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Article

Variations in Temperature, pH, and Osmotic Pressure Contribute to Changes in *Pseudomonas aeruginosa* Autofluorescence via Mechanisms Involving Regulation of the *pvdD/pvdS* Gene

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Abstract: *Pseudomonas aeruginosa* is a bacteria that is frequently detected in chronic wounds. Bacterial infection can impede wound healing and, in severe cases, result in fatalities. Bacterial fluorescence imaging is a technique used to detect the cyan fluorescence emitted by *P. aeruginosa*, thereby assisting clinicians in improving wound treatment. Our previous observations revealed an intriguing phenomenon whereby bacteria exhibited distinct fluorescence characteristics despite the presence of an identical bacterial load in the wound. To investigate whether these alterations in fluorescence intensity were influenced by varying bacterial environments, we conducted a study using image J software to analyze the changes in fluorescence intensity of *P. aeruginosa* colonies under different conditions, including temperatures (25°C, 31°C, 37°C, 43°C), pH levels (5, 6, 7, 8, 9), and osmotic pressures (0.9%, 1.8%, 2.7%, 3.6%). Additionally, we assessed the expression levels of pyoverdine synthetase genes (*pvdD*) and the sigma factor of pyoverdine genes (*pvdS*), which determine the fluorescence intensity of *P. aeruginosa* under these varying conditions. Our experimental findings demonstrated that the bacterial fluorescence intensity was significantly influenced by the culture environment, with the ability to be enhanced or attenuated by modifying the growth conditions. Moreover, there was a strong correlation between the fluorescence intensity of the bacteria and the expression level of key enzyme genes. Consequently, when implementing fluorescence imaging systems in clinical settings, it is essential to consider the changes in the wound microenvironment.

Keywords: *Pseudomonas aeruginosa*; temperature; pH; osmolality; autofluorescence

1. Introduction

Chronic wounds are commonly caused by underlying conditions (such as diabetes, venous diseases, and immune system disorders), local pressure, and ischemia. The healing time for these wounds typically ranges from several months to years[1]. According to data from the World Health Organization, there are approximately four million people worldwide affected by chronic wound diseases annually. These wounds often result in discomfort symptoms such as pain and itching, and may even lead to severe complications including infection and deep tissue damage. Such complications have a significant impact on patients' health and quality of life, imposing a substantial economic burden on healthcare systems [2–4].

Bacterial infection is a crucial factor contributing to the non-healing of wounds. Bacteria proliferate on the surface and within wounds, forming biofilms and producing virulence factors [5]. Biofilms consist of bacteria, extracellular matrix, and other substances that protect bacteria from external influences, provide nutrients and oxygen, and enhance bacterial resistance to antibiotics[6–8]. *P. aeruginosa* is one of the most prominent pathogens found in the chronic wound environment [9]. In a retrospective analysis by Jockenhöfer et al., *P. aeruginosa* was detected in one-third of the patients with lower limb venous ulcers[10]. Similarly, Gjødsbøl et al. found *P. aeruginosa* in more than half of the patients with persistent venous leg ulcers in their longitudinal study[11]. *P. aeruginosa* commonly exists in the natural environment and adheres to wound surfaces through flagella, adhesins, and polysaccharides when the skin barrier is compromised, rapidly multiplying and forming biofilms[12]. If left untreated, the virulence factors produced by bacteria can further invade tissues, leading to more severe complications such as sepsis and infective endocarditis, posing a greater threat to patients' health[13,14].

In diagnosing wounds, clinicians primarily rely on observing patients' clinical signs to make decisions. Signs of infection include abnormal granulation tissue, purulent exudate, elevated temperature, edema, and erythema[15,16]. This method heavily relies on the clinical experience of the attending physician. Wound culture is considered the gold standard for infection diagnosis. Traditional culture methods include swab culture, needle aspiration, and tissue biopsy techniques, which are relatively inexpensive and have high diagnostic efficiency. However, these methods require a longer culture time, cannot provide immediate feedback on wound infection, and are invasive, causing discomfort to patients[17]. Various laboratory biomarkers, including C-reactive protein, procalcitonin, presepsin, and bacterial protease activity, have been quantified to assist in diagnosing infection [18–21]. Additionally, imaging techniques such as computed tomography, magnetic resonance imaging, ultrasound imaging, spatial frequency-domain imaging, thermography, automated fluorescence imaging, and biosensors have emerged for real-time diagnosis of wound infection, demonstrating unique advantages in deeper wound infection diagnosis [22–26]. While these methods are non-invasive and highly accurate, they tend to be expensive and require specialized technicians to operate.

An instant handheld bacteria fluorescence imaging device can provide real-time monitoring of bacterial information within wounds, assisting clinicians in making precise clinical decisions, with *P. aeruginosa* showing as green fluorescence in the images. According to a prospective clinical trial by Raizman et al., 26 out of 28 wound samples exhibited green fluorescence, and *P. aeruginosa* was confirmed by culture-based diagnosis, with a positive predictive value exceeding 90% [27]. Hurley et al. examined chronic wounds at an outpatient plastic surgery center and found that all wounds displaying green fluorescence contained *P. aeruginosa*, with a sensitivity and specificity of 100%[28]. In previous studies, pyoverdine was considered the source of green fluorescence production[29]. Its primary function is iron uptake, and once secreted, it forms stable complexes with extracellular free ferric ions, transporting iron ions into the bacterial cells[30]. Other studies have shown that pyoverdine plays an important role in immune evasion and biofilm formation of *P. aeruginosa* [31]. The synthesis level of pyoverdine is crucial for the localization of *P. aeruginosa* in wounds. According to a recent study by Emily Pham, the synthesis of pyoverdine is closely associated with pyoverdine synthetase and sigma factor of pyoverdine expression. When the genes encoding these two factors are knocked out, the green fluorescence of *P. aeruginosa* is almost completely abolished, confirming their contribution to the green fluorescence[32].

Different types of wounds and local treatments result in diverse and complex microenvironments, reflected by changes in wound temperature, pH, and osmotic pressure [33–36].Studies by Tayiba Gillani et al. showed that temperature and pH would affect the production of pyoverdine synthesis by *P. aeruginosa*. Under the optimal temperature of 37℃ and the optimal pH of 7, the production of pyoverdine was the highest, and low temperature and acidic environment would reduce the synthesis of pyoverdine[37]. Studies have shown that the growth and biofilm formation of *P. aeruginosa* PAO1 are inhibited in acidic environments. In pH 6.8 and weakly alkaline

environment, pH has no significant effect on the growth of PAO1, but these conditions have a positive effect on the formation of biofilm[38].

In a recent clinical observation using the MolecuLight i:X, some interesting phenomena were discovered, where wounds with the same bacterial load exhibited different intensities of green fluorescence, as shown in Figure 1 (Photos were provided by the First Affiliated Hospital of Anhui Medical University), Under the same conditions of *P. aeruginosa* infection load, the fluorescence intensity of wounds treated with 0.25% acetic acid wet gauze was significantly weaker than that of wounds not treated with 0.25% acetic acid wet gauze(Fig.1A and Fig.1B). Although the autofluorescence intensity of wound C is higher than that of wound D, bacterial load measurements indicate that both wounds harbor equivalent levels of bacteria. The distinction lies in the fact that the temperature of wound D is lower due to local vascular pathology (Fig.1C and Fig.1D).
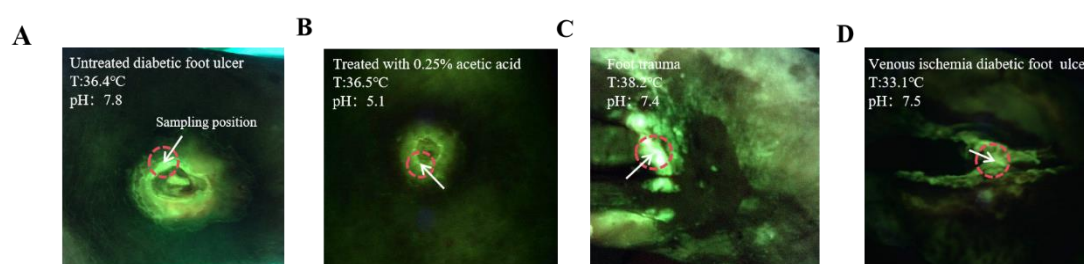
Figure 1. Under varying infection microenvironments, equivalent quantities of *P. aeruginosa* demonstrate disparate autofluorescence intensities. (A) Fluorescent image of diabetic foot patients infected with *P. aeruginosa.* (B) Fluorescent image of diabetic foot patients infected with *P. aeruginosa* after treatment with 0.25% acetic acid wet gauze. (C) Fluorescent image of the patient's wound infected with *P. aeruginosa* due to trauma. (D) Diabetic foot ulcer wound infected with *P. aeruginosa* in local venous ischemia.

Based on this patient's clinical observation and previous research, whether different wound microenvironments affect the fluorescence intensity of *P. aeruginosa* is unclear. In this study, an in vitro bacterial culture model was established to investigate the effect of culture temperature, pH, and osmotic pressure on the fluorescence intensity of *P. aeruginosa*. The expression levels of pyoverdine synthetase and sigma factor of pyoverdine genes under different conditions were analyzed. The results of this study elucidate the impact of changes in wound infection microenvironment on bacterial fluorescence, thereby enhancing the accuracy and treatment effectiveness of this technology in future clinical applications.

2. Results

2.1. Autofluorescence of P. aeruginosa Under Normal Growth Conditions

To investigate the temporal changes in bacterial fluorescence under standard culture conditions (temperature: 37 ℃, pH: 7, osmolality: 0.9%), the autofluorescence images were captured using the MolecuLight i:X at 12, 20, 28, 36, 44, 52, 60 and 68 hours post-inoculation, respectively. The entire process was conducted in a dark environment, and the results are presented in the Figure 2A. A quantitative analysis of the average fluorescence intensity at different time points revealed that the autofluorescence intensity of *P. aeruginosa* exhibited a gradual increase with the extension of the incubation time, reaching a peak at 36 hours. Following this, the fluorescence gradually decreased, as illustrated in the right Fig. (Figure 2B).

A

B
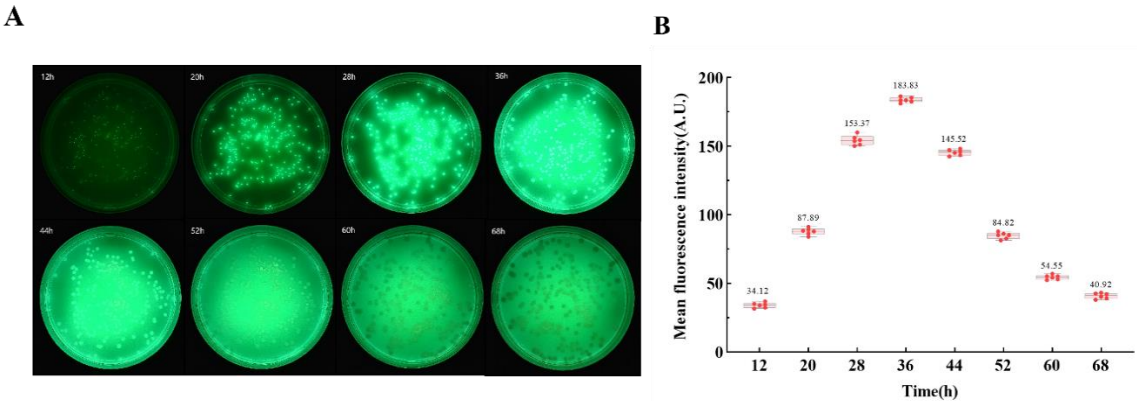
Figure 2. Green fluorescence emitted from *P. aeruginosa* over an 68h period.

2.2. Effect of Temperature on Autofluorescence of P. aeruginosa

The inoculated *P. aeruginosa* was cultivated at ambient temperatures of 25, 31, 37, and 43°C. Autofluorescence images of *P. aeruginosa* were captured at various time points, with the results depicted in Figure 3A. An analysis of the average fluorescence intensity over time indicated that the fluorescence of *P. aeruginosa* gradually increased, ultimately reaching a peak. Compared with the control group, the final fluorescence peak of *P. aeruginosa* was not significantly different at 37°C and 43°C, the fluorescence intensity of *P. aeruginosa* at 25°C and 31°C decreased significantly compared with that at 37°C Fig. However, higher culture temperatures correlated with shorter times to reach the fluorescence peak (Fig. 3B). Before achieving the peak fluorescence intensity, a linear regression of time against fluorescence intensity under different temperature conditions was performed. The slope of this regression provides an indication of the fluorescence production rate under varying temperatures. The data suggests that as the culture temperature rises, the rate of fluorescence production by *P. aeruginosa* also increases (Fig. 3C).
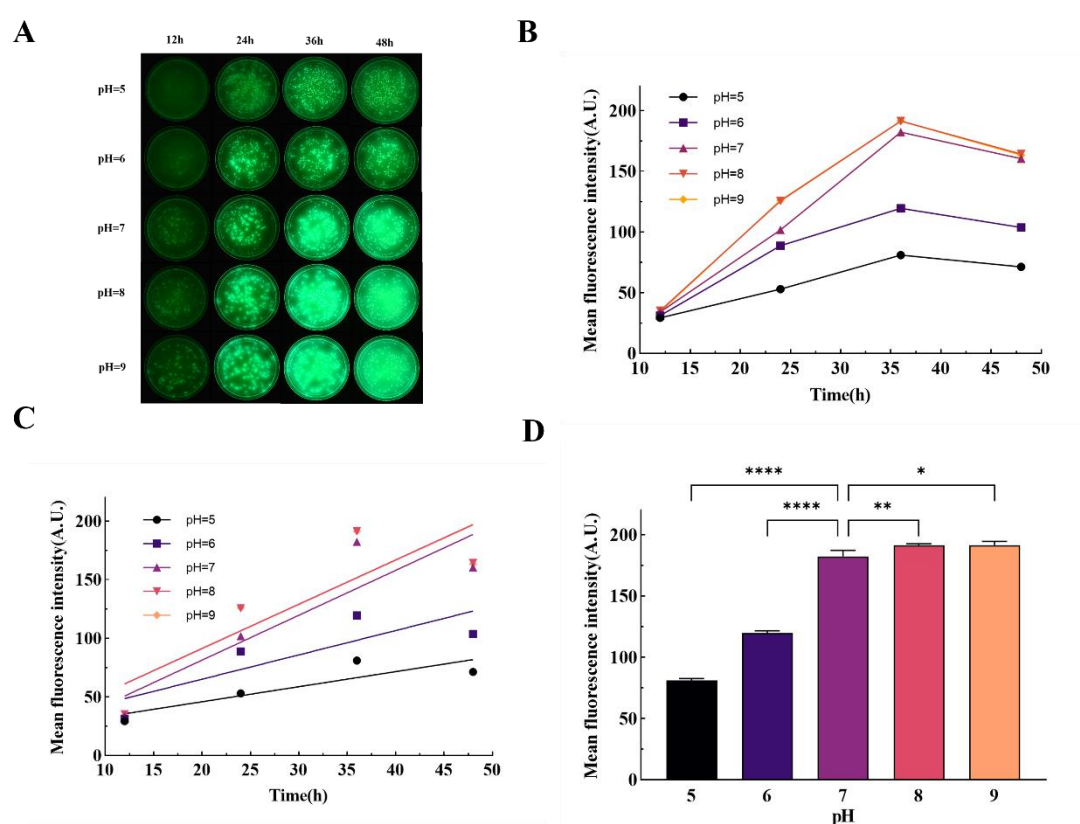
A   **B**

C   **D**
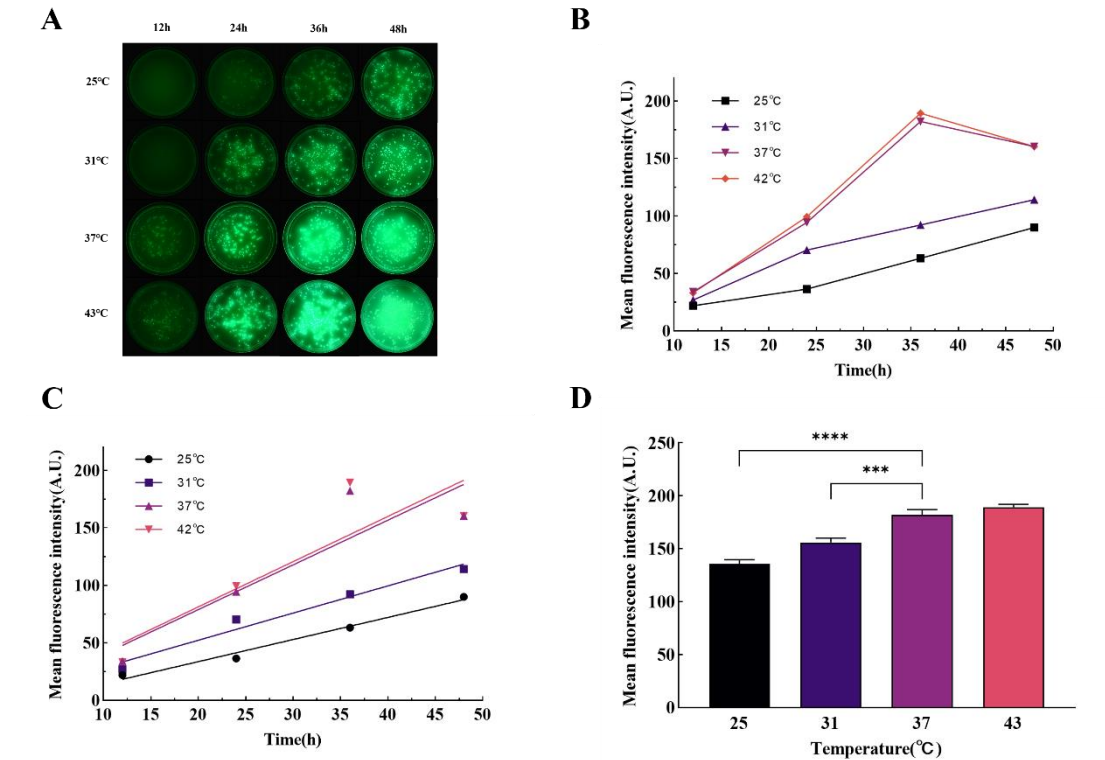
Figure 3. Effect of temperature on *P. aeruginosa* autofluorescence. (A) Variation of bacterial autofluorescence over time. (B) Trends in bacterial autofluorescence intensity changes. (C) Linear fitting results of fluorescence intensity

and time. (D) Maximum fluorescence intensity of *P. aeruginosa* at different temperatures. ***$P < 0.001$, ****$P < 0.0001$ vs. 37℃ normal temperature.

2.3. Effect of pH on Autofluorescence of P. aeruginosa

P. aeruginosa was inoculated into solid media with varying pH levels, and fluorescence images were captured at different time intervals using a fluorescence imaging system. The findings are presented in Figure 4A. *P. aeruginosa* is capable of growing within a pH range of 5 to 9, and all strains of this bacterium can produce fluorescence. In response to diverse pH conditions during cultivation, the autofluorescence intensity of *P. aeruginosa* demonstrated a consistent increase over time until 36 hours, after which it declined from its peak (Fig. 4B). The maximum fluorescence intensity of *P. aeruginosa* gradually increased with the increase of pH value, and the fluorescence intensity under alkaline conditions was significantly higher than that under acidic conditions (Fig. 4B and Fig. 4D). As pH decreases, the rate of fluorescence synthesis slows down. when pH is below 5, the rate of fluorescence increase is greatly slowed down (Fig. 4C).
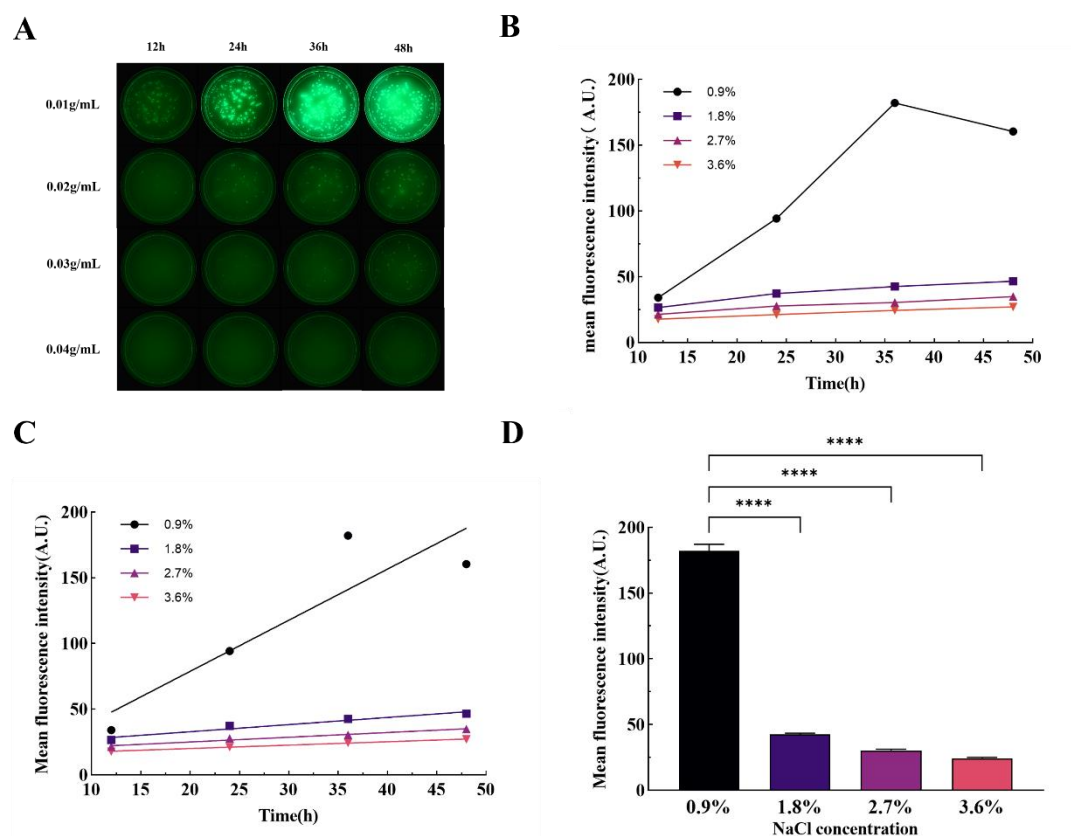

Figure 4. Effect of pH on *P. aeruginosa* autofluorescence. (A) Variation of bacterial autofluorescence over time. (B) Trends in bacterial autofluorescence intensity changes. (C) Linear fitting results of fluorescence and time intensity before peak fluorescence intensity. (D) Differences in peak fluorescence intensity. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ vs. pH=7 normal saline.

2.4. Effect of Osmotic Pressure on Autofluorescence of P. aeruginosa

The autofluorescence of *P. aeruginosa* was monitored over time by inoculating the bacteria in solid medium with NaCl concentration values of 0.9, 1.8, 2.7, and 3.6% (mass fraction). The changes in autofluorescence of *P. aeruginosa* over time were recorded under different osmolality conditions at different time points, as shown in Figure 5A. By analyzing the fluorescence intensity at different time points, it can be observed that the time required to reach the peak fluorescence intensity under different osmolarity conditions remained consistent at 36 hours. The average autofluorescence intensity of *P. aeruginosa* under the same time-point condition of incubation exhibited a notable decline with the increase of osmolarity, when the concentration of sodium chloride exceeds 2.7%,

fluorescence is almost entirely suppressed (Fig.5B and Fig.5D). Prior to reaching the peak fluorescence intensity, a linear fit between time and mean fluorescence intensity was performed for different osmolarity conditions. The slopes of this fit could be used to reflect the rate of fluorescence production under different osmolarity conditions. The results demonstrated that the rate of fluorescence production by *P. aeruginosa* obviously decreased as the osmolarity of the culture increased (Fig.5C)

Figure 5. Effect of osmolality on *P. aeruginosa* autofluorescence. (A) Variation of bacterial autofluorescence over time. (B) Trends in bacterial autofluorescence intensity changes. (C) Linear fitting results of fluorescence intensity and time before peak fluorescence intensity. (D) Differences in time required to reach peak fluorescence intensity. ****P＜0.0001 vs. 0.9% normal saline.

2.5. Effects of Temperature, pH and Osmotic Pressure on pvdD and pvdS Expression in P. aeruginosa

The *pvdD* and *pvdS* genes encode pyoverdine synthetase and sigma factor of pyoverdine, respectively. Both play important roles in the synthesis of pyoverdine. We investigated the effects of temperature, pH, and osmotic pressure at different time points on the expression of the *pvdD* and *pvdS* genes. The expression levels of these two genes were detected by real-time fluorescence quantitative PCR at 12 and 24 hours in *P. aeruginosa*. Compared to the control group, low temperature inhibited the expression of the *pvdD/pvdS* gene, with the greatest inhibition observed at 25°C (Fig. A and Fig. D). The expression level of the *pvdD/pvdS* gene was significantly reduced under acidic conditions, with the lowest expression observed at pH 5(Fig. B and Fig. E). Osmotic pressure had a significant impact on the expression of the *pvdD/pvdS* gene, Under the condition of high osmotic pressure, the expression of *pvdS* gene decreased significantly. When the concentration of sodium chloride exceeded 2.7%, the expression of both genes remained at a very low level (Fig. C and Fig. F). Interestingly, It seems that pH had a greater effect on *pvdD* gene expression than on *pvds* gene expression. The genes *pvdD* and *pvdS* exhibit characteristics of coordinated expression under various influencing factors, potentially related to ensuring the production and secretion of pyoverdine.
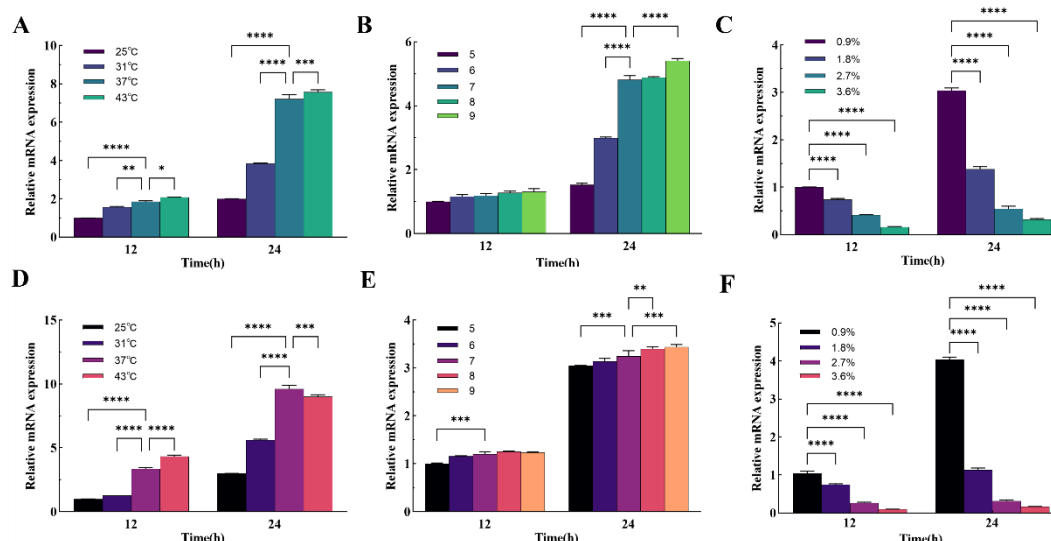
Figure 6. The expression levels of *pvdD* gene and *pvdS* gene under different temperature, pH and osmotic pressure. (A) *pvdD* gene expression under different temperature. (B) *pvdD* gene expression under different pH. (C) *pvdD* gene expression under different osmotic pressure. (D) *pvdS* gene expression under different temperature. (E) *pvdS* gene expression under different pH. (F) *pvdS* gene expression under different osmotic pressure. $^{***}P < 0.001$ vs. 0.9% normal saline or other group, $^{****}P < 0.0001$ vs. 0.9% normal saline or other group.

3. Conclusions

Chronic wound infection is a common problem globally, especially in developing countries and resource-limited areas where medical conditions and sanitation facilities are relatively poor. Patients in these regions often have low awareness of wound cleanliness and proper management, making infection occurrence and transmission more likely [39]. Previous studies have shown that *P. aeruginosa* is one of the most common pathogens in chronic wounds. Early detection and clearance of *P. aeruginosa* in wounds can significantly shorten wound healing time and improve patients' quality of life. Compared to traditional bacterial detection techniques, bacterial fluorescence imaging has the advantages of instant, rapid, and non-invasive application for *P. aeruginosa* detection. Based on the intuitive images provided by the instrument, doctors can make accurate diagnoses and treatment decisions[27]. Surface and internal temperature, pH, and osmotic pressure vary among different types of wounds. Acute wounds exhibit local inflammatory reactions with higher temperature than surrounding skin. Chronic wounds typically have local tissue damage, ischemia, or inflammation, resulting in lower surface temperature than surrounding tissues. Burn wounds, due to impaired local blood circulation, also exhibit lower surface temperature [40]. Studies have shown that the pH range of open wounds is 5.45 to 8.65, while non-healing chronic wounds mostly exhibit alkaline pH ranging from 7.15 to 8.90, and some even as high as 9.25 [41]. Furthermore, research indicates that changing wound pH value is an effective strategy for wound treatment. Currently, acidic wound dressings and cleansing solutions are commonly used to change wound pH, promoting wound acidification, which is beneficial for controlling bacterial proliferation and stimulating granulation tissue growth [42,43]. For localized wound infection, it has been found that using high osmotic saline as a disinfectant can control bacterial proliferation and promote granulation tissue growth[44]. Therefore, the wound infection microenvironment is complex and diverse.

In a recent experimental clinical observation, we found that under the same bacterial load, *P. aeruginosa* exhibited different fluorescence intensities in different types of wounds. The observed phenomenon is undeniably intriguing, and the findings of this experiment suggest that the bacterial microenvironment may exert an influence on fluorescence intensity. *P. aeruginosa* in infected wounds is identified and located by emitting green fluorescence. In previous studies, pyoverdine has been considered the source of green fluorescence, and the *pvdD* and *pvdS* genes play important roles in fluorescence production[32]. There have been few reports on the effects of temperature, pH, and

osmotic pressure on pyoverdine synthesis. In this experiment, an in vitro bacterial culture model was established, and the dynamic changes of *P. aeruginosa* under different conditions were analyzed using Image J software. Real-time fluorescence quantitative PCR was used to detect the expression levels of key genes involved in pyoverdine synthesis, and the experimental results were subjected to correlation analysis. The results showed that temperature influenced the production of *P. aeruginosa* green fluorescence, with a gradual decrease in fluorescence production rate and peak intensity at temperatures below 37°C. Real-time fluorescence quantitative PCR results showed that the expression levels of the *pvdD* and *pvdS* genes were consistent with the fluorescence results, indicating that temperature may regulate pyoverdine synthesis by affecting the expression of these genes. The fluorescence of *P. aeruginosa* was influenced by the pH level of the culture medium. When the pH of the medium decreased from 7 to 5, the fluorescence intensity showed a weakening trend, while alkaline conditions slightly enhanced fluorescence intensity. The expression levels of the *pvdD* and *pvdS* genes exhibited the same trend as fluorescence intensity, with the expression of the *pvdD* gene more significantly affected by pH. This suggests that the fluorescence intensity of *P. aeruginosa* may not be affected by alkaline environments as the wound pH becomes alkaline. Although acidic solutions cannot kill bacteria, the fluorescence intensity may still weaken after acidic treatment of the wound. The osmotic pressure of the culture medium significantly affected the production of *P. aeruginosa* fluorescence. When the sodium chloride concentration in the medium exceeded 1.8%, the colony area significantly decreased, indicating poor salt tolerance of *P. aeruginosa*, making it difficult to survive under high osmotic conditions. At the same time point, the fluorescence intensity decreased sharply, and real-time fluorescence quantitative PCR results showed a significant decrease in the expression levels of the *pvdD* and *pvdS* genes, highly consistent with the changes in fluorescence intensity.

Bacterial fluorescence imaging technology demonstrates unique advantages in wound bacterial detection, and changes in the bacterial microenvironment may lead to changes in the fluorescence intensity of *P. aeruginosa*. Our experimental results indicate that the production of *P. aeruginosa* fluorescence is influenced by cultivation temperature, pH, and osmotic pressure, and corresponding gene expression patterns. The underlying mechanisms of how temperature, pH, and osmotic pressure affect pyoverdine synthesis are not clear, and the bacterial microenvironment is complex and diverse. It is still unknown whether other factors in the bacterial microenvironment, such as antibacterial adjuncts and antibiotics used, can affect bacterial fluorescence. Further research is needed to elucidate these aspects. In conclusion, our study demonstrates the dynamic changes of *P. aeruginosa* fluorescence under in vitro conditions influenced by temperature, pH, and osmotic pressure, and reveals the expression patterns of the *pvdD* and *pvdS* genes under different conditions. The results of this study provide valuable reference data for real-time bacterial fluorescence imaging for wound infection detection.

4. Materials And Methods

4.1. Lysogeny Broth (LB) Preparation and Bacterial Liquid Culture Method

In the preparation of Lysogeny Broth (LB), 1g of beef extract (Basebio, Hangzhou, China) and 2g of sodium chloride (Sangon, Shanghai, China) were meticulously weighed and subsequently dissolved in 200 mL of purified water. This aqueous solution was then sterilized using an autoclave (Zealway, Delaware, USA) at a temperature of 121° C for a duration of 30 minutes. Post-sterilization, the solution was cooled to ambient room temperature. A volume of 10 mL of the sterilized LB was transferred aseptically into a 15 mL centrifuge tube. To this tube, 50 μL of *P. aeruginosa* bacterial suspension−obtained from the General Microbial Species Preservation and Management Center, China−was added. The centrifuge tube containing the bacterial inoculum was then placed on a benchtop shaker (Boxun, Shanghai, China) and incubated at a constant temperature of 37° C for a period of 36 hours. Following incubation, optical density measurements were conducted at a wavelength of 600 nm (OD600) to determine the bacterial culture's growth density.

4.2. The Absorbence-Concentration Standard Curve of Bacteria Was Established

A 50 μL of the bacterial solution was introduced into 10 mL of LB and incubated in a benchtop constant temperature oscillator for a duration of 36 hours. Subsequently, the bacterial solution was diluted in a series from 1 to 5 times using an equal gradient with 1× Phosphate Buffered Saline (PBS) solution (Solarbio, Beijing, China). These dilutions resulted in three parallel groups of bacterial suspensions across five distinct concentrations, which were then subjected to the dilution coating plate technique to ascertain the bacterial concentration in each group. For quantitative analysis, 200 μL of each bacterial suspension was dispensed into individual wells of a 96-well plate. The optical density measurements at a wavelength of 600 nm were obtained using a multifunctional enzyme labeling instrument (Tecan, Switzerland). To ensure reliability, three iterative measurements were conducted for each sample group, and the results were averaged. The OD600 values corresponding to each sample group were plotted on the x-axis, while the respective bacterial solution concentrations were plotted on the y-axis, thereby establishing a linear standard curve that correlated bacterial solution concentration with absorbance. This curve serves as a calibration tool to calculate the concentration of bacterial suspensions based on their OD values in subsequent experimental procedures.

4.3. Image Acquisition Method

Mount the MolecuLight i:X on the carrier table such that the instrument's camera is positioned 12 cm above the desktop. Securely fasten the Petri dish in place using label paper, ensuring that the imaging area of the Petri dish is centered within the screen. Activate the UV mode of the instrument and proceed to capture images of the samples at designated time points. It is crucial to conduct the entire image acquisition process in an environment devoid of light to eliminate any confounding influence of ambient light on the experimental outcomes.

4.4. Fluorescence Imaging Under Different Culture Temperature

A mixture consisting of 2 grams of tryptone, 1 gram of beef extract powder, 2 grams of sodium chloride, and 3 grams of agar powder (all obtained from Solarbio, Beijing, China) was accurately weighed and dissolved in 200 milliliters of purified water. This solution was subsequently sterilized using an autoclave. Upon completion of the sterilization process, the inverted plate technique was employed when the solution's temperature decreased to approximately 50°C. The bacterial suspension's absorbance was ascertained after 36 hours of incubation. A volume of 100 μL from the diluted suspension, post 10^6-fold dilution, was introduced onto the solidified medium and spread uniformly. The inoculated medium was then placed into an electrically heated thermostatic incubator (Jinghong, Shanghai, China) for consistent temperature incubation at 25°C, 31°C, 37°C, and 43°C, respectively. Fluorescence imaging was conducted in a darkened environment every 8 hours, commencing 12 hours following the initiation of incubation, to monitor the progression of bacterial growth and fluorescence expression under varying temperature conditions.

4.5. Fluorescence Imaging Under Different Culture pH

The pH of the medium was modulated following the procedure outlined by Bruce SK et al.[45]. Components including 2 g of tryptone, 1 g of beef extract powder, 2 g of sodium chloride and 3 g of agar powder were meticulously weighed and dissolved in 200 μL of purified water. Subsequent to autoclaving, the pH of the resultant medium was adjusted to values of 5, 6, 7, 8, and 9 respectively, utilizing hydrochloric acid and sodium hydroxide (both sourced from Aladdin, Shanghai, China). *P. aeruginosa* was then inoculated into Petri dishes containing media with varying pH levels according to the previously described method. These dishes were subsequently incubated in a constant temperature incubator maintained at 37°C. Fluorescence imaging commenced in a darkened environment every 8 hours, starting after a self-incubation period of 12 hours, to monitor the influence of pH on bacterial fluorescence expression over time.

4.6. Fluorescence Imaging Under Different Culture Osmotic Pressure

The osmotic pressure adjustment of the medium was performed according to the method described by Broekman JH [46]. Constituents including 2 g of tryptone, 1 g of beef extract powder, and 3 g of agar powder were accurately weighed, added to 200 μL of pure water, and subsequently, sodium chloride (NaCl) concentrations were adjusted to 0.9%, 1.8%, 2.7%, and 3.6% respectively. Following this adjustment, the medium was sterilized using an autoclave. Bacterial inoculation was then performed on solid media with varying osmotic pressures, ensuring an even distribution of the bacterial suspension. These inoculated media were incubated at a consistent temperature of 37° C. Fluorescence imaging was initiated in a darkened environment every 8 hours, commencing after an auto-incubation period of 12 hours, to monitor the impact of osmotic pressure on the fluorescence emission of the bacteria over time.

4.7. Image Processing and Fluorescence Intensity Calculation

For the acquired image, the edge detection algorithm within the OpenCV software (Intel, USA) was employed to identify the perimeter of the Petri dish present in the image. Subsequently, the Hough circle algorithm was utilized to determine the coordinates and radius of the Petri dish's center, which were then outputted. Utilizing the Circle function, a circular area was isolated with a specified center coordinate (1924, 1100) and a defined radius of 770 pixels. A mask was created and superimposed over the original image to eliminate interference from reflections on the Petri dish wall. In the experimental results, post-processing of the image was carried out using ImageJ software. This involved subtracting the background associated with the Petri dish by adjusting the threshold value, followed by the computation of the average fluorescence intensity within the colony area.

4.8. Bacterial RNA Extraction Procedure

The RNA extraction procedure was conducted in accordance with the guidelines provided by the RNA/DNA extraction kit (Beyotime, Shanghai, China). Initially, bacteria were harvested from the solid Petri dish and collected via centrifugation after three thorough washes with 1× PBS solution. Subsequently, the bacterial suspension was adjusted to a concentration of $1×10^7$ CFU/mL. From this suspension, 1 mL was centrifuged, the supernatant discarded, and 1 mL of lysis buffer was added. The mixture was then vortexed appropriately and incubated at room temperature for 5 minutes to ensure complete lysis of the samples. Following incubation, 0.2 mL of chloroform (Aladdin, Shanghai, China) was introduced to the lysate, mixed thoroughly through vortexing, and allowed to incubate at room temperature for an additional 3 minutes. The mixture was then centrifuged at 12000×g for 15 minutes at 4°C, resulting in the separation into a colorless aqueous phase (upper layer) and a red organic phase (lower layer). At this juncture, 0.5 mL of the lower phase was transferred to a new centrifuge tube and combined with 0.5 mL of anhydrous isopropanol (Aladdin, Shanghai, China). The sample was inverted several times to mix and left to precipitate at room temperature for 10 minutes. Afterwards, it was centrifuged at 12000g for 10 minutes at 4°C. The supernatant was discarded, and 1 mL of Wash Solution I was added, mixed by inversion. A final centrifugation step was performed at 7500g for 5 minutes at 4°C. The supernatant was again discarded, and the tube was subjected to a brief centrifugation to remove residual liquid, which was carefully aspirated. The RNA pellet was allowed to air dry slightly before being reconstituted in 50 μL of DEPC-treated water. The concentration and purity of the extracted RNA samples were assessed using a microspectrophotometer (Nanodrop 2000, Thermo Fisher, USA).

4.9. Real-Time Fluorescence Quantitative Polymerase Chain Reaction (RT-PCR) Detection of pvdD and pvdS Gene Expression Levels

The cDNA synthesis and genomic DNA elimination reactions were conducted in accordance with the protocols outlined in the PrimeScript FAST RT reagent Kit with gDNA Eraser (Takara, Japan) Reverse Transcription Kit. To initiate the process, a centrifuge tube was prepared by sequentially adding 2 μL of 8× gDNA Eraser Premix, 2 μL of RNA sample, and 12 μL of RNase-free H_2O. This mixture was incubated at 42°C for 2 minutes to eliminate genomic DNA. Subsequently, 4 μL of 5× RT Premix was incorporated, and upon gentle mixing, the reverse transcription reaction proceeded

under the following thermal cycle conditions: 37°C for 10 minutes, followed by 85°C for 5 seconds. This procedure yielded a cDNA product free from genomic DNA contamination. Bidirectional primers for the *P. aeruginosa* 16s gene, *pvdD* gene and *pvdS* gene were designed using Primer Express software. The forward primer sequence for the 16s gene was 5'-CCAGACTCCTACGGGGAGGC-3', and the reverse primer sequence was 5'-TCCTACGGGAGGCAGCAGT-3'. For the *pvdD* gene, the forward primer sequence was 5'- CGCTCCTCCAGGCATTCG-3', and the reverse primer sequence was 5'- GACCACATCTTCGGCTCGC-3'. For the *pvdS* gene, the forward primer sequence was 5'-CATGACACCGAGCAGTTCCAC-3', and the reverse primer sequence was 5'-CGGTCAGGGTCGAAGCGT-3'.The RT-PCR amplification was performed according to the instructions provided with the TB Green Premix Ex Taq™ II FAST qPCR (Takara, Japan) kit. Into each PCR tube, a reaction mix consisting of 10 μL of TB Green Premix Ex Taq™ II Fast qPCR (2×), 1 μL of PCR Forward Primer, 1 μL of PCR Reverse Primer, 2 μL of cDNA template, and 6 μL of sterilized water was assembled. Each sample set was allocated to three replicate wells, and the reaction was executed on a LightCycler 96 (Roche, Basel, Switzerland). The Real Time PCR conditions were as follows: initial denaturation at 95°C for 30 seconds; followed by 40 cycles of a two-step PCR involving denaturation at 95°C for 5 seconds and annealing/extension at 60°C for 10 seconds.

4.10. Statistical Methods

All experiments were done with 3 replications. SPSS 25.0 was used to analyze the data statistically; one-way ANOVA was used to compare the differences between multiple groups, and two-by-two comparisons within groups were made using the LSD-t test; pearson correlation analysis was used between the mean fluorescence intensity and the bacterial content. Differences were expressed as statistically significant at $P < 0.05$.

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