 rpm for 3 m, the pellet was suspended in 0.5 ml of TRizol TM reagent, then homogenized the cell lysate by pipette it well up and down for several times.

**Separation into three phases**: To all tubes containing lysate, 0.2 ml of chloroform was added, then closed with caps. Incubated the mixture for 2-4 m, then centrifugated at 13000 rpm for 10 m to separate it into three phases, lower organic phase, inter phase and upper colorless aqueous phase; the latter was transferred into the new tubes.

**Precipitation of RNA**: A volume of 0.5 ml of isopropanol was added to the colourless aqueous phase in the tubes, incubated for 10 m, then centrifugated at 13000 rpm for 9 m. Precipitation of total RNA was formed and appeared in the bottom of the tubes as a white gel like pellet, the supernatant was removed.

**RNA washing**: For all tubes 0.5 ml of ethanol (70%) and vortex well added, then centrifugated at 1300 rpm for 5 m. Aspirated the ethanol, left pellet to dry in air.

**Solubility of RNA**: Pellet in all tubes were rehydrated by adding 50 µl of nuclease free water to it, then incubated at 55-60 ºC for 15 m in a water bath.

**Detection of *PilA* gene expression by RT-PCR**

In the **Fluorescence technique**, a quantum fluorometer was used for detecting the concentration of extracted RNA to determine the quality of these samples for downstream applications. For 1 µl of RNA, 199 µl of diluted Quanti Flour Dye was added and mixed well. After incubation for five minutes at room temperature in a dark place, RNA concentration values were detected.

**Primer used in RT-PCR Primers preparation**: These primers were supplied by the Macrogen Company in a lyophilized form. Stock solutions of these primers were prepared by dissolving the lyophilized primers in nuclease free water to get final concentration loop mol/ml.The working solution of these primers was prepared by adding 10 µl of primer stock solution (stored at freezer -20 ºC) to 90 µl of nuclease-free water to geta working primer solution at a concentration 10 p mol/ µl.

**Reaction setup and thermal cycling protocol**: The final volume of RT-PCR mixture for each isolate was 10 µl which consisted of 5µl of master mix, 0.5 µl forward primers, 0.5 µl reverse primers, 1µl extracted RNA, $MgCl_2$ (0.25 µl), RT mix (0.25 µl), and 2.5 µl nuclease free water as shown in table 6.

**Table 6. The volumes of qPCR reaction mixture.**

| Component | Volume (µl) | Component | Volume (µl) |
|---|---|---|---|
| qPCR Master Mix | 5 | Reverse primer | 0.5 |
| RT mix | 0.25 | Nuclease Free Water | 2.5 |
| $MgCl_2$ | 0.25 | RNA | 1 |
| Forward primer | 0.5 | Total volume | 10 |

The Mic tubes were placed in PCR device that turned on to start the reactionstages for RT-PCR program as shown in table 7.

**Table 7. Real Time PCR Program for detection of pilA gene.**

| **Step** | RT. Enzyme Activation | Initial Den. | Denaturation | Annealing | Final extension |
|---|---|---|---|---|---|
| **Temp °C** | 37 | 95 | 95 | 55 | 72 |
| **Time m: s** | 15 min | 5 min | 20 sec | 20 sec | 20 sec |
| **Cycle** | 1 | | 40 | | |

**Gene expression calculations**

The calculated data of qRT-PCR were a direct comparison of Ct values between the target *pilA* gene and the reference 16s

rRNA (housekeeping) gene. Genes were analyzed by the relative quantification gene expression levels (fold change) by using ΔΔCt method (Yasaman *et al*., 2023) as seen in the following equations:

**ΔCT = CT gene - CT housekeeping gene (16S rRNA)**

**ΔΔCT = ΔCT Treated - ΔCT Control**

**Fold change =$2^{-\Delta\Delta CT}$**

Thus, the relative changes in mRNA expression levels were determined by used the comparative threshold cycle (CT) value method ($2^{-\Delta\Delta Ct}$) (Parsa *et al.,* 2020).

**Statistical analysis**

The statistical analysis system applied by one-way ANOVA test that is used for analyzing the data for *pilA* gene expression at different times. Graphpprim v 7.0 test was used to compare significantly between the percentage in the paper.

## 3. Results and Discussion

### 3.1 Sample collection and isolation of bacteria

103 (65%) of isolates showed identical morphological characteristics and biochemical tests that belong to *Pseudomonas aeruginosa*. The high isolation rate was from burn infections followed by wound infections at 40.62% and 23.75% respectively, the rest 27 isolates (35%) were related to other pathogenic bacteria like *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Putida*, *Staphylococcus aureus* and *Enterobacter cloacae* as shown in table 7.

**Table 7. number and isolation ratio for *P. aeruginosa* isolated from clinical cases.**

| source of specimens | No. of | No. and isolation ratio for | |
|---|---|---|---|
| | | *P. aeruginosa* % | other bacteria (%) |
| burns | 80 | 65 (40.62) | 15 (9.37) |
| wounds | 50 | 38 (23.75) | 12 (7.50) |

The results of the isolation have been consistent with results reported by Genan *et al*. (2022) who collected out of 170 clinical specimens. Out of 144 isolates have been identified in which 66 (45.8%) were Gr- bacteria, 57 (39.6%) were Gr+, 21 (14.6%) related to fungus. Predominant Gr- isolated bacteria was *P. aeruginosa* at ratio 50 (34.72%), followed by *Escherichia coli* 41 (28.47%), *S. epidermidis* 34 (23.61%), *S. hemolyticus* 11 (7.63%) and 8 (5.55%) related to *Candida* spp. Another results by Tafase *et al.* (2022) identified 58 isolates from a total of 65 clinical specimens taken from burns and wounds ~~and otitis media~~. The isolated bacteria were related to *Escherichia coli, P. aeruginosa, K. pneumoniae* and *Proteus vulgaris* at ratio 21 (36.20%), 19 (32.75%), 11 (18.96%), 7 (12.06%) respectively.

### 3.2 Detection of biofilm formation by *P. aeruginosa* isolates

The strength of biofilm development is determined by the location and type of infection, length of bacterial invasion, host immune response, composition of EPS, and environmental adaptation (Elmanama *et al*., 2020). In this paper, 40 *P. aeruginosa* isolates from (Ps1-Ps40) were screened to detect their ability for biofilm production by using microliter plate (Table 8). This result revealed that from a total of 40 isolates from Ps1-Ps40 (85%) were a biofilm producer, strong biofilm was detected in 21 (52.5%) of isolates within OD value range from (0.65-0.98), 2(5%) revealed a moderate biofilm formation, within OD value 0.45, also 11(27.5%) revealed a weak biofilm production, 6(15%) of isolates were detected as non- biofilm producer, as shown in table 8. This result has been consistent with the result by Alaa (2022) who reported that 96% of *P. aeruginosa* isolated from burns, and wounds, ~~ear~~, ~~urine~~, ~~sputum~~, ~~blood~~ in Baghdad hospitals were biofilm producer at three categories in which 68% of isolates were strong biofilm producer, 18% were moderate, and 14% weak biofilm production. Another result (Zena 2021) revealed that *P. aeruginosa* isolated from burns, and wounds ~~and respiratory system infections~~ can produce a biofilm at three grades strong, moderate and weak at ratio 60%, 30%, and 10% respectively. Also, the result was nearly to the result reported by Wedad (2020) who revealed that *P. aeruginosa* isolated from different clinical cases in Egypt produce a biofilm at three categories strong, moderate,and weak (43%, 33%, and 11%) respectively. Isolates have resistance ability to different antibiotics (Obaid 2024).

**Table 8. Types of *P. aeruginosa* isolates according to biofilm production.**

| Biofilm strength groups | Samples | OD values | Samples | OD values | Mean ± SE |
|---|---|---|---|---|---|
| Non biofilm (OD < ODc) | PS4 | 0.12 | PS22 | 0.11 | 0.1067 ± 0.0033 |
| | PS18 | 0.10 | PS1 | 0.10 | |
| | PS21 | 0.10 | PS2 | 0.11 | |
| Weak biofilm (ODc < OD < 2xODc) | PS3 | 0.17 | PS26 | 0.19 | 0.21454 ± 0.0047 |
| | PS5 | 0.25 | PS28 | 0.27 | |
| | PS6 | 0.19 | PS30 | 0.18 | |
| | PS13 | 0.18 | PS34 | 0.25 | |
| | PS14 | 0.19 | PS40 | 0.29 | |
| | PS16 | 0.23 | | | |
| Moderate biofilm (2xODc < OD < 4xODc) | PS17 | 0.45 | PS39 | 0.45 | 0.45 ± 0.0 |
| Strong biofilm (4xODc < OD) | PS7 | 0.75 | PS25 | 0.80 | 0.8066 ± .039 |
| | PS8 | 0.80 | PS27 | 0.65 | |
| | PS9 | 0.73 | PS29 | 0.91 | |
| | PS10 | 0.88 | PS31 | 0.94 | |
| | PS11 | 0.76 | PS32 | 0.93 | |
| | PS12 | 0.68 | PS33 | 0.58 | |
| | PS15 | 0.98 | PS35 | 0.81 | |
| | PS19 | 0.66 | PS36 | 0.73 | |
| | PS20 | 0.98 | PS37 | 0.68 | |
| | PS23 | 0.66 | PS38 | 0.84 | |
| | PS24 | 0.78 | | | |
| Negative control | broth only | | | | 0.126848 |

**ODc=Negative control means OD+(3 x negative control SD)**

This result consistent with result by Al-Kabi *et al.* (2022) who reported that 50% of *P. aeruginosa* isolates produced a strong biofilm that linked with resistance to many antibiotics; because biofilm development in chronic infections caused by *P. aeruginosa* leads to highly limited in the efficacy of antibiotics treatments, beside the persistence of biofilm specific genes gave a transient protection against antibiotics therapy leads to develop of resistance (Swapna *et al*., 2022).

### 3.3 Detection of biofilm associated genes by colony PCR

The results of the amplification in *P. aeruginosa* isolates revealed that 28/30 (93%) have *PilA* gene that detected by using *PilA* -F and *PilA* -R primers and size of amplified fragment for *PilA* gene was 160bp as shown in Figure 2.

**M ps1 ps2 ps3 ps4 ps5 ps6 ps7 ps8 ps9 ps10 ps11ps12 ps13 ps14 ps15 ps16**

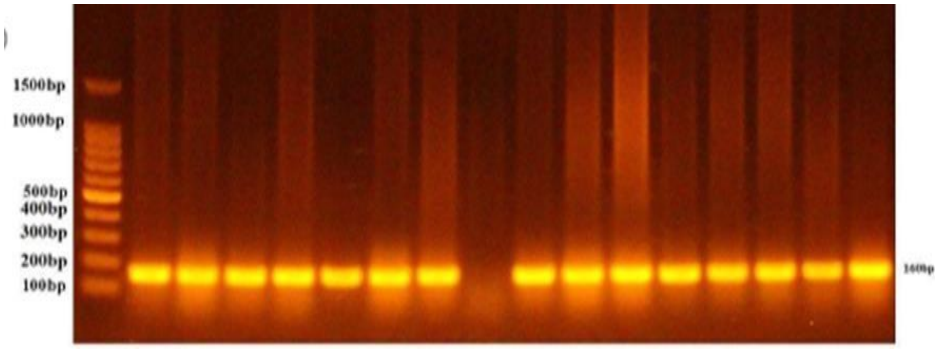


**Figure 2. Detection of *PilA* gene in *P. aeruginosa* isolates in agarose gel at concentration 1%, M: DNA ladder 100bp, lines from Ps1-Ps16 represent bacteria have a *PilA* gene with amplified product size 160bp.**

The result of detection for *PilA* gene in this paper was consistent with result by Noor and Ashwak (2022) who showed that (95%) of *P. aeruginosa* isolated in Baghdad from burns and wounds, ~~otitis media, diabetics foot and urine~~ have *PilA* gene. Also, in accordance with Nitz *et al*. (2021) who reported that *P. aeruginosa* isolated in Brazil from different clinical cases have *PilA* gene at ratio 98%. Result was consistent with result by Kiyaga *et al.* (2022) who reported that *P. aeruginosa* strains isolated from six medical centers in Kenya were positive for *PilA* gene at ratio 96%, that is the same by Kamali *et al*. (2020) who revealed that *P. aeruginosa* isolates have *PilA* gene at ratio 92%. Extracellular appendages type IV pili play an important role in twitching motility, and has an adhesive role in cell-surface interactions as well as information of microcolony in biofilms (Kamali *et al*., 2020). Genes present in pilMNOPQ operon are essential for both assembly of T4P and formation of bacterial movement (Holbrook *et al*., 2018).

The result of detection for *PslA* gene in *P. aeruginosa* isolates revealed that 24/30 (80%) of isolates have *PslA*. *PslA* gene was detected by using *PslA*-F and *PslA*-R primers and the size of amplified fragment for *PslA* gene was 230bp as shown in figure 3.

**M ps1 ps2 ps3 ps4 ps5 ps6 ps7 ps8 ps9 ps10 ps11 ps12 ps13 ps14 ps15**

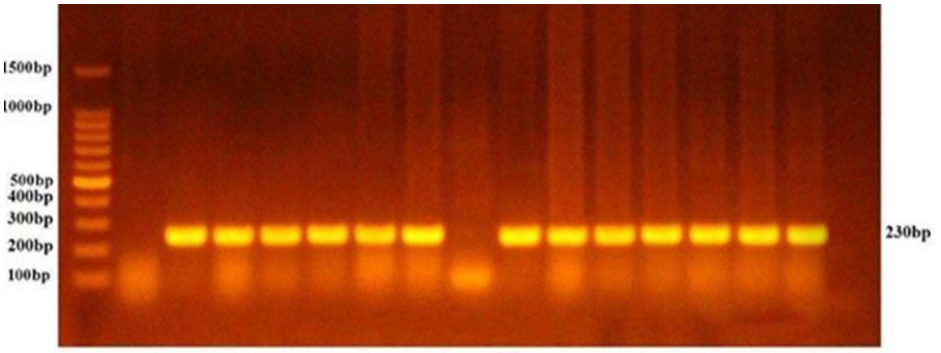


**Figure 3. Detection of *PslA* gene in *P. aeruginosa* isolates in 1% agarose gel, M: DNA ladder 100bp, lines from Ps1-Ps15 represent bacteria have a *PslA* gene with amplified product size 230bp.**

The result for *PslA* gene in this paper was consistent with result by Kamali *et al.* (2020) who reported that from a total of 80 *P. aeruginosa* strains isolated from ~~endotracheal~~ ~~secretion, urine~~, wounds, ~~CSF and ears~~, *PslA* gene was present in 87.5%. This result was in accordance with result by Ahmed *et al.* (2023) who showed that (82%) of *P. aeruginosa* isolated in Iraq from burns and wounds, ~~otitis media and urine~~ have *PslA* gene.

The results of amplification for *PslB* gene in *P. aeruginosa* isolates revealed that 27/30 (90%) of isolates have *PslB* gene. *PslB* gene was detected by using *PslB*-F and *PslB*-R primers and the size amplified fragment for *PslB* gene 220bp as shown in figure 4.

**M ps1 ps2 ps3 ps4 ps5 ps6 ps7 ps8 ps9 ps10 ps11 ps12 ps13 ps114 ps15**

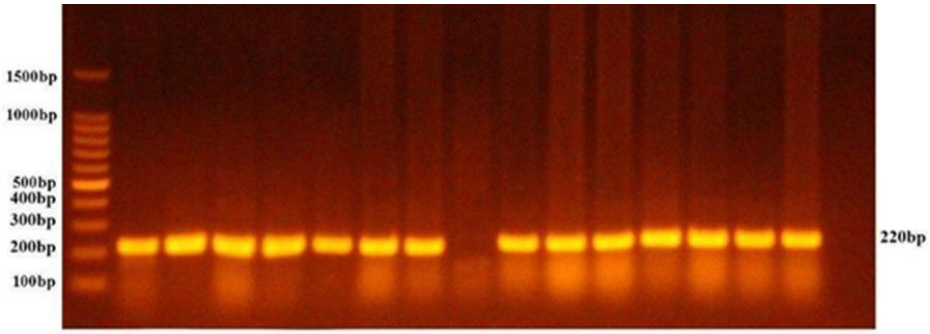


**Figure 4. Detection of *PslB* gene for *P. aeruginosa* isolates in 1% agarose gel, M: DNA ladder 100bp, lines from Ps1-Ps15 represent bacteria have a *PslB* gene with amplified a product size 220bp.**

This result was consistent with result by Farhan *et al.* (2019) who recorded that *PslB* gene was found in 94% of *P. aeruginosa* isolates producing a biofilm in Iraq. The same result by Elnegery *et al.* (2021) who reported that *PslB* gene was detected in *P. aeruginosa* isolates in Egypt revealed a different categories of biofilm development at ratio 91.4%. The same result by Mohammad *et al*. (2017) who showed that from a totalof 37 *P. aeruginosa* isolated in Iraq from burn ~~and otitis~~ infections, 34/37 (91.89%) have *PslB* gene. Both Pel and Psl can serve as a primary structure scaffold for biofilm development and are involved at early stages of biofilm formation (Magalhães *et al*., 2020). The Psl genes encoded a protein involved in the biosynthesis of Psl polysaccharide (Correia *et al.*, 2017). The *PslA* protein may vary slightly depending on the specific strain of *P. aeruginosa*, between 15- 20 KDa. Similarly, the molecular weight of *PslB* protein can vary, generally range from 20-25 KDa (Impey *et al.,* 2020). These genes and their encoded protein play an essential role in maintenance of biofilm and are regarded as a key factor in pathogenicity and persistence of *P. aeruginosa* infections (Jakubovics *et al*., 2021).

The results of amplification for *RHII* gene in *P. aeruginosa* isolates revealed that 27/30 (90%) have *RHII* gene. The *RHII* gene was detected by using *RHII*-Fand *RHII*-R primers and size amplified fragment for *RHII* gene was 245 bp as shown in figure 5.

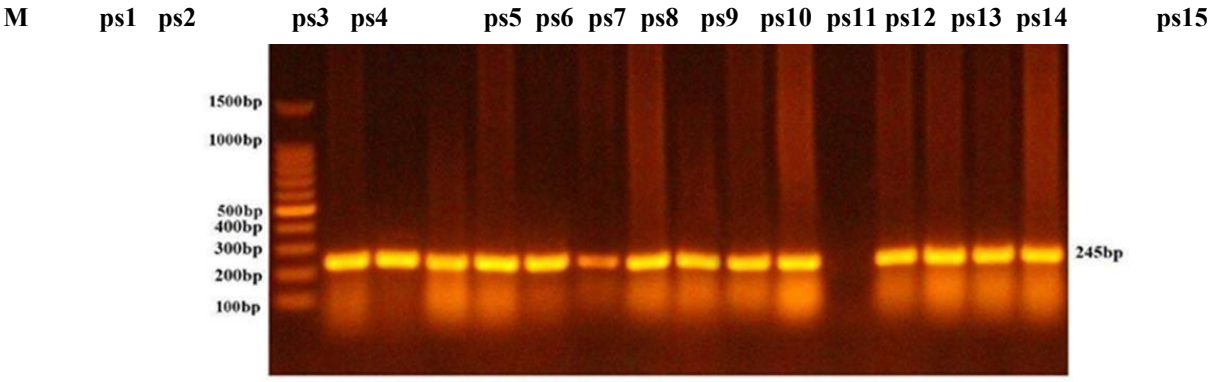


**Figure 5. Detection of *RHII* gene in *P. aeruginosa* isolates in 1% agarose gel, M: DNA ladder 100bp, lines from Ps1-Ps15 represent bacteria have a *RHII* gene with amplified product size 245bp.**

This result was consistent with result by Al-Kilabi *et al.* (2020) who recorded that, 31 *P. aeruginosa* isolated from clinical cases contained genes *Las, LaR, rhII* and *RhIR* at ratio (87%,80.6%, 83.5% and 91.7%), respectively. The result of this paper was in accordance with Abdullah *et al.* (2022) who reported that specific primers were used for detection the genes *LasI, RhII, LasR* and *RhIR* and showed in Iraq that these genes were present in *P. aeruginosa* isolates at percentage 100%, 95%, 85% and 87% respectively. *Pseudomonas aeruginosa* possesses three main QS systems that are involved in biofilm formation (Nathwani *et al.,* 2018, Paller *et al.,* 2019).

The results of amplification for *rhIAB* gene in *P. aeruginosa* isolates revealed that 26/30 (86.66%) have *rhIAB* gene. The *rhIAB* gene was detected by using *rhIAB*-F and *rhIAB*-R primers and the size amplified fragment for *rhIAB* gene was size 151bp as shown in figure 6.

**M ps1 ps2 ps3 ps4 ps5 ps6 ps7 ps8 ps9 ps10 ps11 ps12 ps13 ps14 ps15**

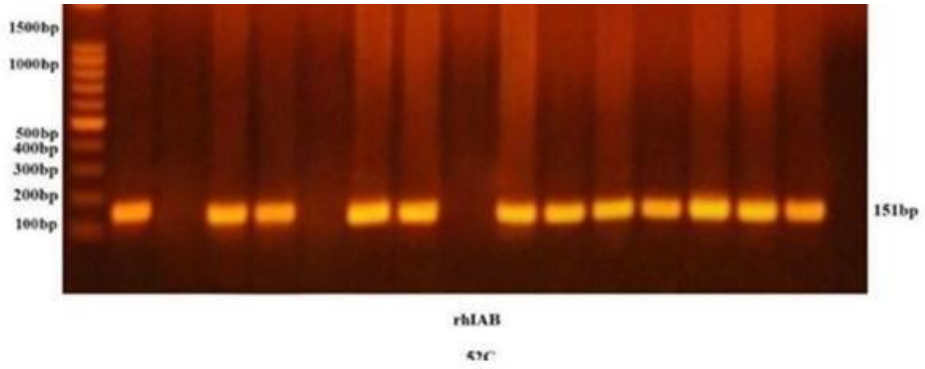


**Figure 6. Detection of *rhIAB* gene in *P. aeruginosa* isolates in 1% agarose gel, M: DNA ladder 100bp, lines from Ps1-Ps15 represent bacteria have a *rhIAB* gene with amplified product size 151bp.**

The result of this paper was consistent with result by Neha *et al.* (2014) who recorded that *rhIAB* gene was found in India in 87% of *P. aeruginosa* isolates producing a biofilm. Also consistent with result by Leila et al., (2021) who revealed that 100 *P. aeruginosa* isolated in Iran from skin, ~~urine, sputum, blood~~ and wound were screened for the presence of seven genes included *rhII, rhIR, rhIAB, LasB, LasI, LasR* and *aprA* and showed that these isolates have the seven genes in which the ratio of *rhIAB* gene was 93%. The rhI system controlled the expression of rhIAB operon, the molecular study for *rhIAB* operon revealed that it encoded two key enzymes responsible for rhaminolipid production which is controlled by *RhIRI* (Hemmati 2020, Corley 2022). Ouyang (2020) reported that *RhIRI* was encoded the gene required for *rhIAB* transcription.

### 3.4 Gene expression

A total RNA was extracted from three *P. aeruginosa* Ps10, Ps15, and Ps29, isolated from burns and wounds ~~and UTIs infections~~. These isolates contained *PilA* gene and revealed a strong biofilm production, also multidrug resistance MDR to 7 antibiotics and more. The concentrations of RNA were 474 - 720 ng/µl for **Ps10** isolate (A), 417 - 635 ng/µl for Ps15(B), 396 - 720 ng/µl for Ps29(C) as shown in table 7. The result was consistent with result by Zina (2024) who reported that *P. aeruginosa* isolated in Baghdad from burns and wounds, ~~respiratory sputum,~~ ~~and otitis media infection~~ have a total RNA concentration ranged from 369 - 503 ng/µl. Also, in accordance with the results of Noor Al-Huda (2022) who recorded a total RNA from *P. aeruginosa* have a concentration ranged from 356 - 690 ng/µl.

We investigated the effect of time on ***PilA* gene expression** that detected at times 6, 12, 18, 24 and 36 hrs. Results revealed that at 6 hrs., there is a low transcript level (down regulation of *PilA* gene) then, a there was a slight change in gene expression at 12 hrs., there is a notable increased in gene expression at 18 hrs while a significant increase of *pilA* genes fold changes in the strong biofilm *P. aeruginosa* isolates occurred at time 18 - 24 hrs. (P-value =0. 6741) as shown in table 7.

The result of this paper was consistent with result of Abdelraheem *et al*., (2020) who showed effect of different time 5, 10, 15, 25, and 35 hrs. on *PilA* gene expression in *P. aeruginosa* isolated in Iraq from clinical cases, and revealed that a maximum *PilA* gene expression was detected at time between 15 - 25 hrs. Also, in accordance with result by Murray and Kazmierczak (2007) who isolated *P. aeruginosa* in UK with a strong biofilm producer, examined the gene expression of *PilA* gene, at three times 6, 10, 16, 20 and 24 hrs., and found that high gene expression were detected between 16 - 24 hrs. Another result reported by Noor and Ashwak (2022) that *PilA* gene were highly expressed at time between 15 – 25 hrs. in *P. aeruginosa* isolated in Baghdad city from wound and burns, ~~ear and diabetic foot swabs~~ ~~infections~~. The increased in gene expression for *PilA* gene in three *P. aeruginosa* isolates between 18 – 24 hrs. may be related to different factors such as the amount of RNA or proteins were made at different time, in addition to the presences of nutrient availability that may affect *pilA* gene expressed (Schreiber *et al.,* 2007). *P. aeruginosa* begin to transition at the beginning of exponential phase between 18 - 24 hrs., and synthesize a specific protein required for survival or replication. Also, some regulatory mechanisms are initiated at this phase (Shariati *et al.,* 2019).

**Table 7. The folding of gene expression for *PilA gene* in *P. aeruginosa* isolates at different times.**

| Sample group | Time (hr) | Sample | CT (16SrRNA) | CT pilA Gene | ΔCT | ΔΔCT | Folding | Mean ± SE |
|---|---|---|---|---|---|---|---|---|
| (Ps10) A | 6 | A1 | 20.18 | 20.53 | 0.35 | 0 | 1 | 1.962 ± 0.55 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 12 | A2 | 20.19 | 21.47 | 1.28 | 3926 | 0.924 | |
| | 18 | A3 | 20.87 | 20.09 | -0.78 | -4946 | 1.388 | |
| | 24 | A4 | 22.18 | 20.66 | -1.52 | -1.87 | 2.155 | |
| | 36 | A5 | 20.49 | 19.55 | -3927 | -1.29 | 0.845 | |
| (Ps15) B | 6 | B1 | 19.18 | 20.08 | 0.90 | 0 | 1 | 1.362± 0.64 |
| | 12 | B2 | 19.07 | 24.03 | 4.96 | 2.06 | 0.859 | |
| | 18 | B3 | 19.28 | 21.23 | 1.95 | 1.05 | 1.529 | |
| | 24 | B4 | 22.15 | 21.15 | -1.00 | -1.90 | 2.432 | |
| | 36 | B5 | 22.54 | 22.82 | 0.28 | -0.62 | 0.806 | |
| (Ps29) C | 6 | C1 | 21.03 | 22.14 | 1.11 | 0 | 1 | 1.280 ± 0.55 |
| | 12 | C2 | 21.44 | 27.59 | 6.15 | 5.04 | 0.901 | |
| | 18 | C3 | 23.34 | 25.4 | 2.06 | 0.95 | 1.747 | |
| | 24 | C4 | 20.39 | 19.82 | -0.57 | -1.68 | 2.200 | |
| | 36 | C5 | 22.21 | 28.6 | 0.39 | -0.72 | 0.715 | |
| P-value | | | 0.6741NS | | | | | |

**NS: Non-significant**

## 2. CONCLUSIONS

*Pseudomonas aeruginosa* was the main isolated bacteria from burn and wound infections. *P. aeruginosa* isolated from different clinical cases, was able to produce a biofilm at a high percentage.

The molecular analysis for a biofilm associated gene in *P. aeruginosa* isolates revealed that they percent at a high percentage.

The detection of gene expression for *PilA* gene at different time revealed that high gene expression occurred between 18-24 hrs.

## 3. RECOMMENDATIONS

The molecular studying for other biofilm associated gene in multidrug resistance *P. aeruginosa* isolates.

*In-vivo* studying of the pathogenicity of biofilm producer *P. aeruginosa* isolatescontaining a biofilm gene compared with a mutant defective isolate.

Studying the effect of other factors like temperature and pH on *PilA* gene expression.

Studying the effect of resistance antibiotics on *PilA* gene expression

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