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Effects of Temperature on the Antibiogram, Biofilm, and Biopigment Production in *Pseudomonas aeruginosa* Clinical Isolates

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Pseudomonas aeruginosa **is a notorious nosocomial pathogen owing to its resistance to multiple antibiotics and biofilm formation. This study investigated the effects of 1-wk exposure at different temperatures on the antibiogram and biofilm in *P. aeruginosa*. A total of 30 isolates from clinical specimens – 14 from wound, six from urine, four from endotracheal aspirate, four from sputum, one from a tissue sample, and one from a cranial cerebrospinal fluid – were collected from a tertiary hospital. The pre-exposure minimum inhibitory concentration (MIC) was measured against the eight antibiotics – namely amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), piperacillin-tazobactam (100/10 µg), ceftazidime (30 µg), cefepime (30 µg), imipenem (10 µg), and meropenem (10 µg). The isolates were then exposed for 1 wk at 24, 37, and 42 °C, after which post-exposure MICs were taken. The biofilm-forming activities after 48 h were determined using the crystal violet assay. MDR- and XDR–*P. aeruginosa* were recorded in 20 and 17% of the isolates, respectively, mostly to the beta-lactam antibiotics. One isolate was resistant to all eight antibiotics; two were hepta-resistant and hexa-resistant. High carbapenem resistance was observed among isolates (30–43%). Data showed that increasing the temperature from 24 to 42 °C did not significantly affect the MIC ($p > 0.05$) but had a significant effect on the biofilm formation, with lower temperature (24 °C) favoring enhanced biofilm. In addition, pigment production was observed at 24 °C and became more pronounced at 37 °C but inhibited at 42 °C. In conclusion, exposing *P. aeruginosa* to increasing temperatures for 1 wk did not cause a significant change in its antibiogram profile. However, the ability of *P. aeruginosa* to produce bio-pigment and biofilm was found to be temperature dependent. This study underscores the importance of routine antibiotic surveillance for a directed and more efficacious treatment of *P. aeruginosa* infection and the need to explore effective biofilm-disrupting strategies to mitigate *P. aeruginosa* colonization in hospital settings.**

Keywords: antibiogram profile, biofilm-forming activity, crystal violet assay, minimum inhibitory concentration, *Pseudomonas aeruginosa*

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INTRODUCTION

Antibiotic resistance has remained a global health concern, most especially in the clinical setting (WHO 2024; Sanusi *et al.* 2019). One of the notable antibiotic-resistant pathogens that is popularly acclaimed as an agent of nosocomial infection is *Pseudomonas aeruginosa* (Imanah *et al.* 2017; Palavutitotai *et al.* 2018). Natural resistance to antibiotics, as well as acquired resistance (mostly plasmid-mediated), have been reported with this bacterial species, which poses a serious threat (Planet 2017; CDC n/d). The response of the microorganism to an array of antimicrobial drugs is defined in the antibiogram. An antibiogram is an overall profile of antimicrobial susceptibility testing results of a specific microorganism to a battery of antimicrobial drugs (Truong *et al.* 2021).

One mechanism that *P. aeruginosa* employs to survive in the presence of antibiotics is the formation of biofilms, which are communities of bacteria embedded in an exopolysaccharide matrix (Cepas *et al.* 2019; Goltermann and Tolker-Nielsen 2017). Biofilms act as protective barriers that shield bacteria from adverse conditions such as extreme temperature, fluctuating pH, high salinity and pressure, as well as nutrient depletion (Bisht *et al.* 2021). In clinical settings, biofilm-producing bacteria confer survival advantage as compared to other planktonic pathogens (Prasad *et al.* 2020), thus contributing to persistent and chronic infections (Jamsheera and Suman 2018; Samad *et al.* 2019).

One of the Sustainable Development Goals of the United Nations Educational, Scientific, and Cultural Organization is climate change, which was pointed out as a key factor in the emergence of infectious diseases (WHO 2024; MacFadden *et al.* 2018). Moreover, the World Meteorological Organization in 2021 reported that a 1.5 °C temperature increase within the next five years is anticipated. As global warming intensifies, there is an urgent need to investigate the potential impact of rising environmental temperature on various biological processes including the response of bacteria to antibiotics as well as their ability to form biofilm and biopigment, which are both related to virulence (Cavicchioli *et al.* 2019). Environmental factors such as rising temperature may facilitate gene exchange, regulate bacterial growth, and induce the spread of antibiotic-resistant strains (MacFadden *et al.* 2018; Rodríguez-Verdugo *et al.* 2020). Antibiotic resistance was reported by MacFadden *et al.* (2018) to increase from 2.2 to 4.2% among common clinically important pathogens for every increment of 10 °C in local temperature. Moreover, it is projected that in the coming years, such a direct association between temperature and antibiotic resistance will persist in a broader antibiotic category and in a more diverse species of clinical pathogens.

P. aeruginosa is implicated with a number of diseases such as wound infection, urinary tract infection, cystic fibrosis, otitis media, meningitis, and pneumonia. It is a notorious hospital pathogen demonstrating remarkable adaptability and resilience when exposed to antiseptics or antibiotics treatment and environmental stressors such as rising ambient temperature (Imanah *et al.* 2017). While existing literature suggests a possible direct link between higher temperatures and antibiotic resistance, there is limited evidence of the impact of elevated temperatures on the biofilm-forming activities of common hospital pathogens. This study sought to address this gap by investigating the interplay between increased temperature, antibiotic resistance profiles, and biofilm-forming activities, with a specific focus on the opportunistic pathogen – *P. aeruginosa* – isolated from clinical specimens.

MATERIALS AND METHODS

Research Method

This study made use of descriptive research to determine the antibiogram and the biofilm-forming activity of *P. aeruginosa* clinical isolates at varying temperatures.

Sources of *Pseudomonas aeruginosa* Clinical Isolates

P. aeruginosa clinical isolates were obtained from a tertiary hospital (Victoriano Luna Medical Center) between August 2022–January 2023. A total of 30 clinical isolates of *P. aeruginosa* were used in this study. They were sourced from a variety of clinical specimen types – including 14 from the wound, six from urine, four from endotracheal aspirate, four from sputum, one from a tissue, and one from a cranial cerebrospinal fluid (CSF).

Isolation and Purification of *Pseudomonas aeruginosa* Clinical Isolates

Patient specimens were inoculated onto the following selective culture media: blood agar plate or BAP, chocolate agar plate or CAP, and McConkey agar or MAC for primary isolation. Plates were then incubated at 37 °C for 24 h and observed for growth. Isolated colonies growing on MAC were picked out for identification using the VITEK 2 automated identification system. Using a sterile loop, an isolated colony from the MAC plate was transferred into a sterile tube containing saline. The turbidity of the suspension was adjusted to 0.5 McFarland standard using a spectrophotometer set at 600 nm. This standardized culture was loaded on the VITEK 2 card for automated bacterial identification. Each confirmed *P. aeruginosa* isolate was subcultured onto a sterile nutrient agar (NA) medium. The subculturing process was done to

obtain pure colonies. This was then used in preparing for the pure working culture. A pure stock culture was also maintained in a NA slant overlaid with sterile mineral oil.

Ethical Consideration

The study complied with the required protocols, including the submission of documents such as the Material Transfer Agreement, Transport Plan, Material Safety Data Sheet, and Ethics Approval with Protocol Number 002/01-22 from Victoriano Luna Medical Center.

Cultivation and Maintenance

NA was used as the culture medium for the preparation of stock cultures and sub-cultures of clinical isolates. Two types of stock cultures were prepared: a "working" stock for sub-cultures during experimentation and a "permanent" stock culture, which was opened only once for the generation of subsequent stock cultures.

Controls

P. aeruginosa ATCC 27853 strain was used as a positive control for the biofilm-formation assay. This was shared with the researchers through the generosity of the Centro Escolar University (CEU) Clinical Laboratory. A microorganism-free control composed of only sterile Mueller-Hinton broth with antibiotics was used as a negative control. To ensure that there is no growth of microorganisms in the negative control, triplicate tubes of Mueller-Hinton broth with antibiotics were incubated at 37 °C for 24 h and checked for the absence of turbidity. Both positive and negative controls were included alongside the test clinical isolates in running the biofilm-formation assay.

Growth and Maintenance of Test and Control Bacteria

Following the protocol of Al-Kafaween *et al.* (2019), the sub-cultures of *P. aeruginosa* clinical isolates and the ATCC 27853 strain (positive control) and negative control were incubated at 37 °C for 24 h to facilitate bacterial growth before analysis and temperature interventions.

Test Antibiotics

The antibiotic susceptibility testing was carried out against eight different antibiotics in five categories – namely piperacillin-tazobactam (β-lactam combination agent), ceftazidime and cefepime (cephalosporins), imipenem and meropenem (carbapenems), amikacin and gentamicin (aminoglycosides), and ciprofloxacin (fluoroquinolones). The Department of Health's national antibiotic guidelines (DOH 2017) for treating *Pseudomona*s-related infections in the Philippines served as the basis for the choice of test antibiotics. The interpretation of results followed the guidelines of the Clinical Laboratory Standards Institute (CLSI) M100 performance standard for antimicrobial susceptibility testing (CLSI 2020).

Determination of Baseline Antibiogram

The baseline antibiotic susceptibility profile or antibiogram was determined by measuring the minimum inhibitory concentration (MIC) using automated *VITEK® 2* technology by Biomerieux, following the CLSI standard protocol.

Determining the Effects of Different Temperatures on the Antibiogram

The antibiogram was repeatedly taken after exposing the culture of 30 bacterial isolates for 7 d (Marajan *et al.* 2018) at three different temperatures. Post-exposure MICs were generated by the automated sensitivity testing machine (*VITEK® 2* technology), following the CLSI standard. The selection of temperature range used in this study was based on prior research conducted by Paksanont *et al.* (2018) and Eze and El Zowalaty (2019). In this study, samples were incubated at temperatures of 24, 37, and 42 °C, which reflect the temperature ranges encountered in different clinical settings – 24 °C representing the controlled environment of hospitals and clinical laboratories, 37 °C mirroring normal physiological body temperature, and 42 °C being the highest possible external temperature at which *P. aeruginosa* can survive.

Microtiter Biofilm Formation Assay

The crystal violet assay was conducted to quantify biofilm formation, following the methods of ImQuest BioSciences (2016), Kamali *et al.* (2020), and Rodríguez-Lázaro *et al.* (2018) with modification on the temperature utilized during the 48-h incubation period. Overnight cultures of *P. aeruginosa* were diluted in assay media, the turbidity of which was standardized at 0.5 McFarland to ensure inoculum density of 1.5×10^8 CFU/mL before they were seeded in the microtiter plate. From this, 100 µL were transferred to a 96-well microtiter plate, with each plate including three replicates for each *P. aeruginosa* strain. Clinical isolates for each microtiter plate were inoculated and allowed to grow for 48 h at different temperatures – namely 24, 37, and 42 °C – to induce biofilm formation within the microtiter plates. To eliminate planktonic or non-adherent cells, the contents of each well were gently pipetted, and the wells were washed three times with phosphate-buffered saline. Biofilms formed in the wells were fixed by adding 200 µL of 100% ethanol to each well. The plates were incubated for 15 min. Ethanol was completely aspirated, and the microtiter plates were inverted to allow them to air-dry thoroughly. A 1% solution of crystal violet was added to each well and allowed to

stand for 15 min at room temperature. Wells were rinsed 3–4 times with water and then blotted with paper towels to remove excess water. The microtiter plates were left to dry for 1–2 h. A 30% acetic acid solution (150 µL) was added to each well to elute the bound crystal violet from the biofilm. The plates were incubated for 1 h at room temperature. The absorbance was obtained at $OD_{600\ nm}$ using the microtiter plate reader. To determine biofilm formation, the optical density or OD of each strain was calculated as the arithmetic mean of the absorbance readings from the three wells. These values were then compared to the mean absorbance of positive and negative controls, following the classification criteria established by Kamali *et al.* (2020).

Statistical Treatment

Statistical analysis was made using SPSS version 21. The mean MIC for each antimicrobial agent was determined to evaluate changes in the antibiogram profile of *P. aeruginosa* clinical isolates. The one-way ANOVA was used to analyze the antibiogram profile after 7 d of incubation and biofilm formation after 48 h of incubation at varying temperatures. Paired t-tests were employed to compare antibiogram profiles during pre-exposure and post-exposure varying temperature interventions. Pearson's r correlation was utilized to examine the relationship between temperature, MIC, and biofilm formation.

RESULTS

Baseline Antibiogram Profile of *Pseudomonas aeruginosa* Clinical Isolates

Table 1 presents the antibiogram of the 30 clinical isolates from a wide range of patients' specimens. On top of the list is PA008, a wound isolate, showing a resistance pattern to all the eight test antibiotics (octa-resistant). Two isolates, namely PA011 from urine and PA020 from the wound, were resistant to seven antibiotics. PA016 and PA017, both from urine samples, were resistant to six antibiotics and intermediate to gentamicin. PA005 and PA018 were resistant to five antibiotics. PA001 and PA012, in turn, showed resistance to four antibiotics, and PA019 demonstrated resistance to three antibiotics.

Interestingly, 20% of the *P. aeruginosa* or PA isolates were found to be multiple drug resistant (MDR), defined by the Centers for Disease Control and Prevention (CDC) as resistance to ≥ 1 agent in ≥ 3 antimicrobial categories. Extensively drug-resistant (XDR) PA, defined as susceptibility limited to ≤ 2 categories, was seen in 17% of the isolates examined. Many of the MDR-PA and XDR-PA noted in this study are those isolated from wound and urine specimens. Only 33% (10 out of 30) isolates were sensitive to all eight test antibiotics: six from the wound, one from urine, one from tissue, one from endotracheal aspirate, and one from sputum.

Most isolates were sensitive to aminoglycosides (amikacin and gentamicin). It is worth mentioning that most of the isolates exhibited resistance to beta-lactam antibiotics such as piperacillin-tazobactam (46.67%), ceftazidime (43.33%), imipenem (43.33%), cefepime (30%), and meropenem (30%).

Antibiotic Resistance Profile (MIC) and Duration of Exposure

A comparison of the resistance profile of the isolates at 1-d exposure (baseline MIC) and at 7-d incubation at 37 °C was made. Results are presented in Table 2. It is interesting to note that a 20–50% increase in the number of isolates resistant to PIP-TAZ, ceftazidime, cefepime,

Table 1. Baseline antibiogram profile of the 30 *Pseudomonas aeruginosa* isolated from the clinical specimens.

Resistance pattern	Number of antibiotics isolates are resistant to	Number of resistant isolates	Percentage
Sensitive to 8 test antibiotics	0	10	33.3%
Sensitive to 6 test antibiotics; intermediate results to two Antibiotics	0	2	6.7%
MONOresistant	1	4	13.3%
BIresistant	2	4	13.3%
TRIresistant	3	2	6.7%
TETRAresistant	4	2	6.7%
PENTAresistant	5	2	6.7%
HEXAresistant	6	2	6.7%
HEPTAresistant	7	2	6.7%
OCTAresistant	8	1	3.3%

Table 2. Comparison of the number of resistant *Pseudomonas aeruginosa* clinical isolates at 1- and 7-d exposure at 37 °C.

	n (%) 1-day exposure	n (%) 7-day exposure	% increase/decrease in the resistance
Amikacin	4 (13.33%)	2 (6.67%)	(50)
Gentamicin	1 (3.33%)	2 (6.67%)	50
Ciprofloxacin	7 (23.33%)	7 (23.33%)	0
Piperacillin-Tazobactam	14 (46.67%)	20 (66.67%)	20
Ceftazidime	13 (43.33%)	19 (63.33%)	20
Cefepime	9 (30.00%)	18 (60.00%)	50
Imipenem	13 (43.33%)	13 (43.33%)	0
Meropenem	9 (30.00%)	9 (30.00%)	0

Table 3. Comparison of the antibiogram profile of *Pseudomonas aeruginosa* clinical isolates on baseline and post-exposure conditions at varying temperatures.

Antibiotic	Pre-exposure MIC (mean)	Post-exposure MIC at 24 °C (mean)	Post-exposure MIC at 37 °C (mean)	Post-exposure MIC at 42 °C (mean)	*p*-value
Amikacin	12.77	12.39	11.03	12.45	> 0.05**
Gentamicin	3.32	3.55	3.45	3.42	> 0.05**
Ciprofloxacin	1.11	0.91	0.81	0.9	> 0.05**
Piperacillin-Tazobactam	70.29	78.14	88.69	78.9	> 0.05**
Ceftazidime	27.61	33.29	33.81	30.26	> 0.05**
Cefepime	21.45	30.1	30.81	25.97	> 0.05**
Imipenem	7.67	6.77	6.64	6.35	> 0.05**
Meropenem	5.13	4.78	4.24	4.32	> 0.05**

Note: *significant; **not significant

and gentamicin was observed when isolates were exposed at 37 °C for 1 wk as compared to Day 1 incubation. However, the duration of exposure did not change the number of resistant isolates against the antibiotics, ciprofloxacin, imipenem, and meropenem. It is worth noting that the number of isolates showing resistance to amikacin was reduced by 50% when exposed to 37 °C for a 1-wk duration.

Comparison of Antibiogram across Temperatures

Figure 1 shows the comparison of the responses of *P. aeruginosa* clinical isolates against the eight test antibiotics at three different temperatures. It can be surmised from the graph that the MIC of carbapenems, meropenem, and imipenem – whose actions are to interfere in the cell wall synthesis – demonstrated decreasing MIC at increasing temperatures.

For the other beta-lactam antibiotics (cefepime, ceftazidime, and piperacillin-tazobactam) a slight increase in MIC was seen when the isolates were shifted from 24 to 37 °C incubation temperature. However, increasing further the temperature to 42 °C had led to a corresponding decrease in the MIC.

Comparison of Pre- and Post-exposure Antibiotic Resistance Profile

The comparison of the antibiogram in pre-exposure and post-exposure samples at variable temperatures is shown in Table 3. While there are slight changes in MIC values after exposure to different temperatures, these changes are not statistically significant ($p > 0.05$).

This suggests that the antibiogram profile of *P. aeruginosa* clinical isolates remains relatively stable for some antibiotics across the tested temperature conditions.

Comparison of Biofilm Formation at Varying Temperatures

Table 4 shows the comparison of biofilm formation of *P. aeruginosa* across temperatures. It is worth noting that

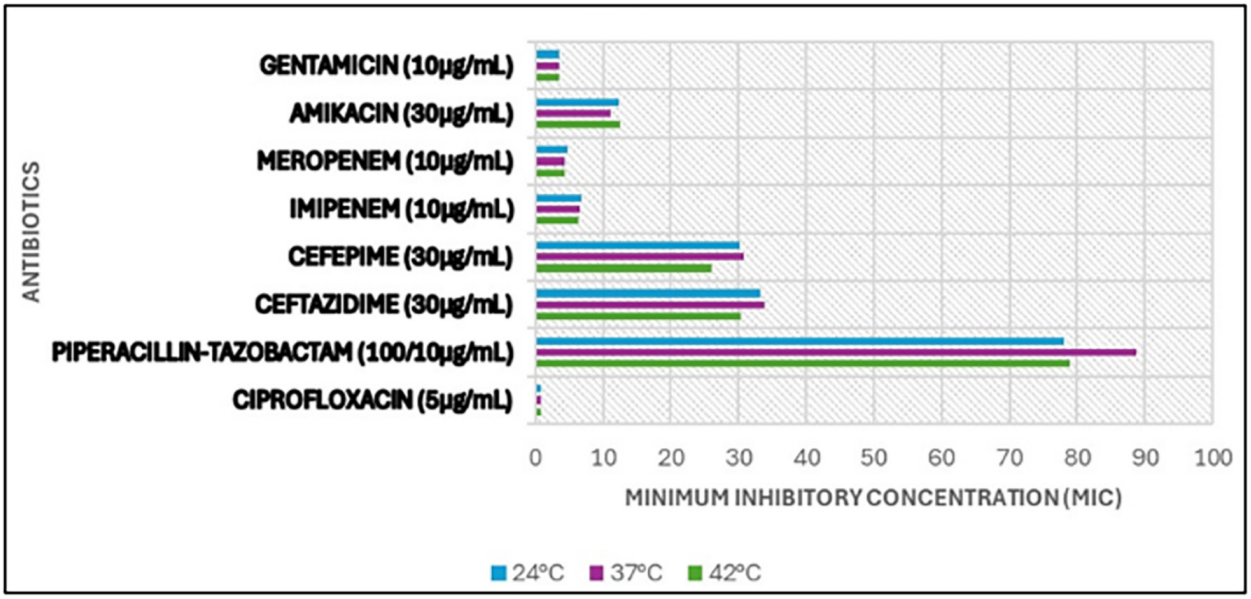

Figure 1. Comparison of the MIC of the eight test antibiotics against *Pseudomonas aeruginosa* clinical isolates (n = 30) across different temperatures.

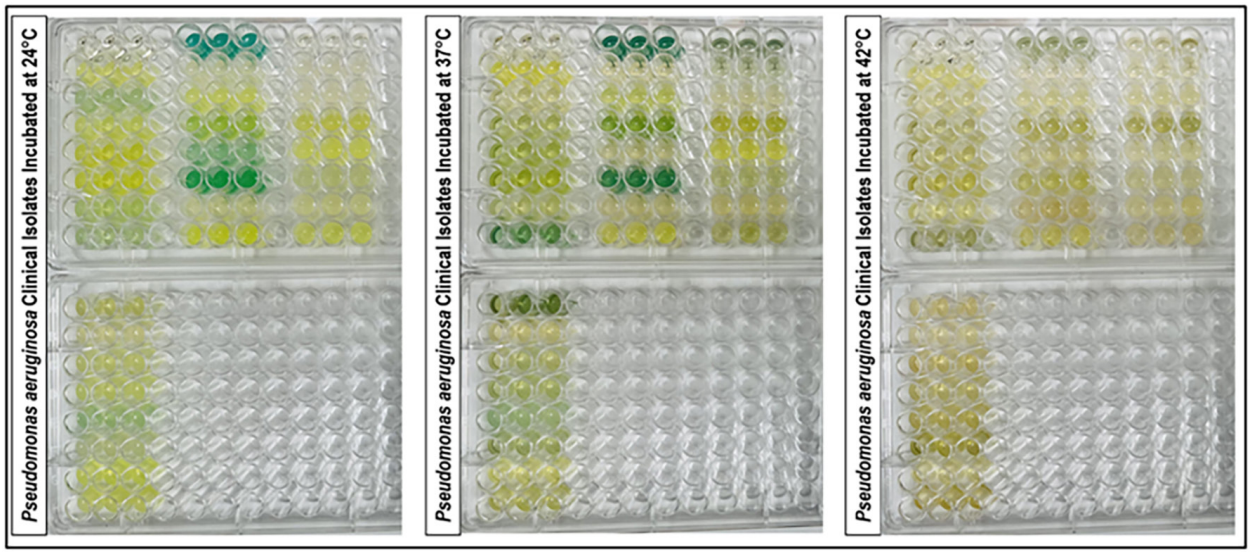

Figure 2. Pigment production of *Pseudomonas aeruginosa* clinical isolates after 48 h of incubation at 24, 37, and 42 °C.

all the 30 clinical isolates and the positive control were found to be strong biofilm-formers. Biofilm, however, is significantly more pronounced at 24 °C than at 37 and 42 °C (p = 0.042).

Pigment Production at Varying Temperatures

One interesting result of the study while doing the biofilm assay was the observation that at a 48-h incubation period, the blue-green pigment (pyocyanin) produced by *P. aeruginosa* was notable at 24 °C and became more intense at 37 °C (see Figure 2). Pigment production, however, was lost when the temperature was raised to 42 °C. This may suggest a potential temperature dependence of pigment production in *P. aeruginosa.*

Relationship Between Biofilm-forming Activity of *Pseudomonas aeruginosa* and Temperature

A moderate negative correlation (r = –0.5361) was obtained between temperature and biofilm formation. Although statistically, the relationship is not significant

Table 4. Biofilm formation assay result of *Pseudomonas aeruginosa* clinical isolates at varying temperatures.

Temperature (°C)	Mean (OD/ABS) (SD)	*p*-value
24 °C	1.07 (0.57)	0.042*
37 °C	0.66 (0.61)	
42 °C	0.85 (0.71)	

Note: *significant; **not significant

Table 5. Relationship between the rising temperature and antibiotic resistance profile of *Pseudomonas aeruginosa* clinical isolates.

Antibiotic	Correlation coefficient	*p*-value
Amikacin	0.0374	0.9762**
Gentamicin	–0.9459	0.1919**
Ciprofloxacin	–0.0908	0.9421**
Piperacillin-Tazobactam	–0.0646	0.9589**
Ceftazidime	–0.7902	0.4199**
Cefepime	–0.7901	0.4201**
Imipenem	–0.9767	0.1378**
Meropenem	–0.7892	0.4210**

Note: *significant; **not significant

(p = 0.6398), the negative correlation, nonetheless, is consistent with the observation that an enhanced biofilm was observed at a lower temperature (24 °C) than at higher temperatures (37 and 42 °C), as earlier mentioned. The small sample size and the heterogeneous sources of clinical isolates may account for this finding.

Relationship between Antibiogram of *Pseudomonas aeruginosa* and Temperature

The relationship between the MIC of *P. aeruginosa* clinical isolates and temperature is presented in Table 5. A strong negative relationship between temperature and the MIC was seen in the antibiotics, gentamicin (r = –0.9459), imipenem (r = –0.9767), meropenem (r = –0.7892), cefepime (r = –0.7901), and ceftazidime (r = –0.7902). Thus, increasing temperature can lower the minimum concentration required for these antibiotics to inhibit or kill *P. aeruginosa*. While a strong negative relationship between temperature and MIC was observed in all these five antibiotics, this, however, is not statistically significant ($p > 0.05$).

DISCUSSION

According to the CDC, MDR-PA remains a serious public health threat, with at least 32,600 cases and 2700 deaths reported (Kunz Coyne *et al.* 2022). It is one of the ESKAPE pathogens – an acronym for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., which are known for their high virulence and antibioticresistance. The findings of this present study once again verified the nature of *P. aeruginosa* as a strong biofilm-forming and antibiotic resistance pathogen in the hospital setting.

The present study recorded a high rate of MDR-PA and XDR-PA from clinical samples. The rates obtained in this present study – namely 20% MDR-PA and 17% XDR-PA – are close to those reported in the 2018 Philippine Antibiotic Resistance Surveillance Program (ARSP 2018) Annual Report at 21–23% MDR and 13–18% XDR but a little lower than those reported in the 2023 ARSP Report at 28.3 and 18.2% (ARSP 2023). Considering that the sources of the isolates were from a tertiary hospital, these data are quite alarming, as this implies a limited therapeutic option for *P. aeruginosa* infections. So far, of the eight test antibiotics, aminoglycosides, amikacin, and gentamicin appear to be effective for this kind of bacterial infection. These antibiotics kill bacteria by inhibiting bacterial protein synthesis, specifically targeting the 30s ribosomal subunit (Krause *et al.* 2016).

Among the eight antibiotics tested, beta-lactam antibiotics showed high levels of resistance and, hence, may no longer be effective in treating infections caused by Gram-negative bacteria such as *P. aeruginosa.* This is expected since the beta-lactams work best for Gram-positive bacteria, with their thick peptidoglycan cell wall as the target (Moradali *et al.* 2017). The variable pattern of antibiotic resistance from isolates coming from clinical specimens emphasizes the need for an individualized treatment plan. This is in support of the World Health Organization (WHO) directives on antibiotic stewardship that aim to minimize the further spread of antimicrobial resistance (AMR) genes not only in clinical settings but also in the environment and livestock. Determining the antibiogram of a specific bacterial species is, therefore, recommended for a more directed and efficacious treatment of infectious diseases for optimal patient care.

Of the beta-lactam group of antibiotics used in this study, the highest observed resistance (46.67%) was seen in piperacillin-tazobactam, (a kind of penicillin/ beta-lactamase inhibitor) and ceftazidime (a kind of cephalosporin) with resistance at 43.33%, rates higher than those reported in 2023 at 23.25 and 17.33% for piperacillin-tazobactam and ceftazidime, respectively.

The increasing resistance to cephalosporins may be attributed to the overexpression of chromosomal AmpC cephalosporinase (Oliver 2024).

Interestingly, the present study identified a high occurrence of carbapenem-resistant *P. aeruginosa*, which ranges from 30–43%. This is higher as compared to the 15–19% resistance rate reported in the 2023 ARSP for this group of antibiotics. This finding is in support of the recent report by WHO, wherein carbapenem-resistant *P. aeruginosa* was included on the list of high-priority pathogens (WHO 2024). The increasing resistance of this opportunistic pathogen to a wide number of beta-lactam and carbapenem antibiotics may be ascribed to the reported acquisition of resistance genes coding extended-spectrum beta-lactamases and metallo-beta-lactamases carbapenemases. Such enzymes are both encoded in the bacterial chromosome and in plasmid (Chilam *et al.* 2021). These antibiotic-resistance genes are reported to be carried by various mobile gene elements such as integrons, transposons, and plasmids (Chilam *et al.* 2021) and disseminated to *P. aeruginosa* bacteria *via* horizontal gene transfer (HGT) mechanisms. Specifically, carbapenem resistance was described by Chilam *et al*. (2021) and Hasan and Al-Harmoosh (2020) as due to the loss of function of the OprD porin and acquisition of the metallo-β-lactamase gene bla_{VIM}.

It must be pointed out that of the different specimens used as sources of *P. aeruginosa*, those from the wound and urine samples showed the highest number of resistant results in the antibiogram. This is consistent with the report that *P. aeruginosa* is a major colonizing pathogen on infected wounds and in the urinary tract (UT) – causing catheter-associated UT infection, which may be attributed to the ability of this bacteria to colonize patient's tissues and form robust biofilms on various hospital instrument. Thus, patients who are in the ICU with mechanical ventilators, intubators, and indwelling catheters are at high risk of acquiring biofilm-forming *P. aeruginosa* strain, an observation that accounts for its high mortality and morbidity. Similarly, immunocompromised patients and those previously exposed to carbapenems and fluoroquinolones are at higher risks of acquiring infections caused by MDR-PA and XDR-PA (Kunz Coyne *et al.* 2022).

The challenge of treatment of *P. aeruginosa* infection in hospital settings is made more difficult as 100% of the clinical isolates tested in this study were found to be strong biofilm-forming strains. In this study, a more robust biofilm was seen at 24 °C than at 37 and 42 °C. This is consistent with the findings presented by Kim *et al.* (2020), reiterating the inverse relationship between temperature and microbial biofilms. Although the exact mechanism as to how the temperature influences biofilm formation remains unclear, Kim *et al.* (2020), highlighted the role of temperature in modulating cyclic-di-GMP (c-di-GMP), which is reported as the molecular mediator orchestrating the thermoregulation of biofilm formation in *P. aeruginosa*. Moreover, Bisht *et al.* (2021) support the thermal regulation of genes associated with biofilm formation. It is postulated that at lower temperatures (such as 24 °C), the biofilm matrix is more dispersed, whereas at higher temperatures (37 °C), a more dense, intricately intertwined structure forms the biofilm matrix.

The effects of increasing temperatures on various biological processes such as antibiotic resistance in major human pathogens have become an interesting research topic in health and the environment. MacFadden *et al.* (2018) suggested that increasing temperature may facilitate HGT and the assimilation of extrinsic genetic material from environmental DNA (eDNA) trapped within a biofilm matrix. Biofilm, therefore, may facilitate the transfer of virulence genes, including the one that encodes pigment production in *P. aeruginosa*, as cells in the microbial consortia are glued together by the extracellular polymeric substance. Bisht *et al.* (2021) identified the temperature-regulated phage genes – CoaB protein of Pf1 bacteriophage – as responsible for the development of biofilm and antibiotic resistance in *P. aeruginosa*. These discoveries provide bases for a better understanding of *P. aeruginosa* pathogenesis, explore potential therapeutic approaches, and elucidate the dynamic relationship between biofilm formation, virulence, and AMR.

The effect of increasing temperature on the biofilm formation varies from one bacterial species to another. For instance, *Listeria monocytogenes* (Russo *et al.* 2018) and *Serratia marcescens* (Harimawan *et al.* 2017) exhibited enhanced biofilm at elevated temperatures. These results, however, were contrary to what the present study observed, wherein lower temperature (24 °C) favors more intense *P. aeruginosa* biofilm than at higher environmental temperatures. The same behavior was seen by Mizan *et al.* (2018) in *Aeromonas hydrophila*, wherein a heightened biofilm was evident at low temperatures (20–25 °C).

As biofilm is positively linked with AMR, so is microbial virulence (Tutulan 2015). Biofilm creates a microenvironment that promotes variations in antibiotic susceptibility and resistance mechanisms among bacteria (Abebe 2020; ASM 2023). *P. aeruginosa* biofilm formation, its inherent resistance mechanism, and the acquired resistance and virulence genes all contribute to its category as a serious threat by health organizations (CDC n/d; Prasad *et al.* 2020).

One of the interesting findings of the present study is the temperature-dependent bio-pigment production in *P. aeruginosa* in clinical isolates, with blue-green pigmentation seen more intensely at lower temperatures. Pyocyanin (blue-

green pigment) has been linked with biofilm formation and virulence, both being regulated by quorum-sensing (Behzadi *et al.* 2022; Debritto *et al.* 2020; Zhao *et al.* 2020; Subramani and Jayaprakashvel 2019). Similar to those reported in *Serratia* where the scarlet red pigment, prodigiosin, is more pronounced at room temperature than at 37 °C, this study also noted that *P. aeruginosa* blue-green pigment (pyocyanin) is evident at 24 °C, which is also the temperature where biofilm was evident.

According to Das *et al.* (2016), a robust biofilm is composed of a pyocyanin-eDNA complex. In this study, the intense blue-green pigment seen at 24 and 37 °C disappeared when the temperature was raised to 42 °C (see Figure 2). This observation is in sync with those reported by Elbargisy (2021) identifying 37 °C as the optimal temperature for the pigment production of *P. aeruginosa.* As pigment production is controlled by quorum sensing, which in turn is dependent on microbial population, it is expected that at 42 °C fewer viable cells are available to elicit pigment production. *P. aeruginosa*, being a mesophilic bacterium is likely to die when temperature goes beyond 40 °C.

It has been a common notion that fever in patients may sometimes be protective as mesophilic pathogens are likely to die at 40–42 °C. Moreover, Finlayson and Brown (2011) suggested that *P. aeruginosa* pigment production may be associated with MDR and the presence of virulence-associated genes. It was observed that pigment-producing *P. aeruginosa* is more virulent and more resistant to antibiotics compared to those that do not produce pigment (Abdelaziz *et al.* 2023; Finlayson and Brown 2011). Pyocyanin, being a secondary metabolite of *P. aeruginosa*, was reported in the paper of Numan *et al.* (2018) as an antibacterial, antioxidant, and anticancer.

Global warming and the occurrence of MDR and XDR strains in the hospital setting are two great challenges that the world is currently facing. The WHO has identified climate change as a significant driver of emerging infectious diseases on a global scale (MacFadden *et al.* 2018). Changes in temperature and severe weather events can lead to the expansion of diseases and the potential for disease outbreaks in non-traditional locations and seasons as described by Wu *et al.* (2016). Hence, studying the impact of varying environmental temperatures on microbial resistance is a relevant and compelling topic. The increasing ambient temperature due to global warming and the rampant presence of MDR-PA and XDR-PA in the hospital environment pose a significant challenge to public health.

Although in this study temperature was found to be negatively correlated with the biofilm-forming activity of *P. aeruginosa,* increasing the temperature, however, did not significantly change the MIC of some antibiotics such as ciprofloxacin and amikacin. However, for most of the beta-lactam antibiotics used in this study (ceftazidime, cefepime, and piperacillin-tazobactam), a negative relationship between temperature and MIC was observed though not statistically significant. One reason is the heterogeneous nature of the biological specimens where *P. aeruginosa* was isolated. Also, MIC results are usually quite small; hence, it is likely to have a negligible effect on the *p*-value. Exposing the pathogen isolated from the same biological specimen to an extended period at a much higher environmental temperature may produce a pronounced change in the MIC and the antibiogram. This may provide a more defined and clearer relationship between the drug resistance-associated biofilm and temperature.

P. aeruginosa – a strong biofilm-forming bacterium – may contaminate the hospital bed, linen, sink, and toilet flooring and even colonize the hospital devices (*e.g.* ECG machine, ventilators, intubators, catheters, trolleys, *etc.*). As such, exposure to MDR- and XDR-PA may place immunocompromised patients and those belonging to vulnerable groups (geriatric and pediatric) at greater risk of developing more severe and life-threatening infections. The response demonstrated by drug-resistant pathogens such as *P. aeruginosa* to a changing environmental temperature, as observed in this study, may guide health officials in navigating strategies to mitigate the spread of infections in hospital wards. Infection control may focus on disrupting the ability of this pathogen to build a robust biofilm at low temperatures. Thus, increasing temperature in the hospital wards may weaken the biofilm formed *by P. aeruginosa.* This strategy may be complemented by using antiseptic with antibiofilm activities by healthcare personnel, as this may curtail the spread of MDR and XDR in a hospital environment. Similarly, hospital table surfaces, sinks, toilets, and indwelling instruments must be sterilized with disinfectant with biofilm-inhibiting action. Moreover, increasing environmental temperature may disrupt biofilms on inert surfaces in the hospital environment, in the same manner when fever may be protective to the patients as elevated body temperature may destroy mesophilic bacteria that cause infections in patients. On top of this, handwashing with soap and water remains an effective health practice in warding off microbial infections.

CONCLUSION

In high occurrences, biofilm-forming MDR- and XDR-PA were isolated mostly from wound and urine samples in a tertiary hospital. Antibiotics belonging to the beta-lactam, carbapenem, and cephalosporin groups had the highest

recorded resistance profile. A strong negative relationship between temperature and MIC was noted, mostly among beta-lactam antibiotics. The biofilm formation and pigment production in *P. aeruginosa* clinical isolates were found to be temperature-dependent with enhanced activity at lower temperatures.

STATEMENT ON CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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The complete appendices section of the study is accessible at https://philjournsci.dost.gov.ph

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APPENDICES

Baseline Antibiogram Profile of the 30 *Pseudomonas aeruginosa* Clinical Isolates

Code	Source	Antibiotic resistance patterns
PA001	Endotracheal aspirate	Tetra-resistant (CTZ^R, MEM^R, IMP^R, TZP^R) FEP^I, AMK^S, GEN^S
PA002	Endotracheal aspirate	Mono-resistant (TZP^R) AMK^S, CTZ^S, GEN^S, MEM^S, FEP^S, $CPFX^S$, IMP^S
PA003	Urine	AMK^S, CTZ^S, GEN^S, MEM^S, FEP^S, $CPFX^S$, IMP^S, TZP^S
PA004	Wound	AMK^S, CTZ^S, GEN^S, MEM^S, FEP^S, $CPFX^S$, IMP^S, TZP^S
PA005	Urine	Penta-resistant (CTZ^R, MEM^R, $CPFX^R$, IMP^R, TZP^R) AMK^I, GEN^I, FEP^I
PA006	Sputum	Tri-resistant (CTZ^R, FEP^R, TZP^R) AMK^S, GEN^S, MEM^S, $CPFX^S$, IMP^S,
PA007	Sputum	AMK^S, CTZ^S, GEN^S, MEM^S, FEP^S, $CPFX^S$, IMP^S, TZP^S
PA008	Wound	Octa-resistant (AMK^R, CTZ^R, GEN^R, MEM^R, FEP^R, $CPFX^R$, IMP^R, TZP^R)
PA009	Wound	Bi-resistant (AMK^R, IMP^R) MEM^I, CTZ^S, GEN^S, FEP^S, $CPFX^S$, TZP^S
PA010	Tissue	AMK^S, CTZ^S, GEN^S, MEM^S, FEP^S, $CPFX^S$, IMP^S, TZP^S
PA011	Urine	Hepta-resistant (AMK^R, CTZ^R, MEM^R, FEP^R, $CPFX^R$, IMP^R, TZP^R) GEN^I
PA012	Wound	Tetra-resistant (MEM^R, $CPFX^R$, IMP^R, TZP^R) CTZ^I, FEP^I, AMK^S, GEN^S
PA013	Endotracheal aspirate	CTZ^I, FEP^I AMK^S, GEN^S, MEM^S, $CPFX^S$, IMP^S, TZP^S
PA014	Endotracheal aspirate	Bi-resistant (CTZ^R, TZP^R) AMK^S, GEN^S, MEM^S, FEP^S, $CPFX^S$, IMP^S
PA015	Sputum	AMK^S, CTZ^S, GEN^S, MEM^S, FEP^S, $CPFX^S$, IMP^S, TZP^S
PA016	Urine	Hexa-resistant (CTZ^R, MEM^R, FEP^R, $CPFX^R$, IMP^R, TZP^R) GEN^I, AMK^S
PA017	Urine	Hexa-resistant (CTZ^R, MEM^R, FEP^R, $CPFX^R$, IMP^R, TZP^R) GEN^I, AMK^S
PA018	Wound	Penta-resistant (CTZ^R, MEM^R, FEP^R, IMP^R, TZP^R) $CPFX^I$, AMK^S, GEN^S
PA019	Sputum	Tri-resistant (CTZ^R, FEP^R, TZP^R) AMK^S, GEN^S, MEM^S, $CPFX^S$, IMP^S
PA020	Wound	Hepta-resistant (AMK^R, CTZ^R, MEM^R, FEP^R, $CPFX^R$, IMP^R, TZP^R) GEN^I
PA021	Wound	AMK^S, CTZ^S, GEN^S, MEM^S, FEP^S, $CPFX^S$, IMP^S, TZP^S
PA022	Cranial CSF	Mono-resistant (IMP^R) GEN^I, MEM^I, $CPFX^I$, TZP^I, AMK^S, CTZ^S, FEP^S
PA023	Wound	AMK^S, CTZ^S, GEN^S, MEM^S, FEP^S, $CPFX^S$, IMP^S, TZP^S
PA024	Wound	AMK^S, CTZ^S, GEN^S, MEM^S, FEP^S, $CPFX^S$, IMP^S, TZP^S
PA025	Wound	Mono-resistant (IMP^R) MEM^I, AMK^S, CTZ^S, GEN^S, FEP^S, $CPFX^S$, TZP^S
PA026	Wound	Bi-resistant (CTZ^R, TZP^R) FEP^I, AMK^S, GEN^S, MEM^S, $CPFX^S$, IMP^S
PA027	Wound	Bi-resistant (CTZ^R, FEP^R) AMK^S, GEN^S, MEM^S, $CPFX^S$, IMP^S, TZP^S
PA028	Urine	Mono-resistant (IMP^R) MEM^I, AMK^S, CTZ^S, GEN^S, FEP^S, $CPFX^S$, TZP^S
PA029	Wound	AMK^S, CTZ^S, GEN^S, MEM^S, FEP^S, $CPFX^S$, IMP^S, TZP^S
PA030	Wound	AMK^S, CTZ^S, GEN^S, MEM^S, FEP^S, $CPFX^S$, IMP^S, TZP^S

Legend: [AMK] amikacin; [GEN] gentamicin; [CTZ] ceftazidime; [MEM] meropenem; [IMP] imipenem; [CPFX] ciprofloxacin; [TZP] piperacillin-tazobactam; [FEP] cefepime

APPENDIX II

Zone Diameter and MIC Breakpoints for *Pseudomonas aeruginosa*

Group	Antimicrobial agent	Interpretative categories and MIC breakpoints (µg/mL)		
		S	I	R
β-lactam combination agent	Piperacillin-Tazobactam (100/10 µg)	≤ 16/4	32/4–64/4	≥ 128/4
Cephalosporins	Ceftazidime (30 µg)	≤ 8	16	≥ 32
	Cefepime (30 µg)	≤ 8	16	≥ 32
Carbapenems	Imipenem (10 µg)	≤ 2	4	≥ 8
	Meropenem (10 µg)	≤ 2	4	≥ 8
Aminoglycosides	Amikacin (30 µg)	≤ 16	32	≥ 64
	Gentamicin (10 µg)	≤ 4	8	≥ 16
Fluoroquinolones	Ciprofloxacin (5 µg)	≤ 0.5	1	≥ 2