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A Phenotypic and Molecular Study of Biofilm Production in *Pseudomonas aeruginosa* Isolated from Some Selected Hospital Wastewater Samples in Baghdad, Iraq

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Abstract

Hospital wastewater represents a unique type of waste that is extremely harmful to the general public it contains a wide variety of microbial species and related genomes. This work aimed to characterize the production of biofilm in hospital wastewater isolates of *Pseudomonas aeruginosa* at the genotypic and phenotypic levels. The strains were identified with cultural and biochemical characteristics, microscopic patterns, and the Vitek 2 System. The isolates were assessed for the measurement of the adhesion of cells to the microtiter plates. Polymerase Chain Reaction was used to determine quorum sensing (QS) genes. The current findings indicated that of the 70 *P. aeruginosa* isolates, 78.57% (55/70) were biofilm producers: 27 (47.27%) were classified as weak producers, and the rates of moderate and strong producers were 19 (34.54%) and 11 (20%), respectively. Furthermore. All examined isolates positively produced the QS genes. According to the findings, biofilm served as a survival mechanism for bacteria in hospital wastewater, providing bacteria with more favorable habitats than planktonic forms. It also implied the significance of QS in the production and persistence of *P. aeruginosa* biofilms by allowing the density of populations to be recognized. Therefore, the detection of these genes and their correlation with the production of biofilm may be useful in restricting the spread of pathogenic bacteria in wastewater.

Keywords: Hospital wastewater, Environmental Surveillance, *Pseudomonas aeruginosa*, Microbial Biofilms, Quorum sensing

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Introduction

Hospital wastewater poses a significantly bigger danger to ecosystems and ecological balance compared to urban sewage (1). Various units and services in medical facilities demand a considerable quantity of water volume for the numerous tasks within hospitals, resulting in an immense quantity of wastewater, which serves as a reservoir for a variety of pathogenic microbes and viruses, radioactive elements, toxic organic pollutants, and pharmaceutical compounds (2,3). The abundance of harmful bacteria in hospital wastewater highlighted the possible risk to the aquatic ecosystem caused by effluent release into receiving water (4).

Indeed, in the majority of developing countries, including Iraq, Hospital wastes are frequently poured into city sanitation systems and released into aquatic ecosystems without any type of management intended to lower the dangers to the public's health

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in the majority of developing countries, including Iraq (5).

Recently, environmental isolates have become the center of attention. The water environment, particularly wastewater, serves as a pool for antibiotic- and biofilm-forming drug-resistant microorganisms and their genes (6). An important source is clinical wastewater, where discharge from various units contains high concentrations of nutrients that can enable resistance genes to transfer between human pathogens and microorganisms in the environment (7). Excessive microbial load and the release of antibiotics at sub-therapeutic doses in wastewater may affect resistance selection (8).

Several previous research investigations demonstrated that *P. aeruginosa* was the most strongly predominant microbe in untreated hospital wastewater and is rapidly spreading in various environmental compartments, confirming that in the absence of an efficient hospital wastewater treatment system, resistant microbes are released into public sewage networks and could act as potential conduits for the genetic exchange and development of antibiotic resistance (9,10,11). Nevertheless, it tends to be more prevalent in hospital environments due to versatile characteristics that enable their persistence, such as developing resistance to different antibiotics, withstanding antiseptics, temperature, and high salt concentrations (12,13).

The significance of *P. aeruginosa* as emergent waterborne pathogens with health, economic, and ecological significance is predicated mainly on their capacity to thrive in a biofilm (14). Biofilms are referred to as complex communities of microorganisms adhered to surfaces by the self-production of an extracellular polymeric matrix, which often serves as a remarkable feature of this bacteria that boosts survival capacity and adaptation (15). Growth within biofilms results in a high genetic variety, which raises the possibility of adaptation to environmental challenges (16). This clarifies why Pseudomonas species persist and thrive in the water system despite the disinfection.

In current years, it has been demonstrated that biofilm formation is a highly controlled and stepwise mechanism managed by cell-cell signaling commonly referred to as a quorum sensing (QS) mechanism (17,18). The two identified QS systems present the *rhl* and *las* (19). The synthesis of the homoserine lactone (3-oxo-C12) is controlled by the *LasI* system, which is crucial for the production of biofilms. It engages in interaction with the *LasR* activator, producing positive feedback for the activator and the activation of numerous virulence genes such as *aprA, tox, lasB*, and *lasA*. Likewise, when combined with *RhlR*, diffusible signal C4-HSL, which the *rhlI* product produces, induces the expression of *rhlI* and *rhlAB* genes (20,21,22).

Even though many research studies have been conducted on clinical isolates there is limited data on environmental isolates of *P. aeruginosa*. Characterizing the variety of pathogenic bacteria in hospital wastewater, particularly *P. aeruginosa*, is crucial for environmental evaluation and monitoring and is also a fundamental first step in comprehending how resistant bacteria develop and spread in environments, which enables the establishment of strategies to mitigate this process (23). Therefore, it was critical to evaluate both the phenotypic biofilm production as well as the prevalence of the previously mentioned QS genes in environmental isolates from selected hospital wastewater. This work aims to detect the frequency and existence of *P. aeruginosa* in selected government hospital wastewater samples and to examine the biofilm formation and various QS genes of these strains.

Material and Methods

Collection of Samples

Eight samples of untreated wastewater were obtained from the main outlet pipes of two public hospitals, namely Al-Yarmouk Teaching Hospitals

and Al-Kindy Teaching Hospitals, located in Baghdad, Iraq, during the period from June 2023 to October 2023. Wastewater treatment systems are not equipped in the selected hospitals, and therefore, untreated effluents are dumped directly into the municipal sewer network. The samples were obtained from the respective sources in 100 ml sterile glass containers and transferred in a cooling box to the laboratory for microbiological examination for 2 hours.

Isolation and Identification of *P. aeruginosa*

An aliquot of 10 mL of hospital effluent has been mixed with 90 mL of Tryptone Soya. Broth (Liofilchem, Italy) and incubated aerobically for 24 hours at 37 ºC. Subsequently, a 100 µl aliquot of bacterial suspension serially diluted ten-fold was streaked on Cetrimide agar (Oxoid, Hampshire, England) and left to incubate aerobically at 37 ºC for a period of 24 to 48 hours. Afterward, nonselective blood agar (Oxoid, Hampshire, England) was used for the cultivation of presumed flat, big, blue-green-colored colonies with irregular edges (24,25). Six presumed colonies (or all colonies if fewer than six were observed) have been transferred to blood agar from all positive Cetrimide agar plates. Gram staining and biochemical tests were used to identify suspect colonies on blood agar. The Vitek 2 System was implemented to complete the identifying process.

Biofilm Assay

The formation of biofilm was investigated following the methodology outlined by a previous study (26), with minor modifications. A medium consisting of trypticase soy broth that was enriched with 1% glucose was used to cultivate bacteria that form biofilms. Following 24 hours of incubation at 37 degrees Celsius, the suspensions were diluted at 1/100 with sterile, 1% glucose-enriched trypticase soy broth. 200 microliters of a diluted suspension were dispensed into each well of sterile 96-well microplates (SPL, Korea). However, negative controls consisted only of sterile TSB media. Each isolated biofilm was assayed in triplicate. Following that, the plates were encased and subjected to incubation again. After discarding the solution content, two hundred microliters of sterilized phosphate-buffered saline were used to rinse the wells. Subsequently, absolute methanol was used to fix the generated biofilms. The plates were washed with PBS after fifteen minutes, and then they were allowed to dry completely before being utilized. Using 200 microliters of a crystal violet solution with a concentration of 1%, the wells were dyed.

Ultimately, stained biofilms have been dissolved in glacial acetic acid (33% v/v). Using an ELISA reader, each well's optical density (ODw) value was determined, with measurements taken at a wavelength of 570 nm. According to the ODw, there are four distinct groups into which the strains have been classified as shown in Table 1.

Table 1: Bacterial Biofilm Production Categorization.

Cut-Off Value Determination	Mean of OD values	Intensity
$OD \leq 0.075$	$OD \leq 0.075$	None producer
$ODc < OD \leq 2 * ODc$	$0.075 < OD \leq 0.144$	Weak
$2 \times ODc < OD \leq 4 \times ODc$	$0.144 < OD \leq 0.276$	Moderate
$OD > 4 \times ODc$	$OD \geq 0.276$	Strong

PCR assay:

The existence of QS genes was examined in all detected isolates. Specifically, *lasR, rhlI, rhlR, rhlAB, aprA, lasB* and *lasI*. DNA extraction was carried out following a G-spinTM extraction kit (iNtRON, Korea). The primers employed for determining genes are documented in Table 2.

A total volume of 20 µl was used to establish the reaction mixtures. Specifically, 2µl of DNA extracted from wastewater isolates was added to 8 µl of the master mix (BioNeers, USA), 0.5µl of magnesium chloride (MgCl2), and 7.5 µl of deionized water (ddH2O). After that, 2µl of both primers was added to the resultant mixture and then placed in the thermocycler (Biometra, Germany) for 5 minutes at 94°C. PCR programs for detecting respective genes are represented in Table 3.

The PCR products were stained with RedSafe™ (iNtRon Biotechnology, Korea), 1% agarose gel (Merck, Darmstadt, Germany) electrophoresis, and photographed under UV light. Images were digitally captured using the Gel Doc apparatus.

Table 2: The primer sequences

Target	Primer sequence (5′→3′)	Size of Amplicon (bp)	References
lasI	CGT GCT CAA GTG TTC AAG TAC AGT CGG AAA AGC CCA G	295	(27)
rhlAB	TCA TGG AAT TGT CAC AAC CGC ATA CGG CAA AAT CAT GGC AAA C	151	(27)
aprA	ACC CTG TCC TAT TCG TTC C GAT TGC AGC GAC AAC TTG G	140	(27)
lasR	AAG TGG AAA ATT GGA GTG GAG GTA GTT GCC GAC GAC GAT GAA	130	(27)
rhlI	TTC ATC CTC CTT TAG TCT TTC C TTC CAG CGA TTC AGA GAG C	155	(27)
lasB	TTC TAC CCG AAG GAC TGA TAC AAC ACC CAT GAT CGC AAC	153	(27)
rhlR	TGC ATT TTA TCG ATC AGG GC CAC TTC CTT TTC CAG AGA G	133	(27)

Table 3: PCR Protocol

Step	Temperature (°C)	Time	Cycles
Initial denaturation	94 ºC	5 min	1
Denaturation	94 ºC	30 sec	35
Annealing	55 ºC	30 sec	
Extension	72 ºC	30 sec	
Final extension	72 ºC	5 min	1

Results

Bacterial Isolation and Identification

A series of confirming tests were carried out to demonstrate that only 70 (67.96%) of the 103 environmental isolates were positive for *P. aeruginosa* culture. The bacterial smear preparations were Gram-negative rods, non-spore producing, and arranged single or in short chains. On solid media, the colonies exhibited flat edges, a high appearance, and were generally thin, rough, or smooth, while some were mucoid in appearance. All isolates were capable of growing on MacConkey agar, in which *P. aeruginosa* non-lactose ferment grew pale yellowish. After that, re-cultured on nutrient agar and then incubated at 42°C. The grown colony exhibited a regular ring shape and creamy color, and most of them produced pyocyanin (water-soluble pigment). Moreover, the isolates indicated a greenish-yellow color and showed positive for urease (slow hydrolysis of the urea), positive for citrate, and negative for TSI and indole. Finally. The Vitek II compact system's result matched the results from biochemical identifications and the findings demonstrated that 70 (67.96%) of isolates belonged to environmental *P. aeruginosa*.

Phenotypic Analysis of Biofilm Formation

All 70 environmental bacterial isolates were assayed to establish a biofilm model using convenient and quantitative pre-sterilized 96-well polystyrene microtiter plates, which is recognized as a standard technique for detecting biofilm biomass readily and accurately. Out of 70 isolates, 55 (78.57%) were biofilm producers: Of those who could create biofilms, 11 (20%) exhibited a strong ability, 19 (34.54%) experienced a moderate capacity, and 27 (47.27%) possessed a weak capability (Table 4).

Table 4: The percentage intensity in isolates

No. of Isolates	Weak (%)	Moderate (%)	Strong (%)	Total No. of biofilm producers
70	27 (47.27%)	19 (34.54%)	11 (20%)	55 (78.57 %)

Molecular Analysis

In the current data, all 70 isolates (100%) harbored QS genes (*lasI, lasR, rhlR, rhlI, rhlAB, lasB,* and *aprA),* as demonstrated in Fig (1, 2,3,4,5,6,7).

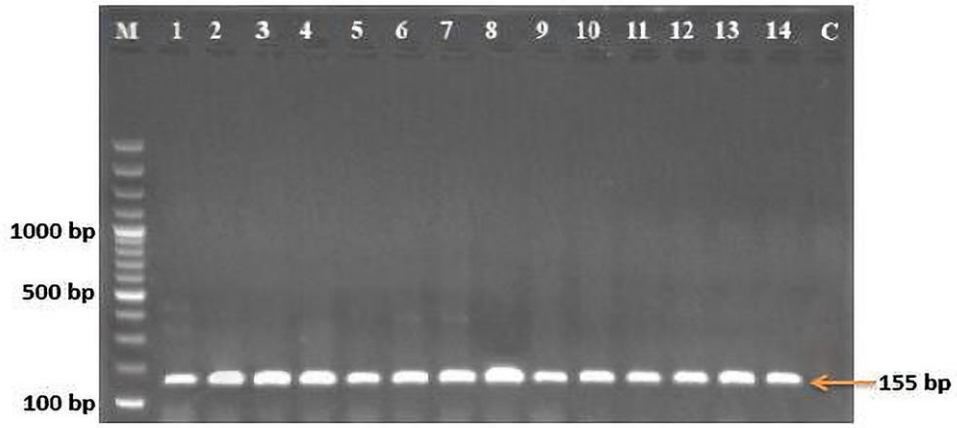

Figure 1: Gene amplified by electrophoresis. Visualization was accomplished using 1% agarose electrophoresis and ethidium bromide staining at 75 V for 1 hour. 100 bps DNA ladder is the marker for Lane M. Positive samples for the *rhlI* gene (155 bp) are shown in lanes 1-14. Lane N: Negative control.

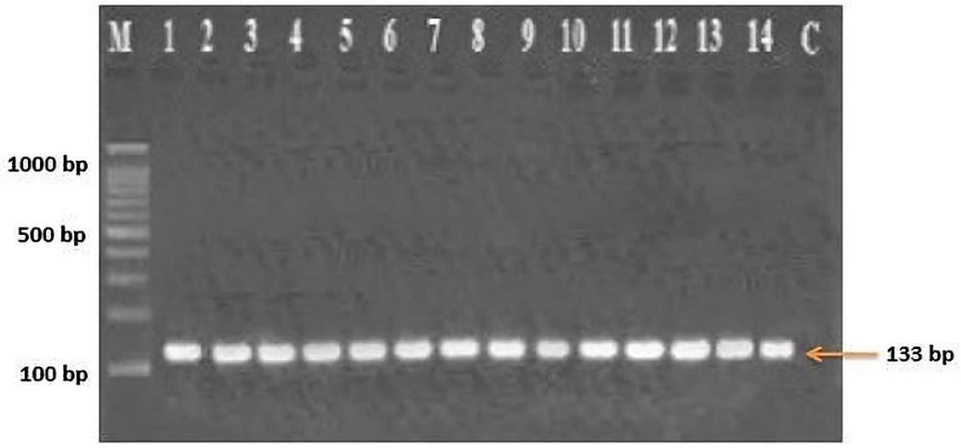

Figure 2: Gene amplified by electrophoresis. Visualization was accomplished using 1% agarose electrophoresis and ethidium bromide staining at 75 V for 1 hour. 100 bps DNA ladder is the marker for Lane M. Positive samples for the *rhlR* gene (133bp) are shown in lanes 1-14. Lane N: Negative control.

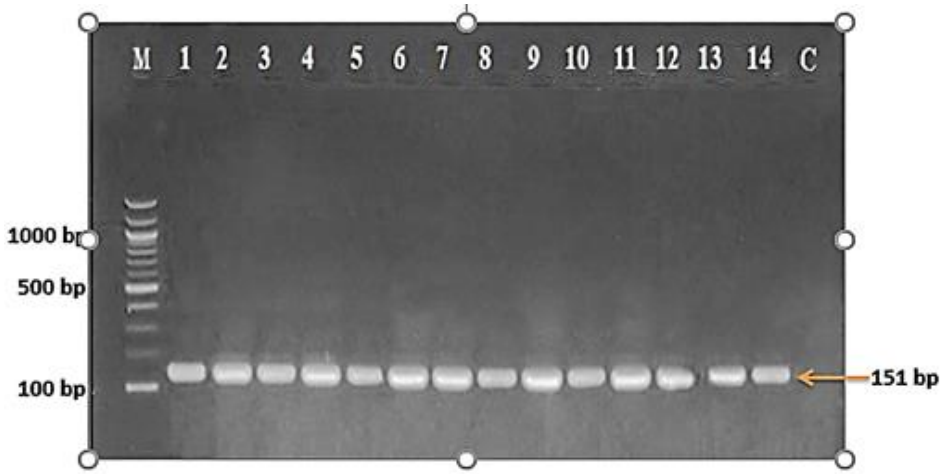

Figure 3: Gene amplified by electrophoresis. Visualization was accomplished using 1% agarose electrophoresis and ethidium bromide staining at 75 V for 1 hour. 100 bps DNA ladder is the marker for Lane M. Positive samples for the *rhlAB* gene (151bp) are shown in lanes 1-14. Lane N: Negative control.

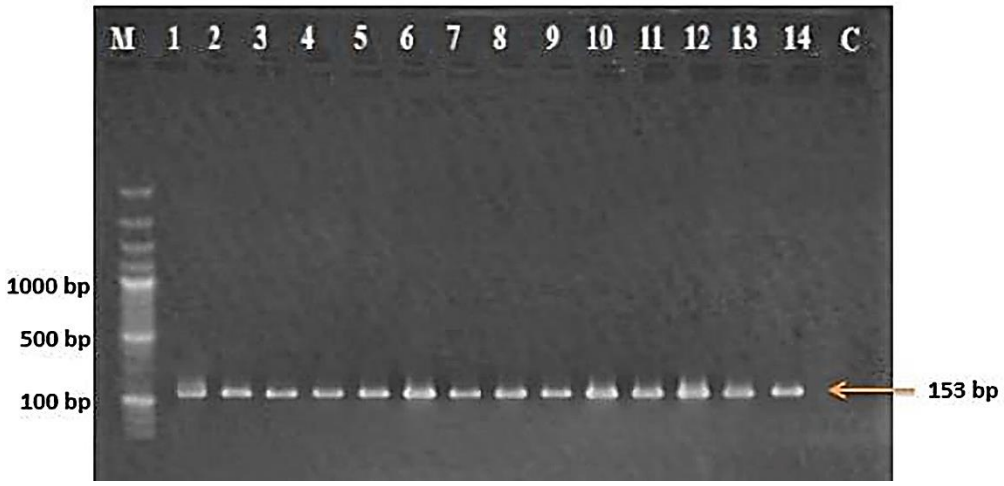

Figure 4: Gene amplified by electrophoresis. Visualization was accomplished using 1% agarose electrophoresis and ethidium bromide staining at 75 V for 1 hour. 100 bps DNA ladder is the marker for Lane M. Positive samples for the *lasB* gene (153bp) are shown in lanes 1-14. Lane N: Negative control.

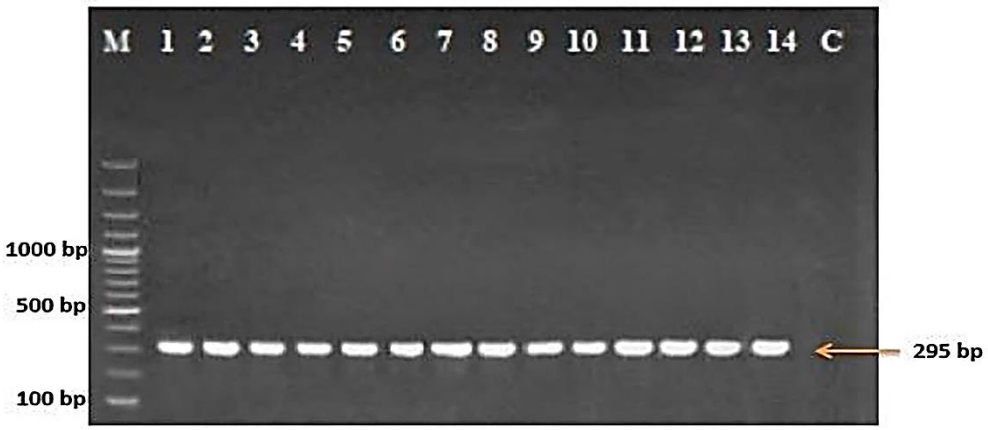

Figure 5: Gene amplified by electrophoresis. Visualization was accomplished using 1% agarose electrophoresis and ethidium bromide staining at 75 V for 1 hour. 100 bps DNA ladder is the marker for Lane M. Positive samples for the *lasI* gene (295bp) are shown in lanes 1-14. Lane N: Negative control.

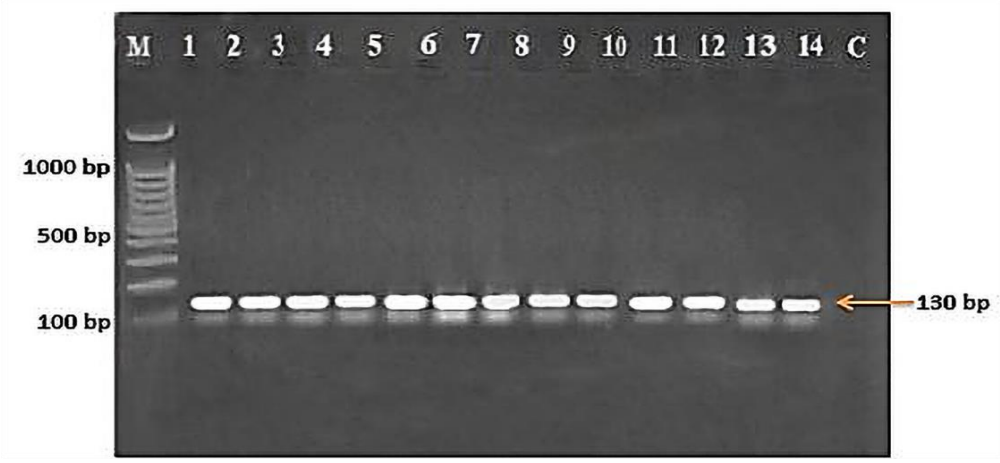

Figure 6: Gene amplified by electrophoresis. Visualization was accomplished using 1% agarose electrophoresis and ethidium bromide staining at 75 V for 1 hour. 100 bps DNA ladder is the marker for Lane M. Positive samples *lasR* gene (130bp) are shown in lanes 1-14. Lane N: Negative control.

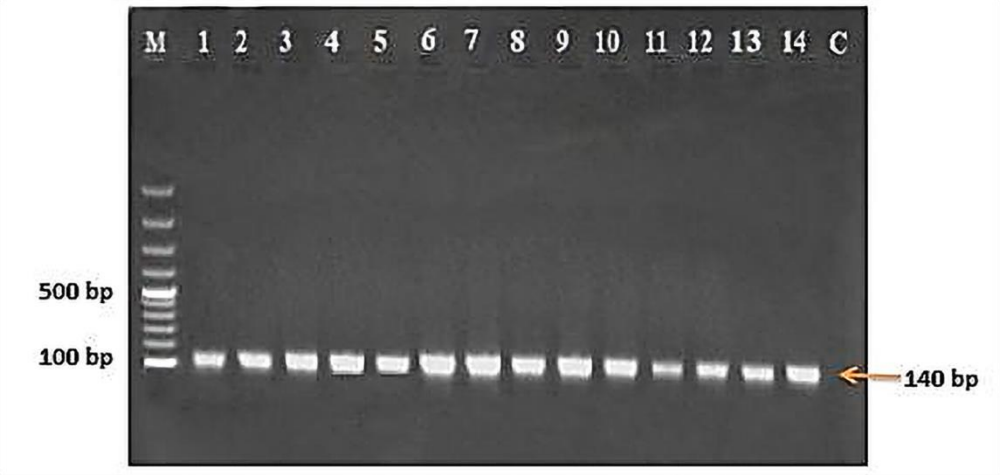

Figure 7: Gene amplified by electrophoresis. Visualization was accomplished using 1% agarose electrophoresis and ethidium bromide staining at 75 V for 1 hour. 100 bps DNA ladder is the marker for Lane M. Positive samples *aprA* gene (140bp) are shown in lanes 1-14. Lane N: Negative control.

Discussion

Hospitals are essential to the well-being of humankind, which includes improvements in health research. The lack of implementation of obligatory wastewater treatment before release into the environment is archaic, as the presence of pathogenic microbes in untreated wastewater above the allowed limit is typically not accidental (28).

Bacterial dispersion, reservoirs, and their environmental dispersal are among the most relevant issues (29). Numerous outbreaks of *P. aeruginosa* disease have been reported, and they have been linked to both environmental and hospital-acquired sources (30, 31, 32).

In the current investigation, the high prevalence of 70 (67.96%) isolates is a concerning situation that reflects the environmental contamination of hospital wastewater by pathogenic bacteria. Our results support another study in Brazil that revealed a high prevalence (68.33%) of isolates in hospital effluents (33). Likewise, Hosu *et al*., (34) and Adesoji *et al*., (35) indicated the highest occurrence rates of *P. aeruginosa* of 65.4% and 66.7%, respectively, from abattoir wastewater, surface water, and sewage. This is expected since healthcare environments represent a unique chance for pathogens to flourish (36,37,38). Nevertheless, the previous studies achieved by, Zhang *et al*., (39) and Mapipa *et al*., (40) on hospital effluents revealed a prevalence rate of 29.4%, and 31%, respectively, which is less than that found in the present finding. This disparity in prevalence rates among research investigations can be linked to variances in sanitary practices and geographical location (41,42,43).

Among the most significant aspects of microbiological pathogenesis is the capacity of the organism to organize itself into a biofilm, which promotes the survival and persistence of bacteria in many environmental niches (44,45). Internal water networks can enhance the establishment of bacterial biofilm under a variety of favorable circumstances, including the availability of nutrients, hot water temperatures, and water (46). The present investigation showed that biofilm production was indicated in 78.57% of the isolates tested.

The sensitivity of the MTP approach to measuring the small quantities generated may be contributing to the high productivity of biofilm production in this

study. It was regarded as a quantitative gold common technique for detecting the earliest stages of biofilm formation (47,48).

The current data are aligned with those carried out by Karami *et al*., (49), who revealed that 80% of environmental isolates were positive for biofilm. Whereas Okafor *et al*., (50) and Divyashree *et al*., (51), revealed that 57.40% and 39.39% of isolates recovered from the environment and wastewater of government hospitals were biofilm producers, which is less than our observation.

The biofilm adhesion characteristics of the current data revealed a different group distribution that was categorized as weak, moderate, and strong. Similar to our findings, Elnegery *et al*., (52) demonstrated that among 96% of the isolates that produced biofilm, 44% created weak biofilm, 14% produced moderate biofilm, and 38% developed strong biofilm. Likewise, Lima *et al*., (53) conducted that 77.5% established biofilm, with 7.5% developing strong biofilm, 27.5% developing moderate biofilm, and 42.5% developing weak biofilm. Contrary, Bahador *et al*., (54) found that strong producers made up 60% of the isolates that formed biofilm.

One complicated virulence mechanism is biofilm production, which necessitates gene regulation by many QS system components. This crucial bacterial virulence factor can be produced less frequently as a result of mutations or altered gene expression in the QS network-related genes (55, 56, 57). The genotypic examination of 69 environmental isolates in this study showed that all seven examined genes exhibited 100% detection rates.

Regarding the existence of QS genes, the aforementioned percentage of isolates is consistent with research done by Rodrigues *et al*., (58), who analyzed 31 isolates and demonstrated that 100 % (*lasR, lasI, rhlR,* and *rhlI*) were detected in the isolates. Furthermore, Dolatshah *et al*., (59) found *aprA, rhlI, lasB, rhlAB, lasR,* and *lasI* genes in 100% of examined isolates. By contrast, the results conducted by Ghanem *et al*., (60) in Egypt, revealed that QS gene occurrence rates were lower than those obtained in our study, which 94% for the *rhlR* gene, 85.6% for the *aprA* gene, 69.6% for the *rhlR* gene and 81.6% for the *lasI* and *lasB* genes.

Concerning the current study, all isolates included the genes required for the organization of biofilms. However, 21.42% of the samples were unable to form biofilms. This might happen mainly because these genes cannot be expressed or because there are mutations in the regulatory genes (61). On the other hand, it has been previously confirmed that the isolates missing the *lasI* and *lasR* genes failed to produce a biofilm, indicating that these genes are critical in QS and biofilm development (62). Furthermore, Persyn *et al*., (63) noted that isolates exhibiting mutations in QS system genes showed a lower level of virulence.

Conclusion

In this study, our data revealed that Hospital effluent could act as a reservoir for pathogenic bacteria. It was also observed that there was a high tendency among *P. aeruginosa* environmental isolates to produce biofilm, with the majority being weak producers. However, QS genes were detected in all tested isolates, which may boost environmental adaptation and biofilm production.

The frequency of genes linked to biofilm-forming bacteria in hospital effluent remains a risk to public health as they persist and spread to other environments. To develop an innovative system for monitoring water quality, we advise government health officials to take the environmental spread of these harmful microorganisms seriously by ensuring that all hospitals have treatment plants for wastewater.

Conflict of interest:

There are no conflicts of interest.

Funding: None

Ethical approval

The project received approval from the local ethical commission at Al-Nahrain University, Iraq.

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