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Microbial profile and biofilm formation of Pseudomonas aeruginosa from clinical samples at a tertiary care hospital in Nepal

Sudiksha Marahatta[1]

sudikshamarahatta.sm@gmail.com

B. P. Koirala Institute of Health Sciences

Anuradha khanal

B. P. Koirala Institute of Health Sciences

Research Article

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Abstract

Background

Pseudomonas aeruginosa is an important opportunistic pathogen associated with healthcare-associated infection. Its intrinsic and acquired antibiotic resistance mechanism, and its ability to form biofilm, makes infection difficult to treat. This study aims to determine the prevalence and antimicrobial susceptibility pattern of *P. aeruginosa* isolates, and to assess phenotypic ESBL, carbapenemase, and metallo-β-lactamase (MBL) production, as well as biofilm formation.

Methods

A descriptive cross-sectional study was conducted at the B. P. Koirala Institute of Health Sciences in Dharan, Nepal, from September 2023 to August 2024. Clinical specimen submitted for bacteriological culture were processed using standard microbiological methods. *P. aeruginosa* was identified by colony morphology, gram stain, and biochemical tests. Antimicrobial susceptibility test was performed by the Kirby-Bauer disc diffusion method and interpreted according to the CLSI 2024 guideline. ESBL production was detected using the combined-disk method, carbapenemase production with the modified carbapenem inactivation method (mCIM), and MBL production with the EDTA-modified carbapenem inactivation method (eCIM). Biofilm formation was assessed using tube adherence and Congo red agar. The statistical analysis was performed using SPSS version 29.

Results

During the study period, 3,595 clinical specimens were culture-positive, with 178 yielding *P. aeruginosa*, with a prevalence of 4.95%. Of the 178 isolates, 63% were obtained from inpatients and 37% from outpatients. Pus aspirates had the highest percentage of isolates (26%), followed by wound swabs (24%), urine (18%), and sputum (15%). The maximum susceptibility was observed to imipenem (74.71%), followed by meropenem (72.47%), ciprofloxacin (71.34%), tobramycin (70.78%), amikacin (67.41%), and gentamicin (65.16%). ESBL production was detected in 45 isolates (25.28%), carbapenemase production in 52 (29.20%), and MBL production in 33 (18.53%). Biofilm formation was found in 143 isolates (80.33%). Biofilm formation was significantly associated with resistance to amikacin, gentamicin, tobramycin, ciprofloxacin, and meropenem.

Conclusions

Increasing trend of antimicrobial resistance and biofilm formation among *P. aeruginosa* is a great threat for the future, making treatment choices more challenging. Strengthening antimicrobial stewardship is crucial to ensure the appropriate use of antimicrobials. Also, enhancing strict adherence to infection

prevention and control practice is necessary to control the spread of resistant strains. Continuous monitoring and surveillance could identify the resistant strains, approaching the more targeted and effective treatment.

Trial registration

Not applicable. This study was not a clinical trial. It was an observational, laboratory-based study involving the isolation and characterization of *Pseudomonas aeruginosa* from clinical specimens and assessment of biofilm formation.

Introduction

Pseudomonas aeruginosa is a Gram-negative, aerobic, non-spore-forming bacteria found throughout the environment, including soil, water, and plants(1). This opportunistic pathogen is a leading cause of healthcare-associated infection, especially in critically ill and immunocompromised patients. Colonisation is more common in hospitalised patients, especially those with burns, tracheostomy, mechanical ventilation, catheterisation, surgery, or other disruptions to normal epithelial barriers. (2)

P. aeruginosa is considered an important healthcare-associated pathogen because of its diverse virulence factors and its ability to develop resistance to multiple antimicrobial classes. (3)Treatment can be challenging, especially for infections produced by multidrug-resistant strains. β-lactamase production is an important mechanism of antimicrobial resistance in *P. aeruginosa*. (4) ESBLs inactivate extended-spectrum cephalosporins, while carbapenemases, particularly metallo-β-lactamases, hydrolyze carbapenems and substantially reduce therapeutic options. (5)MBLs belong to Ambler class B β-lactamases and hydrolyze a broad range of β-lactam antimicrobials, including carbapenems. (6)

Carbapenem resistance in P. aeruginosa may arise through several mechanisms, including reduced permeability, alteration or loss of the OprD porin, overexpression of efflux pumps, chromosomal AmpC β-lactamase production and acquisition of carbapenemase genes. (7) The emergence and dissemination of carbapenem-resistant *P. aeruginosa* is therefore a major clinical and infection-control concern.

Another important characteristic of *P. aeruginosa* is its ability to form biofilms. Biofilms consist of bacterial communities embedded within an extracellular matrix and facilitate persistence on biological and abiotic surfaces. Biofilm-associated organisms may have reduced susceptibility to antimicrobial agents because of limited antimicrobial penetration, altered metabolic activity, and other physiological adaptations. (8, 9)

Methods

Study design and setting

A hospital-based descriptive cross-sectional study was conducted in the Department of Microbiology, B. P. Koirala Institute of Health Sciences (BPKIHS), Dharan, Nepal, from September 2023 to August 2024.

Study population and specimens

Clinical specimens submitted to the microbiology laboratory for bacteriological culture during the study period were processed using standard microbiological procedures. All clinically significant *P. aeruginosa* isolates recovered during the study period were included.

The specimens included pus, wound swabs, urine, sputum, blood, bronchoalveolar lavage, endotracheal aspirates, tissue, device-associated specimens and other clinical specimens.

Isolation and identification

Specimens were inoculated onto appropriate culture media according to specimen type. Pus, aspirates, wound swabs and sputum were inoculated onto blood agar and MacConkey agar, while urine specimens were inoculated onto cysteine lactose electrolyte deficient agar. Blood specimens were processed using the automated blood culture system available in the laboratory.

Presumptive identification of *P. aeruginosa* was based on colony morphology, pigmentation, Gram staining and oxidase reaction, followed by biochemical identification.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the Kirby–Bauer disk diffusion method on Mueller–Hinton agar. A 0.5 McFarland suspension was prepared and used for inoculation. The antimicrobial disks tested were piperacillin-tazobactam, ceftazidime, cefepime, gentamicin, tobramycin, imipenem, meropenem, amikacin and ciprofloxacin. Zone diameters were interpreted according to CLSI 2024 guidelines. (10)

Detection of ESBL production

According to CLSI guideline, the isolates showing reduced susceptibility to at least one of these drugs with zone of inhibition for Ceftazidime (30µg) 22mm and Cefotaxime(30µg) 27mm is considered as possible ESBL producing strains. The confirmation of suspected ESBL producing strains was done by combined discs assay on MHA plate priory lawn cultured.

The antimicrobial discs used were

Ceftazidime(30µg)

Ceftazidime -Clavulanate (30/10µg)

Each disc was placed at least 24 mm apart center to center. After overnight incubation at 37^0C, the result was interpreted. An enhanced zone of diameter

≥ 5 mm for ceftazidime clavulanate in comparison to zone diameter of ceftazidime alone was considered as ESBL positive. (10)

Detection of carbapenemase production

Detection of carbapenemase was done by modified carbapenem inactivation method (MCIM).

To emulsify bacteria from an overnight blood agar plate in 2 mL TSB, 10-L loopful for *P. aeruginosa* for each isolate that is to be tested was taken Then was Incubated at 35°C ± 2°C in ambient air for 4 hours ± 15 minutes. Then was Inoculated an MHA plate with *E. coli* ATCC 25922 as for the routine disk diffusion procedure Meropenem disk was removed from each TSB-meropenem disk suspension and then placed on the MHA plate previously inoculated with the meropenem-susceptible *E. coli* ATCC 25922 indicator strain MHA plates was inverted and incubated at 35°C ± 2°C in ambient air for 18–24 hours Following incubation, zones of inhibition was measured as for the routine disk diffusion method Zone diameter of 6–15 mm or presence of pinpoint colonies within a 16–18 mm zone indicated positive result.

Detection of MBL production

MBL detection was done after the organism was found to be carbapenemase producer.

It was done by EDTA- modified carbapenem inactivation method (ecim) for detection of IMP MBL producing *P. aeruginosa*.

2ml TSB tube was taken and labelled for ecim test.20µl of 0.5 EDTA was added to 2ml TSB to obtain final concentration of 5mM EDTA. Above steps for mcim was followed for mcim and ecim tubes in parallel. Meropenem disk from mcim and ecim was placed on same MHA plate inoculated with Meropenem susceptible *E. coli* ATCC 25922 indicator strain

For the MBL producer, the activity of carbapenem was inhibited in the presence of EDTA such that meropenem in the disk was not hydrolyzed as early as the tube without EDTA. (10)

Detection of biofilm formation

Biofilm formation will be detected by tube adherence test and Congo red agar method.

Tube adherence test

An overnight culture's colony suspension was inoculated into the trypticase soy broth (TSB) medium (HiMedia, Mumbai, India) and incubated for 24 hours at 35°C. After that, the tubes were flipped over and cleaned with pH 7.3 phosphate-buffered saline (PBS). The tubes were dyed after drying with 0.1% crystal violet (HiMedia). After that, the tubes were repeatedly rinsed under running water to eliminate any remaining stains. They were kept upside-down while biofilm development was monitored. It was regarded positive when there was a biofilm that could be seen lining the tube's wall and bottom.

Congo Red Agar method

To prepare CRA, 37 g of brain heart infusion broth with 1 L of distilled water, 50 g of sucrose, 0.8 g of Congo red dye, and 10 g of agar was combined (all from HiMedia). Following inoculation on CRA, isolates were next cultured aerobically at 35°C for 18–24 hours. Non -biofilm forming isolates produced red colonies, bacteria that created biofilm formed black colonies.(11)

Statistical analysis

Data were entered into Microsoft Excel and analyzed using IBM SPSS Statistics version 29. Descriptive statistics were used to summarize the data

Associations between categorical variables were assessed using the chi-square test. A p-value < 0.05 was considered statistically significant.

Results

Prevalence and distribution of P. aeruginosa

During the study period, 35,116 clinical specimens were processed. Of these, 3,595 were culture positive, and 178 yielded *P. aeruginosa*, giving an overall prevalence of 4.95%.

Among the 178 isolates, 109 (61%) were recovered from female patients and 69 (39%) from male patients. Sixty-three percent of isolates were obtained from inpatients and 37% from outpatients.

The largest proportion of isolates was obtained from patients aged 14–60 years (58.4%), followed by patients older than 60 years (35.4%).

Distribution according to specimen

Pus aspirates constituted the largest proportion of isolates (26%), followed by wound swabs (24%), urine (18%) and sputum (15%).

Antimicrobial susceptibility

The highest susceptibility was observed to imipenem (74.71%), followed by meropenem (72.47%), ciprofloxacin (71.34%), tobramycin (70.78%), amikacin (67.41%) and gentamicin (65.16%). Susceptibility

to cefepime and ceftazidime was 46.62% each, while piperacillin-tazobactam demonstrated the lowest susceptibility (34.83%) (Table 1).

Table 1
Antimicrobial susceptibility pattern of P. aeruginosa isolates

Antimicrobial	Susceptible, n (%)
Amikacin	120 (67.41)
Cefepime	83 (46.62)
Ceftazidime	83 (46.62)
Ciprofloxacin	127 (71.34)
Gentamicin	116 (65.16)
Imipenem	113 (74.71)
Meropenem	129 (72.47)
Piperacillin–tazobactam	62 (34.83)
Tobramycin	126 (70.78)

Table 1 *note: Values are presented as number of susceptible isolates and percentage.*

ESBL, Carbapenemase and MBL production

Of the 178 isolates, 45 (25.28%) were phenotypically identified as ESBL producers. Carbapenemase production was detected in 52 (29.20%) isolates, of which 33 (18.53%) were MBL producers.

Twelve isolates (6.74%) demonstrated concurrent ESBL and MBL production; these isolates were also carbapenemase-positive because MBL is a subgroup of carbapenemases (Table 2).

Table 2
Phenotypic β-lactamase production among P. aeruginosa isolates

Resistance mechanism	n (%)
ESBL	45 (25.28)
Carbapenemase	52 (29.20)
MBL	33 (18.53)
Concurrent ESBL + MBL	12 (6.74)

Table 2 *note: MBL producers are a subgroup of carbapenemase producers; concurrent ESBL + MBL denotes isolates positive for both phenotypes.*

Biofilm formation

Biofilm formation was detected in 143/178 (80.33%) isolates by either or both methods. The tube adherence method detected 140 (78.65%) biofilm-positive isolates, while the Congo red agar method detected 93 (52.24%). Ninety isolates (50.56%) were positive by both methods (Table 3).

Table 3
Biofilm formation by detection method

Method	Biofilm-positive isolates, n (%)
Tube adherence method	140 (78.65)
Congo red agar	93 (52.24)
Both methods	90 (50.56)
Either or both methods	143 (80.33)

Table 3 *note: "Both methods" indicates isolates positive by both tube adherence and Congo red agar methods; "either or both methods" indicates positivity by at least one method.*

Biofilm formation was highest among isolates from bronchoalveolar lavage and blood, followed by pus, tissue, wound swabs, urine, sputum and endotracheal aspirates.

Association between biofilm formation and antimicrobial resistance

Biofilm formation was significantly associated with resistance to amikacin ($p = 0.010$), ciprofloxacin ($p = 0.017$), gentamicin ($p = 0.039$), meropenem ($p = 0.036$) and tobramycin ($p = 0.032$) (Table 4).

Table 4
Association between biofilm formation and antimicrobial resistance

Antimicrobial	Resistance among biofilm producers (%)	Resistance among non-producers (%)	p-value
Amikacin	37.66	14.28	0.010
Cefepime	55.24	40.00	0.182
Ceftazidime	55.24	40.00	0.182
Ciprofloxacin	31.46	11.24	0.017
Gentamicin	36.36	17.14	0.039
Imipenem	27.20	14.28	0.238
Meropenem	32.16	14.28	0.036
Piperacillin–tazobactam	61.53	57.14	0.240
Tobramycin	30.06	11.42	0.032

Table 4 *note: Percentages represent resistance among biofilm producers and non-producers; $p < 0.05$ was considered statistically significant.*

Association between ESBL production and antimicrobial resistance

ESBL production was significantly associated with resistance to cefepime and ceftazidime. No statistically significant association was observed with the other tested antimicrobials.

Association between carbapenemase production and antimicrobial resistance

Carbapenemase production was significantly associated with resistance to all nine tested antimicrobials ($p < 0.001$).

The highest resistance among carbapenemase-producing isolates was observed with meropenem (98.07%), cefepime (94.23%), piperacillin-tazobactam (92.3%) and ceftazidime (90.3%).

Association between MBL production and antimicrobial resistance

MBL production was significantly associated with resistance to all nine tested antimicrobials ($p < 0.001$). Resistance among MBL-producing isolates was particularly high for cefepime, meropenem and piperacillin-tazobactam.

Discussion

The study found the prevalence of *P. aeruginosa* to be 4.95%, which corresponded to a previous study (6.98%) done in the same hospital. (12)Our findings are in the same line with other similar studies conducted in Nepal by Sharma et al. 2016 (4.6%), Maharjan et al. 2022 (6.48%), and Dash et al.2014(9.7%)(13–15). A total of 63% of *P. aeruginosa* were isolated from inpatients and 37% from outpatients. Similar to our study, high rates *of P. aeruginosa* were reported from the inpatient departments in many other studies: 73.22% (Gupta et al. al 2019),73.5% (Ansari et al. al 2016), 80% (Agarwal et al. al 2017), and 83.1% (Maharjan et al. 2022). (16–19)

As reported by the National Healthcare Safety Network between 2011 and 2014, 5.7% of surgical site infections were linked to *P. aeruginosa* (20). Its persistence in the hospital environment serves as a potential reservoir of surgical site infection(21). Contamination of healthcare workers' hands (22) and patients' *P. aeruginosa* intestinal carriage (23)could be other sources of transmission.

Our study isolated 18% of *P. aeruginosa* from the urine specimen. There could be various reasons for the higher isolation of *P. aeruginosa* from urine. Indwelling urinary catheters, vulnerable conditions like prolonged hospitalization, use of broad-spectrum antibiotics, and instrumentation could be the predisposing factors for UTI associated with *P. aeruginosa*.

P. aeruginosa is a most common cause of infection that poses a significant risk to burn patients and is linked to sepsis and even mortality(24). This study has isolated *P. aeruginosa* from one burn case. The wet conditions in which burn patients are exposed may be a factor for infection in burn patients. Likewise, the lowest number of isolates was obtained from various body fluids (1%) in our study, which was similar to the study done in India(25)

P. aeruginosa has developed resistance to various antibiotics, including aminoglycosides, quinolones, and β-lactams (Hancock and Speert, 2000) over the years. Nearly every known resistance mechanism is present in *P. aeruginosa* isolates, making the bacteria an emblem of the phenomena of antibiotic resistance. As a result of many resistance mechanisms, *P. aeruginosa* frequently exhibits multidrug-resistant (MDR) phenotypes(26). In our study, among the tested antibiotics, the highest number of isolates were found sensitive to imipenem (74.71%), which is in concordance with the study done earlier by Rinku Sah et al. al in the same setup. This also corresponds with a study that was published in Kathmandu in 2013 by Chander et al., which showed a higher number of isolates sensitive to Imipenem. Our study revealed the highest number of isolates were found resistant to piperacillin-tazobactam, ceftazidime, and cefepime. A study done by Djuikoue et.al. in Central Africa showed a higher number of isolates resistant to piperacillin-tazobactam (27). Likewise, a previous study done in our hospital by Rinku Sah et al. al showed a higher number resistance to ceftazidime and cefepime.

To identify isolates that produced ESBL, the present study found 25.28% of the isolates to be ESBL producers. Similarly, study from India have reported ESBL production in approximately 24% of the *P. aeruginosa* isolates(28). Urine (24.4%), sputum (22.2%), and pus (26.6%) specimens included the highest

number of ESBL producers. In a study done by Nithyalakshmi J et al. 2016, the highest number of ESBL producers was obtained from urine (27.7%), followed by sputum (23.68%) and pus (22.95%). (28) In our study, among the ESBL producers, the majority of isolates were found to be sensitive to meropenem, which was in concordance with the study done by Omer et. al.(29). It has been shown that carbapenem is the most effective antibiotic drug for infections caused by ESBL producers(30, 31). The overuse of antibiotics in recent years has led to a gradual increase in *P. aeruginosa* drug resistance. Multidrug-resistant, pan-drug resistant bacteria have emerged, posing serious challenges for clinical prevention and treatment and even creating a situation where no medication is available(32). According to Zavascki et al. 2010, carbapenems are the preferred medication for treating infections by *Pseudomonas aeruginosa*, which can produce cephalosporinase, AmpC, or extended-spectrum beta-lactamases. Carbapenem use in treating *P. aeruginosa* infections is now in jeopardy due to the emergence of carbapenem-resistant strains of *P. aeruginosa.* Resistance to carbapenem is most commonly caused by impermeability, caused by deletion or modification of the porin OprD, increased expression of an efflux pump, or synthesis of class B metallo-lactamases (MBLs) (Kateete et al. 2016). Production of metallo-beta-lactamase (MBL) enzyme has been one of the major causes of carbapenem resistance in *P. aeruginosa*. Metallobeta-lactamase mediates resistance to beta-lactams by cleaving the amide bond of the beta- lactam ring. In 2017, Carbapenem-resistant *P. aeruginosa* (CRPA) was ranked as the second most critical-priority bacterium among 20 antimicrobial-resistant bacterial species in a survey conducted by the World Health Organization on multi-country antibiotic resistance (Tacconelli et al. 2018). Our study found 29.20% of the isolates to be carbapenemase producers, which is slightly higher than a study conducted by Maharjan et al. (2020). Click or tap here to enter text. Similarly, our study revealed 18.53% of MBL producers, with the majority of them recovered from pus and wound swab aspirate. According to a similar study carried out in Kathmandu, *P. aeruginosa* produced 16.40% (16) and 20.75% (17) of MBL.

P. aeruginosa grows slowly in the deeper layers of dense biofilms, which could account for multidrug resistance as antibiotic molecules don't penetrate further across the biofilm matrix. (33). A higher number of biofilm-producing*P. aerginosa* isolates were recovered from bronchoalveolar lavage followed by blood, drain fluid, pus aspirate, tissue specimen, wound swab and sputum samples. A total of 80.33% of isolates were biofilm positive by either or both methods of biofilm detection. A higher number of biofilm detections was seen in the tube adherence method than in the Congo red agar method. There was a significant association between antibiotic resistance and biofilm formation for aminoglycosides, meropenem, and ciprofloxacin. Isolates that were biofilm producers were also found to be resistant to gentamicin, amikacin, tobramycin, ciprofloxacin, and meropenem. Rewatkar and Wadher (34) observed very high rates of biofilm development among the strains of *P. aeruginosa* (90% by Congo red agar method and 83.33% by tube adherence method), in comparison to our study. Even though our study's tube adherence method discovered more biofilm producers, the Congo red agar method is simpler and less expensive than the tube adherence method, making it more suitable for testing in labs in developing nations like Nepal(35)

Strengths and limitations

The major strength of this study is the inclusion of 178 clinical *P. aeruginosa* isolates collected over one year from a tertiary care hospital. The study evaluated antimicrobial susceptibility together with phenotypic ESBL, carbapenemase, MBL and biofilm formation.

However, several limitations should be considered. First, ESBL, carbapenemase and MBL production were assessed using phenotypic methods only. Molecular detection of resistance genes was not performed; therefore, specific genetic determinants cannot be inferred from the phenotypic findings.

Second, biofilm formation was assessed using tube adherence and Congo red agar methods. A quantitative microtiter plate assay, which may provide a more standardized quantitative assessment of biofilm biomass, was not performed.

Third, this was a single-centre cross-sectional study, and therefore the findings may not be generalizable to all hospitals in Nepal. Finally, the cross-sectional design allows identification of associations but does not establish causal relationships between biofilm formation and antimicrobial resistance.

Conclusions

Clinical *P. aeruginosa* isolates from this tertiary care hospital demonstrated substantial antimicrobial resistance, with phenotypic ESBL production in 25.28%, carbapenemase production in 29.20%, and MBL production in 18.53% of isolates.

Biofilm formation was highly prevalent, being detected in 80.33% of isolates by either or both phenotypic methods. Biofilm formation was significantly associated with resistance to important antimicrobials, including amikacin, gentamicin, tobramycin, ciprofloxacin and meropenem.

The findings highlight the importance of continuous antimicrobial resistance surveillance, antimicrobial stewardship and effective infection prevention and control. Further multicentre studies incorporating molecular characterization of resistance determinants and quantitative biofilm assays are warranted.

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki. Ethical approval for the study was obtained from the Departmental Research Unit (DRU), an authorized wing of the Institutional Review Committee (IRC), B.P. Koirala Institute of Health Sciences (BPKIHS), Dharan, Nepal (Reference No. 148/080/081).

Consent to Participate

Written informed consent to participate was obtained from adult participants. For participants younger than 16 years of age, written informed consent was obtained from their parents or legal guardians.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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No funding was received for this study.

Author Contribution

SM conceived and designed the study. SM contributed to the acquisition of data. SM performed the laboratory investigations and data analysis. SM drafted the manuscript. All authors contributed to the interpretation of the results, critically revised the manuscript for important intellectual content, and approved the final version of the manuscript for publication.

Data Availability

The datasets generated and/or analyzed during the current study are not publicly available due to restrictions related to patient confidentiality and ethical considerations. The data are, however, available from the corresponding author on reasonable request and subject to appropriate permission and applicable ethical and institutional requirements

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Figures

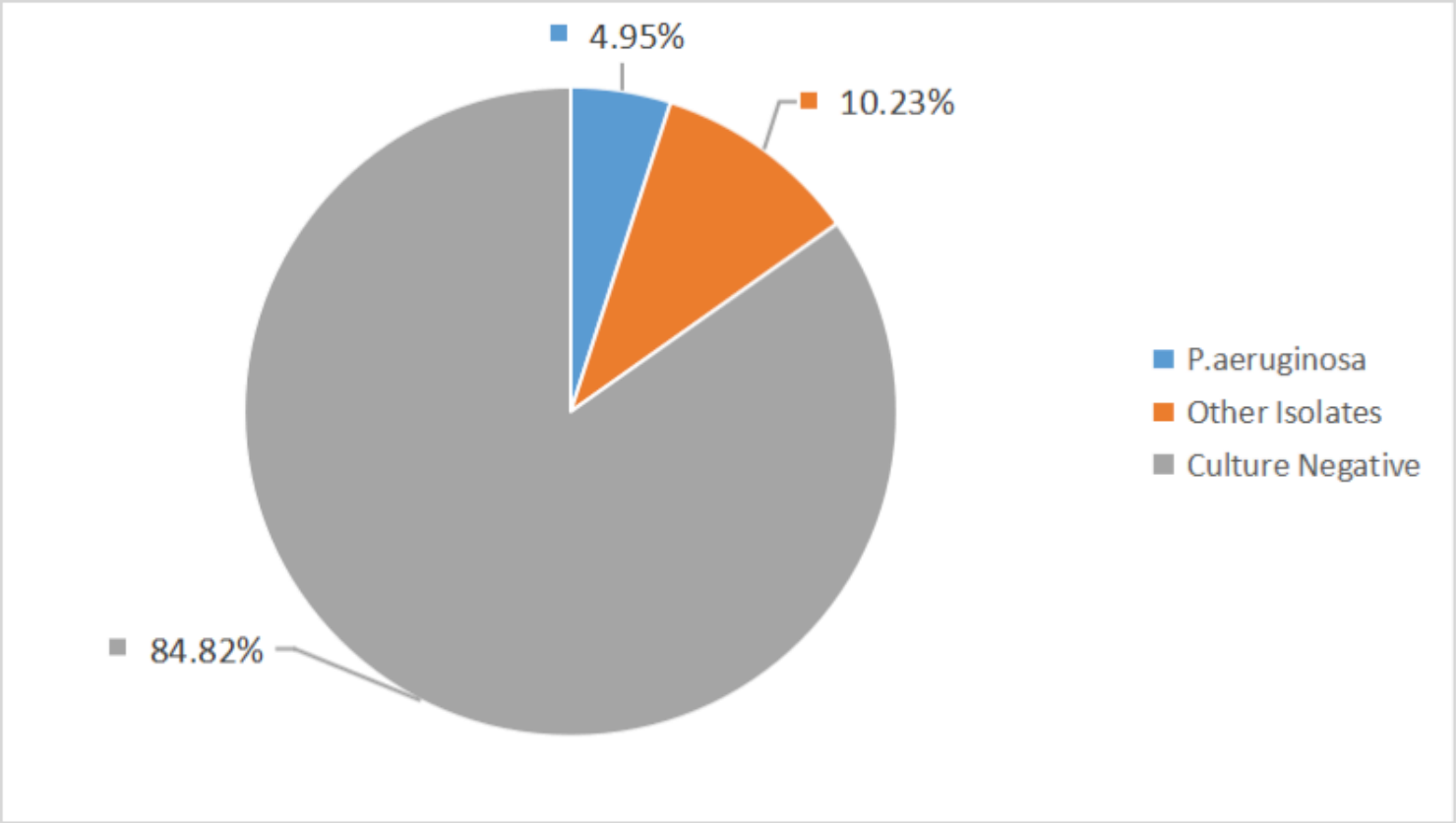


Figure 1

Unnumbered image in the Result section.