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Oral cavity colonization by *Pseudomonas aeruginosa* in patients admitted in the intensive care units (ICUs)

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Patients admitted in the Intensive Care Units (ICUs) often experience various oral health issues during their hospitalization, which can lead to a shift in their oral flora from Gram-positive to pathogenic Gram-negative bacteria, including *Pseudomonas aeruginosa*. **Aim:** This study aimed to identify the colonization of *P. aeruginosa* in the oral cavity of ICU patients, as well as to assess its antimicrobial susceptibility profile, phenotypic resistance mechanisms, and biofilm-forming capacity. **Methods:** Oral cavity samples were collected from patients in ICUs across three public hospitals in Porto Velho, Rondônia, Brazil. Antimicrobial susceptibility testing was performed using the disk diffusion method, phenotypic research on resistance determinants was conducted using a disk method with and without the addition of enzymatic inhibitors, and biofilm formation was assessed by measuring absorbance in microplates. **Results:** A total of 114 oral cavity samples were obtained, with 28% showing colonization by *P. aeruginosa*. The antimicrobial susceptibility profile revealed that 93.7% of the isolates were susceptible to Polymyxin B. The highest rate of non-susceptibility was observed with levofloxacin (46.8%), followed by carbapenems: imipenem (43.7%) and meropenem (40.6%). Additionally, 37.5% of the isolates were classified as multidrug-resistant (MDR). In phenotypic research for carbapenemases, 35.7% of the isolates were positive for plasmid-mediated AmpC, and 14.3% were positive for Metallo-β-lactamases. Regarding biofilm production, 75% of the isolates were classified as moderate/strong biofilm producers. **Conclusion:** Therefore, oral cavity colonization by *P. aeruginosa* in ICU patients is evident, with notable multidrug resistance to antibiotics, highlighting the need for vigilant monitoring and effective oral hygiene practices for these patients.

Keywords: Biofilms. Bacteria. Drug resistance.

Introduction

The human microbiome is a critical factor in maintaining the balance between health and disease, with the oral microbiota being the second most diverse microbial community after the intestinal microbiota. It serves as a primary entry point for various microorganisms[1]. According to the Human Oral Microbiome Database, there are over 774 oral bacterial species, with 58% of these already identified and named.

In a healthy individual, a balanced and stable oral microbiota acts as a barrier that prevents the colonization of opportunistic pathogens and subsequent oral or systemic infections[2]. However, an imbalance between the microbiota and the host can lead to the onset of various pathologies, such as periodontal diseases, tonsillitis, systemic infections, cardiovascular diseases, and pneumonias[3].

Patients admitted to an Intensive Care Unit (ICU) often experience various oral health issues caused by factors such as malnutrition, endotracheal tubes, reduced fluid intake, and oral medication administration. These factors, combined with inadequate oral care, lead to oral mucosal dryness and inflammation, altered salivary flow, increased dental plaque formation, and biofilm accumulation with pathogenic bacteria[4,5]. Consequently, within the first 48 hours after ICU admission, the patient's oral flora is likely to change, being predominantly replaced by pathogenic Gram-negative bacteria such as *Pseudomonas aeruginosa*, which is mainly associated with Ventilator-Associated Pneumonia (VAP)[6].

P. aeruginosa is a Gram-negative, ubiquitous bacillus found in various environments such as water, soil, food, and hospital settings[7]. It is an opportunistic pathogen responsible for Healthcare-Associated Infections (HAIs) in ICUs, primarily affecting immunocompromised patients, those with pulmonary infections, and patients with cystic fibrosis, leading to high morbidity and mortality rates[8,9].

Ventilator-Associated Pneumonia (VAP) is a pulmonary infection that develops after 48 hours of patient hospitalization, accounting for more than 20% of hospital-acquired infections[10]. *P. aeruginosa* is a leading cause of these infections, with mortality rates exceeding 30%[11]. Furthermore, the widespread emergence of multidrug-resistant strains of this pathogen has become a global public health issue, impacting clinical outcomes and increasing morbidity and mortality rates within ICUs[12].

The World Health Organization[13] (WHO, 2024) classifies carbapenem-resistant *P. aeruginosa* (CRPA) as a high group pathogen for which research and development of new antibiotics are urgently needed due to the extensive resistance of this bacterium to antimicrobials. Resistance to carbapenems has become a health emergency, as this class of antibiotics represents a high-potency, broad-spectrum option used as a last resort for infections caused by this species[14].

P. aeruginosa has various mechanisms of antibiotic resistance related to membrane permeability, such as efflux pumps and reduced production of porins, as well as the production of enzymes that hydrolyze antibiotics, including carbapenemases[15]. The emergence and spread of carbapenemases have become a serious issue, as they confer resistance not only to carbapenems but also to other β-lactam antibiotics and β-lactamase inhibitors[16].

A global cohort study conducted by Reyes et al.[17] (2023) to analyze the global frequency and clinical impact of CRPA found that South and Central America had the highest rate of carbapenemase presence, with 69% of isolates. Additionally, data from the SENTRY program reveal that between 1997 and 2016, *P. aeruginosa* had a 44.6% isolation rate in hospitalized patients, with a high tendency towards resistance to carbapenems[18].

Bacterial biofilms are also associated with antibiotic resistance in *P. aeruginosa*. In addition to being an intrinsic biofilm producer, biofilm formation is considered a virulence factor for the species, enhancing its ability to colonize and resist extreme environments and antibiotics[19]. Inadequate oral hygiene in ICU patients leads to increased dental plaque (biofilm), which becomes a reservoir for pathogenic microorganisms, increasing plaque mass and producing more biofilm. This, in turn, reinforces colonization and serves as an entry point for infections in the host[4,6].

Therefore, considering the prevalence, pathogenic role, and high morbidity and mortality rates caused by *P. aeruginosa* in ICU patients, this study aimed to investigate the colonization, antimicrobial susceptibility profile, biofilm formation, and phenotypic detection of carbapenemases in isolates of this pathogen derived from oral cavity samples of patients admitted to ICUs.

Materials and Methods

Sample Collection and Processing

Samples were collected between December 2017 and February 2018, and between December 2018 and January 2019. Oral cavity secretions were obtained using swabs from patients hospitalized in three public hospitals (referred to as Hospital I, Hospital II, and Hospital III) in Porto Velho, Rondônia, Brazil. Following collection, the samples were transported to the Microbiology Laboratory at Fiocruz – Rondônia in thermally controlled containers for processing, isolation, identification, and characterization of microorganisms.

This study was approved by the Ethics Committee of Tropical Medicine Research Center, Porto Velho, Rondônia, Brazil (Process n. 2.368.951).

Bacteriology and Isolation of Isolates

All samples were inoculated onto various culture media, including Blood Agar (BA; HiMedia®, India), MacConkey Agar (MC; FirstLab®, Brazil), Cetrimide Agar (CT; Biolog®), and Luria-Bertani Broth (LB; Kasvi®, Spain). The plates were then incubated in bacterial incubators at approximately 37°C for 18 to 24 hours.

Colonies suggestive of *P. aeruginosa* were subjected to genomic DNA extraction using phenol-chloroform, followed by amplification of the 16S rRNA gene using conventional PCR as described by Arruda et al.[20]. The amplified products were purified using the QIAquick Gel Extraction Kit (QIAGEN®, Germany) according to the manufacturer's protocol. The purified DNA was quantified using NanoDrop1000® (Thermo Scientific®, USA) and sequenced using Sanger methodology.

Sequence analysis and consensus sequence generation were performed using BioEdit Sequence Alignment Editor (version 7.0), and species identification and confirmation were carried out using the Basic Local Alignment Search Tool (BLAST) database.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was conducted using the disk diffusion method on Mueller-Hinton Agar (Kasvi®, Italy), following the Clinical Laboratory Standards Institute (CLSI) 2022 guidelines. The following antibiotics were tested: Piperacillin-tazobactam (TTP – 100/10 µg), ceftazidime-avibactam (CZA – 30/20 µg), ceftolozane-tazobactam (C/T – 30/10 µg), ceftazidime (CAZ – 30 µg), cefepime (CPM – 30 µg), aztreonam (ATM – 30 µg), imipenem (IPM – 10 µg), meropenem (MER – 10 µg), gentamicin (GEN – 10 µg), tobramycin (TOB – 10 µg), amikacin (AMI – 30 µg), ciprofloxacin (CIP – 5 µg), and levofloxacin (LVX – 5 µg). *E. coli* ATCC® 25922 was used as a quality control. Multidrug resistance (MDR) was classified according to the criteria established by Magiorakos et al.[21].

For susceptibility testing to Polymyxin B, the microdilution method was used to determine the minimum inhibitory concentration (MIC) according to the POLICIMBAC kit (Probac®, Brazil). *Pseudomonas aeruginosa* ATCC® 27853 was used as a quality control.

Phenotypic Detection of Carbapenemases

Phenotypic detection of carbapenemases was performed following the methodology outlined in Technical Note No. 01/2013 from the Brazilian National Health Surveillance Agency (ANVISA). A combined disk test was conducted using ertapenem (ETP – 10 µg), imipenem (IPM – 10 µg), and meropenem (MER – 10 µg) with and without the addition of enzyme inhibitors. For enzyme interpretation and classification: for Ambler class A, IPM and MER were impregnated with phenylboronic acid (PBA); for class B, ethylenediaminetetraacetic acid (EDTA) was used; and for class C, PBA or cloxacillin (CLOXA) was used. Quality control for the tests included *E. coli* ATCC® 25922, *Klebsiella pneumoniae* CCBH 16302, *Klebsiella pneumoniae* CCBH 6556, and other strains from the CCBH collection at Fiocruz (http://ccbh.fiocruz.br/).

Biofilm Formation Test

The biofilm formation capacity of the isolates was evaluated in triplicate as described by Alvim et al.[22] with modifications, using 96-well polystyrene microplates.

P. aeruginosa isolates were grown in Luria-Bertani broth (LB; Kasvi®, Spain) in an orbital shaker at 120 rpm at approximately 37°C overnight for 18 to 24 hours. After this period, the culture was diluted 1:20 in sterile LB, and 200 µl of the suspension was transferred in triplicate to the 96-well polystyrene microplates (Costar, USA), which were incubated at approximately 37°C for 24 hours. Subsequently, the plates were washed twice with 200 µl of distilled water to remove LB and non-adhered bacteria. Adhered bacteria were then stained for 5 minutes with 100 µl of crystal violet (0.1% w/v, Laborclin®, Brazil), excess dye was removed with distilled water, and elution was performed with 95% ethanol (Neon®, Brazil). Biomass was quantified by measuring absorbance with a spectrophotometer (Biotek Epoch®, USA) at a wavelength of 570 nm (optical density – OD). *E. coli* ATCC® 25922 was used as a negative control, and *P. aeruginosa* ATCC® 27853 was used as a positive control.

Results were interpreted based on the average of triplicates for each isolate. The cutoff point was calculated by the mean OD of the negative control plus three times the standard deviation of the same. Isolates with OD values equal to or below the cutoff point were considered weak/non-biofilm producers, while those with OD values above the cutoff point were considered moderate/strong biofilm producers.

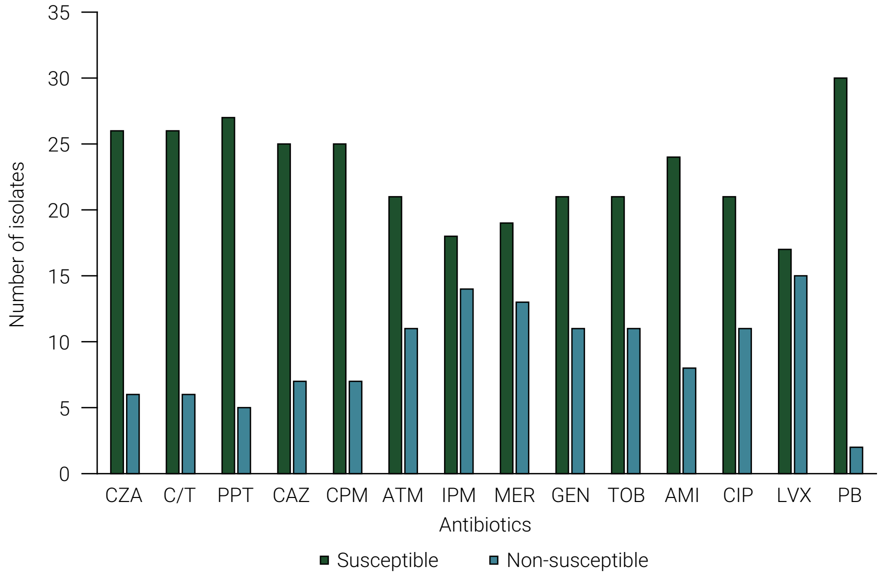
Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5.0 software, employing non-parametric tests such as Fisher's Exact Test and Odds Ratio. Statistical significance was defined as a p-value < 0.05.

Results

A total of 144 oral cavity samples were collected. Stratified by hospital, 42.1% (48/114) of the samples were from Hospital I, 32.4% (37/114) from Hospital III, and 25.5% (29/114) from Hospital II. Of these, 28% (32/114) of the patients were found to be colonized by *Pseudomonas aeruginosa*.

The antibiotic susceptibility profile showed that 93.7% (30/32) of the oral cavity isolates were susceptible to polymyxin B, 84.3% (27/32) to piperacillin-tazobactam, 81.2% (26/32) to ceftazidime-avibactam and ceftolozane-tazobactam, and 78.1% (25/32) to ceftazidime and cefepime. Among the non-susceptible isolates, the highest rate observed was for levofloxacin at 46.8% (15/32), followed by carbapenems, with 43.7% (14/32) for imipenem and 40.6% (13/32) for meropenem. Additionally, two isolates exhibited resistance to polymyxin B. Figure 1 illustrates the susceptibility profile of the oral cavity isolates to all tested antibiotics. In terms of multidrug resistance, 37.5% (12/32) were classified as MDR.



AMI: amikacin; ATM: Aztreonam; CAZ: Ceftazidime; CIP; Ciprofloxacin; CPM: Cefepime; CZA: Ceftolozane-Avibactam; C/T: Ceftazidime-Tazobactam; GEN: Gentamicin; IPM: Imipenem; LVX: Levofloxacin; MER: Meropenem; PB: Polymyxin B; PPT: Piperacillin-Tazobactam; TOB: Tobramycin.

Figure 1. Antimicrobial susceptibility profile of *Pseudomoas aeruginosa* isolates from the oral cavity of patientes admitted in the ICUs.

For the phenotypic detection of carbapenemases, only isolates that exhibited non-susceptibility to carbapenems (imipenem and meropenem) were tested. Thus, 14 oral cavity isolates were assessed, with 50% (7/14) being negative, 35.7% (5/14) positive for plasmid-mediated AmpC, and 14.3% (2/14) positive for metallo-β-lactamases (MBL). Table 1 presents the quantity of detected enzymes and the resistance classification of the isolates.

Table 1. Phenotypic detection of carbapenemases and multidrug resistance classification in *Pseudomonas aeruginosa* isolates from the oral cavity of patients admitted in the ICUS.

Oral cavity	**Resistance classification**		
carbapenemases	**MDR**	**Non-MDR**	**total**
Plasmid-mediated AmpC	60% (3/5)	40% (2/5)	100% (5/5)
MBL	100% (2/2)	0	100% (2/2)
Negative	71,4% (5/7)	28,6% (2/7)	100% (7/7)

Biofilm production was observed in 75% (24/32) of the oral cavity isolates as moderate/strong biofilm producers, while 25% (8/32) were weak/non-producers. Figure 2 displays the average absorbance obtained for each tested isolate.

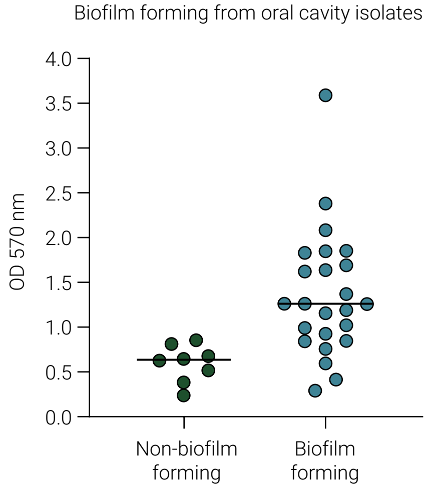


Figure 2. Mean absorbance of *Pseudomoas aeruginosa* isolates subjected to the biofilm formation test.

Analysis of biofilm formation and multidrug resistance (MDR) classification among the isolates, as shown in Table 2, revealed that 37.5% (12/32) of the isolates were both MDR and moderate/strong biofilm producers, which was the same rate observed in non-MDR isolates that were also moderate/strong biofilm producers. Additionally, 25% (8/32) of the isolates were weak/non-biofilm producers and non-MDR. No statistically significant relationship was observed between biofilm production and multidrug resistance.

Table 2. Biofilm formation and multidrug resistance classification of *Pseudomonas aeruginosa* isolates from the oral cavity of patients admitted in the ICUs.

Oral cavity	**Biofilm model**	
MDR classification	**Strong/moderate producer**	**Weak/non producer**
MDR	37,5% (12/32)	0
Non-MDR	37,5% (12/32)	25% (8/32)

Discussion

Patients hospitalized in intensive care units (ICUs) are vulnerable to various complications during their stay, including oral health issues. Factors such as malnutrition, the presence of endotracheal tubes, low fluid intake, poor oral hygiene, and others contribute to susceptibility to pathogenic bacterial colonization, which can lead to multiple infections[4].

In this study, out of 114 oral swabs collected from patients in ICUs across three hospitals, 28% (32/114) were colonized with *Pseudomonas aeruginosa*. A study conducted by Bratic et al.[23] (2021) found a 14.6% (33/225) rate of oral colonization by *P. aeruginosa* among ICU patients in Croatia. These findings are alarming, as oral colonization by Gram-negative bacteria can become a gateway for other types of infections. Furthermore, colonization by *P. aeruginosa* is concerning due to its prevalence in cases of hospital-acquired pneumonia (HAP), which has been associated with aspiration of oral colonization and its multidrug resistance[24,25].

In this study, 37.5% of the isolates were classified as multidrug-resistant (MDR), with the highest rates of non-susceptibility observed to levofloxacin (46.8%), imipenem (43.7%), and meropenem (40.6%). Levofloxacin is part of the fluoroquinolone class, a major group of antibiotics used for treating *P. aeruginosa* infections. It is easily administered, being the only therapy given orally, which contributes to its widespread use[26]. Additionally, the second highest rates of non-susceptibility observed in the study were to carbapenems.

Few studies have addressed the colonization of Gram-negative bacilli, specifically *P. aeruginosa*, in the oral cavity, but some report colonization by this pathogen with non-susceptibility to carbapenems[27,28], which is alarming given that this antimicrobial class is one of the last-resort treatments for infections and resistance to it is associated with adverse clinical outcomes[29]. Moreover, these strains have various resistance mechanisms, such as carbapenemases, which not only confer resistance to carbapenems but also extend to other antimicrobial classes[17].

In this study, antibiotic resistance mechanisms were investigated using phenotypic methods, revealing positivity for AmpC and Metallo-β-lactamases. AmpCs are chromosomal enzymes, also known as *Pseudomonas*-derived cephalosporinases (PDCs), which can induce resistance to β-lactams. While the expression of AmpC alone does not affect carbapenems, it contributes to the resistance phenotype in conjunction with other mechanisms[14]. This study had limitations regarding the detection of resistance mechanisms, as it was limited to phenotypic detection of enzymes, requiring genotypic information for a complete assessment of the resistance profile of *P. aeruginosa* isolates. Additionally, phenotypic tests have limitations in specificity and sensitivity, particularly concerning false positives and indistinction of enzymes[30,31].

Of the 32 oral isolates, 75% were moderate/strong biofilm producers. This result is expected, as *P. aeruginosa* is intrinsically a biofilm producer, considered a virulence factor in the species and associated with horizontal gene transfer of resistance[19]. Furthermore, the presence of *P. aeruginosa* in dental plaques has been reported[32,33]. However, the presence of these species in the oral cavity of ICU patients should be monitored to prevent microaspiration of these pathogens and, consequently, avoid infectious processes such as hospital-acquired pneumonia commonly caused by *P. aeruginosa*[34]. In this regard, the process and quality of oral hygiene in patients should be assessed, as they can help prevent the proliferation of these pathogens and reduce infections, mortality, and length of hospital stay[6].

In conclusion, based on the results presented in this study, monitoring the oral cavity of ICU patients is necessary to track pathogenic microorganisms, reinforce effective oral hygiene guidelines and protocols, and encourage more robust research on the topic to aid in addressing this issue.

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Conflict of Interest

The authors have no conflict of interest to disclose.

Data availability

Datasets related to this article will be available upon request to the corresponding author.

Author Contribution

Marcos Eduardo Passos da Silva: contributed to the laboratory analyzes; data acquisition, performed the analysis, interpretation of data for the work, literature search and revised the manuscript. **Luccas Manoel de Melo Suica**: perfomed the analysis of data for the work and revised the manuscript. **Renata Santos Rodrigues** and **Anjo Gabriel Carvalho**: perfomed the analysis of data for the work and revised the manuscript. **Izabelly Vitória Gotara Ramos**, **Nucia Cristiane da Silva Lima**, **Mayra Gyovana Leite Belém** and **Rosimar Pires Esquerdo** conducted laboratory analyzes. **Najla Benevides Matos**: conceptualized the project, interpretation of data, revised the manuscript and supervised the project. All authors actively revised and approved the final version of the manuscript.

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