

# Original Research Article

## Isolation, Antibiotic Sensitivity Profiling, and Biocide Tolerance of *Pseudomonas aeruginosa* from Canine Otitis Cases

### ABSTRACT

**Aims:** This study aimed to detect *Pseudomonas* from cases of canine otitis and profile their antibiotic sensitivity pattern.

**Study design:** Samples were collected aseptically. Antibiogram was done by disc diffusion test. The broth microdilution method was applied for biocide tolerance against chlorhexidine and Cetyl Trimethyl ammonium bromide.

**Place and Duration of Study:** The study period was from March 2023 to March 2024. Samples collected were by veterinarians treating the dogs presented at Teaching Veterinary Clinical Complex Pookode and Peripheral Veterinary Clinics, Kakkavayal, Wayanad District, Kerala, India and submitted to the lab.

**Methodology:** *Pseudomonas aeruginosa* were identified based on colony characteristics and biochemical tests. Antibiotic sensitivity was estimated by a disc diffusion test in Muller Hinton Agar. Broth microdilution assay for the minimum inhibitory concentration of biocides was done in 250 ulmicrotitre plate and employed cation-adjusted Muller Hinton Broth for culturing bacterial isolates. Statistical analysis using Orange Machine Learning Software and Jamovi statistical application.

**Results:** Nine isolates of *Pseudomonas aeruginosa* were obtained. Ciprofloxacin was most effective against most isolates. The difference was statistically significant. Polymixin B and ceftriaxone-tazobactam had lower median zone sizes and were below the cut-off point for sensitivity. There was variation in the zone diameter for most of the drugs. The distribution of zone diameters was positively skewed for gentamicin, ceftriaxone-tazobactam, levofloxacin, ceftazidime, ciprofloxacin, and ofloxacin. Chlorhexidine had a statistically significant lower

MIC than for CTAB.

**Conclusion:** Ciprofloxacin could be a better therapeutic option for treating canine otitis caused by Pseudoonas. Better environmental sanitation against pathogenic Pseudomonas aeruginosa could be attained by chlorhexidine than CTAB.

*Keywords: Pseudomonas aeruginosa, Biocide tolerance, antibiogram, ciprofloxacin, Chlorhexidine*

## 1. INTRODUCTION

Canine otitis is a prevalent condition that causes significant irritation, scratching, and distress to affected dogs. Among the various pathogens responsible for this condition, Pseudomonas species are often implicated. Pseudomonas are obligate aerobes that thrive in oxygen-rich environments such as soil and are closely associated with increased anthropogenic activities, from where dogs can easily acquire the infection (1). Pseudomonas are part of the ESKAPE group of pathogens, known for their ability to rapidly develop resistance to multiple antibiotics (2). Understanding the sensitivity patterns of Pseudomonas is thus imperative for better antimicrobial stewardship and informed decision-making in the treatment of canine otitis.

Biocides are chemical agents used to control harmful microorganisms through nonspecific mechanisms of action. It was previously believed that resistance to biocides would be unlikely due to their broad-spectrum activity and nonspecific modes of action. However, resistance to biocides has been reported in various bacterial species (3). Despite the widespread use of biocides like chlorhexidine and quaternary ammonium compounds, there is limited data on the biocide tolerance of Pseudomonas, particularly from veterinary sources.

Given this knowledge gap, our study aimed to isolate and identify Pseudomonas from cases of canine otitis, profile their antibiotic sensitivity patterns, and evaluate their tolerance to chlorhexidine gluconate and Cetyl Trimethyl Ammonium Bromide (CTAB), a commonly used quaternary ammonium compound. This research will provide valuable insights into the resistance mechanisms of Pseudomonas, guiding more effective treatment strategies and improving outcomes for canine patients.

## 2. MATERIAL AND METHODS

## 2.1 Study area

The study was conducted during the period from March 2023 to March 2024. Dogs presented at Teaching Veterinary Clinical Complex Pookode and Peripheral Veterinary Clinics, Kakkavayal, Wayanad District, Kerala with clinical signs suggestive of otitis like head shaking, ear scratching, redness of ear and presence of otic discharge studied.

## 2.2 Isolation and identification of *Pseudomonas*

Cases presented were clinically examined for the presence of otitis, which included head shaking, ear scratching, redness of the ear and the presence of otic discharge. Ear swabbing was aseptically collected using a cotton-tipped polypropylene swab (Himedia, Mumbai) and immediately transported to the laboratory for cultural isolation. The swab was streaked on Brain Heart Infusion agar and incubated at 37°C for 24 h for colonies to form. *Pseudomonas aeruginosa* were identified based on Gram staining, oxidase test, catalase test, growth on selective media Cetrimide Agar, and presence of fluorescent pyocyanin(4).

## 2.3 Antibiotic sensitivity profile

The disc diffusion method was performed as per CLSI 2021. Bacterial isolates were inoculated in sterile Muller Hinton Broth and incubated overnight at 37°C and turbidity adjusted to 0.5 McFarland Standard. The broth culture was spread over the Muller-Hinton agar plate using a sterile swab to make a lawn. Plates were allowed to dry, and various antibiotic discs were placed on the surface with enough spaces in between for the antimicrobial to diffuse. Plates were incubated at 37°C for 24 hours and the zone of inhibition of growth of the organism around each disc was measured in millimetres and interpreted as sensitive or resistant by comparing the ranges given by the manufacturer. Antibacterial discs used in the study were Piperacillin-Tazobactam -100/10µg (PIT 100/10), Aztreonam-30µg (AT30), Cefipime- 30µg (CPM30), Imipenem- 10µg (IPM10), Norfloxacin- 10µg (NIX10), Ceftazidime- 30µg (CAZ30), Gentamicin- 10µg (GEN10) Ceftriaxone-Tazobactam- 90µg - (CIT90), Tobramycin- 10µg (TOB10), Ofloxacin- 5µg (OF5), Polymixin- B- 300IU (PB300) and Levofloxacin- 5µg (LE5).

## 2.4 Broth Microdilution Assay

The biocide tolerance of the isolates was evaluated using chlorhexidine and cetyl trimethyl ammonium bromide (CTAB) through a broth microdilution assay on a 96-well U-bottom microtitre plate. The bacterial suspension was prepared at a turbidity of 0.5 McFarland standard. Equal volumes of biocide and bacterial suspension were added to each well.

The procedure began with the preparation of biocide solutions. In the first column of the plate, 100  $\mu$ L of the required drug solution was added to each well. For wells 2 to 12, 50  $\mu$ L of sterile cation-adjusted Mueller Hinton Broth (MHB) was added. A serial dilution was then performed by transferring 50  $\mu$ L from the first well to the second well and continuing this process up to the 11th well, discarding 50  $\mu$ L from the final well to maintain a consistent volume. To ensure sterility control, the 12th column was left containing only the broth without any biocide or bacterial suspension. Following this, 50  $\mu$ L of the bacterial suspension, standardized to 0.5 McFarland unit, was added to each well except those in the sterility control column. The plate was then incubated at 37°C for 24 hours to allow bacterial growth. After the incubation period, 20  $\mu$ L of 1% resazurin dye was added to each well, and the plate was incubated for an additional 30 minutes. Bacterial growth was indicated by a colour change in the dye from blue to pink. The initial concentrations of chlorhexidine and CTAB used were 50  $\mu$ g/mL and 250  $\mu$ g/mL, respectively.

## **2.5 Statistical analysis**

The statistical tests and data analyses were performed using Orange and Jamovi software applications. Orange was utilized for its data mining and machine learning capabilities, while Jamovi was employed for its user-friendly interface and robust statistical testing functions.

The antibiotic sensitivity index was calculated. Box plots and stacked density plots of the zone diameter were prepared to determine the variability in the sensitivity of the isolates to the antibiotics tested. The normality of the distribution of zone size was tested by the Shapiro-Wilks test. Drugs that showed normal distribution were analysed using one-way ANOVA, followed by Tuckey's range test to detect drugs with significant differences. If the distribution was not normal, the Krushak-Wilkis test was applied.

The biocide tolerance of pseudomonas to chlorhexidine (CHX) and Cetyl Trimethyl Ammonium Bromide (CTAB) was tested for normality and then subjected to Brunner Munzel test with full permutation. Pearson's correlation test was performed to assess the relation between the MIC of the two biocides.

### 3. RESULTS AND DISCUSSION

#### 3.1 ISOLATION AND IDENTIFICATION OF PSEUDOMONAS

Out of 28 cases, 14 Gram negative bacteria were isolated. Out of which nine were Oxidase positive. Among these all the nine isolates produced fluorescence on shining UV light- indicating pyocyanins. Samples were identified as *Pseudomonas aeruginosa*.

*Pseudomonas aeruginosa* is often implicated as a pathogen associated with canine otitis, particularly chronic otitis media and otitis externa (5). This pathogen is hard to treat because it is often resistant to many antibacterial agents (6). *Pseudomonas* forms large, flat, spreading colonies with a greenish-blue hue and serrated edges. These colonies are known for their characteristic fruity odour(7). Biochemical tests provide a convenient and cost-effective method to identify *Pseudomonas*. These bacteria are ubiquitous in oxygen-rich environments and lack the enzymes necessary for carbohydrate fermentation, making them obligate aerobes that generate ATP using carbohydrates via the tricarboxylic acid cycle. *Pseudomonas* are oxidase-positive and fermentation-negative. They produce pale colonies on MacConkey agar, as they cannot ferment lactose (8). *Pseudomonas aeruginosa* typically produces pyoverdinin, a fluorescent pigment characteristic of the organism (9). In our study, all isolates were Gram-negative short rods that produced colonies with a bluish-green hue and were catalase and oxidase-positive. All isolates exhibited greenish-blue fluorescence under ultraviolet light and were identified as *Pseudomonas aeruginosa*.

#### 3.2 ANTIBIOGRAM

The antimicrobial sensitivity index (ASI) ranged from 0.61 to 0.84, with a mean value of 0.71, indicating that most isolates were sensitive to the majority of the drugs tested. Isolates COPM7 and COPM3 had the highest ASI and were sensitive to eleven of the thirteen antimicrobials tested. Isolate COPM3 was resistant to ceftriaxone-tazobactam and polymyxin-B, while COPM7 was resistant to ofloxacin and aztreonam. Isolates COPM4 and COPM5 showed resistance to the highest number of antibiotics, being resistant to levofloxacin, polymyxin-B, ofloxacin, and ceftriaxone-tazobactam. Additionally, COPM4 was resistant to tobramycin, and isolate COPM5 to aztreonam. (TABLE 1). Only four of the thirteen antibiotics tested were effective against all isolates: gentamicin, piperacillin-tazobactam, cefepime, and norfloxacin. Eight isolates were sensitive to imipenem, ciprofloxacin, and ceftazidime (CAZ).

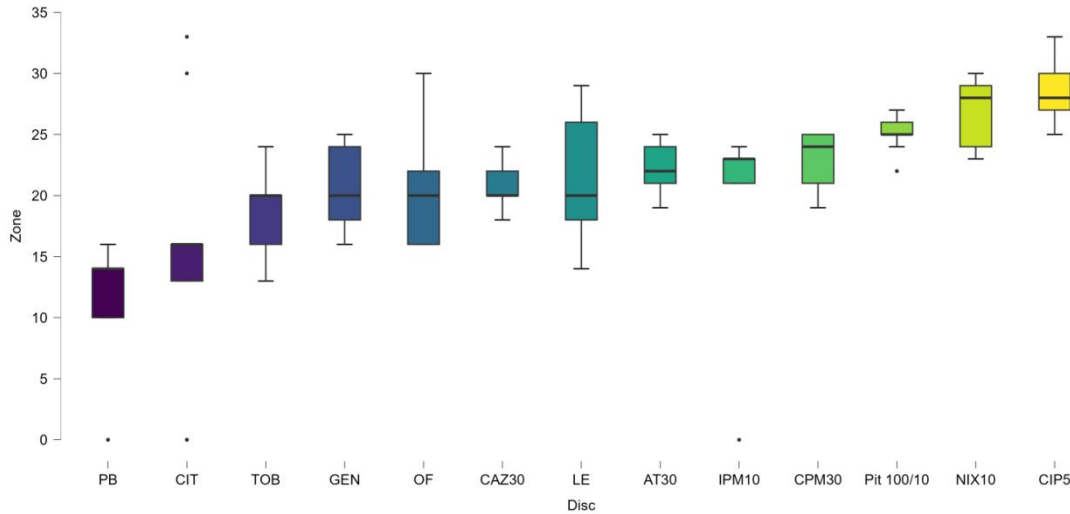
**Table 1: Results of the disc diffusion test**

<b>Sample</b>	<b>PIT</b> 100 /10	<b>AT</b> 30	<b>CPM</b> 30	<b>IPM</b> 10	<b>NIX</b> 10	<b>CIP</b> 5	<b>CAZ</b> 30	<b>GEN</b> 10	<b>CIT</b> 90	<b>TO</b> B 10	<b>OF</b> 5	<b>PB</b> 300	<b>LE</b> 5	<b>Sensitivity index</b>
COPM1	S	S	S	S	S	S	S	S	R	S	S	R	S	0.85
COPM2	S	R	S	S	S	S	S	S	S	S	R	S	S	0.85
COPM3	S	R	S	S	S	S	S	S	R	S	S	S	R	0.77
COPM4	S	R	S	S	S	R	R	S	S	S	S	R	S	0.69
COPM5	S	R	S	S	S	S	S	S	R	S	S	R	R	0.69
COPM6	S	S	S	R	S	S	S	S	R	S	S	R	R	0.69
COPM7	S	S	S	S	S	S	S	S	R	R	S	R	R	0.69
COPM8	S	S	S	S	S	S	S	S	R	R	R	R	R	0.62
COPM9	S	R	S	S	S	S	S	S	R	S	R	R	R	0.62

S= Sensitive; R= resistant

Pseudomonas is a major pathogen and is typically resistant to common antibacterial drugs. It is grouped with ESKAPE pathogens, known for being difficult to treat with antimicrobials. However, the isolates obtained in our study had a higher ASI, indicating that most were sensitive to common antimicrobials. These findings align with existing literature (10), who reported that clinical samples of Pseudomonas are more sensitive to antimicrobials than environmental isolates.

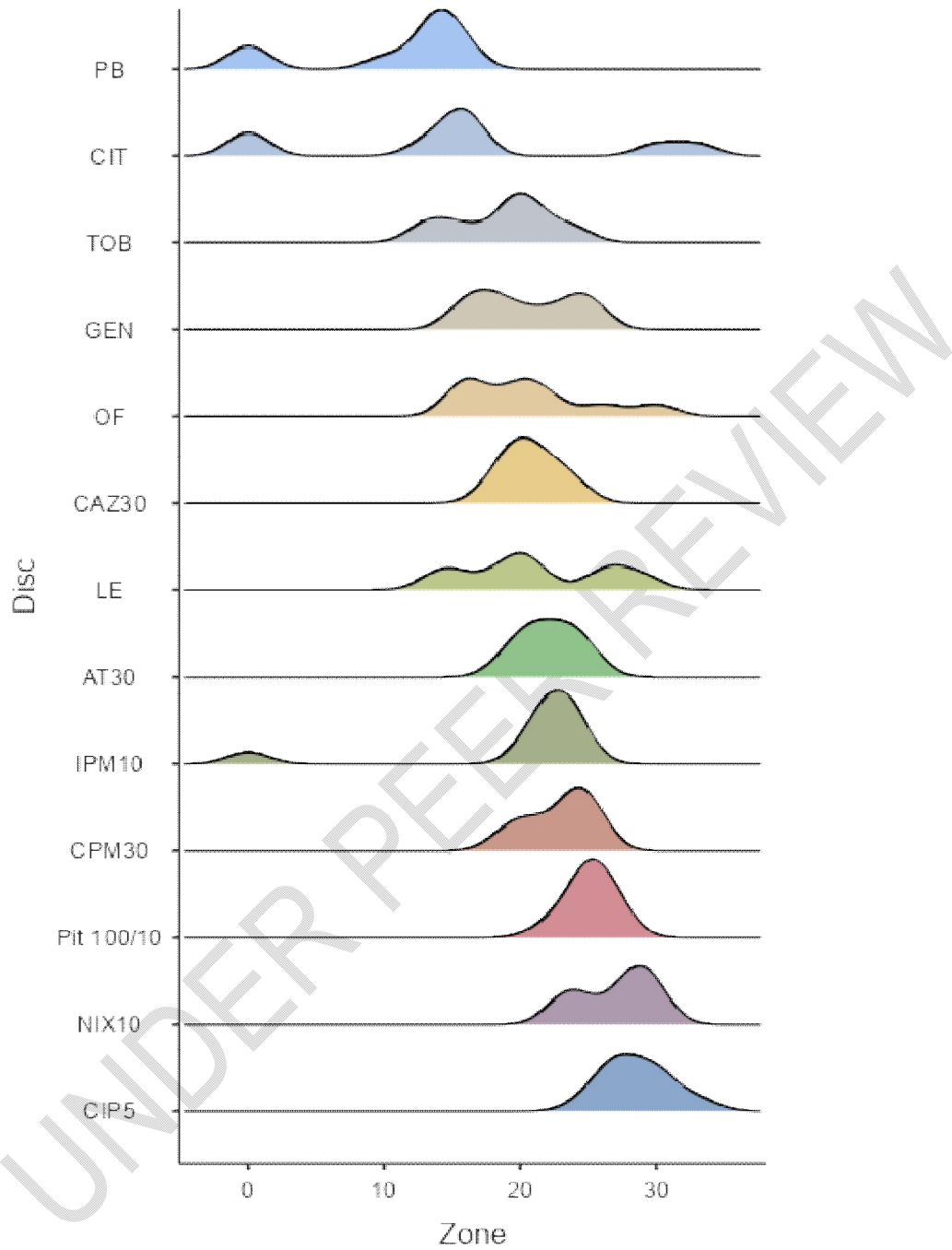
Among the thirteen drugs tested, nine had a median zone diameter greater than the CLSI cutoff limit for sensitivity. Polymyxin-B, ceftriaxone-tazobactam, and levofloxacin had median zone diameters below the CLSI cutoffs. The smallest median zone diameters were observed for polymyxin-B and ceftriaxone-tazobactam, with only two and three isolates being sensitive, respectively. The median diameter of the zone of inhibition was moderate for tobramycin, gentamicin, ofloxacin, ceftazidime, and levofloxacin. The interquartile deviation (IQD) was also moderate for these antibiotics, with levofloxacin showing a wider deviation. Aztreonam, imipenem, cefepime, piperacillin-tazobactam, and ciprofloxacin had higher median zone diameters, and the IQD was least for norfloxacin, ciprofloxacin, and imipenem. Compared to other drugs with higher median diameters, norfloxacin exhibited a wider range of zone inhibition. The data revealed a minimal IQD range and low diversity in antimicrobial sensitivity. Results are depicted as a box and whiskers plot (Fig. 1).



**Fig.1.Box and whiskers plot showing the diameter of zone of inhibition against different antibiotics by the isolates. Y axis indicates the zone diameter in mm.**

The first line of treatment for *Pseudomonas* infections often involves a combination of an aminoglycoside with a beta-lactam. Our findings support the effectiveness of this combination, as gentamicin, traditionally used against *Pseudomonas*, continued to show high efficacy(11). Fluoroquinolones are also effective against *Pseudomonas* by inhibiting bacterial DNA gyrase. Although the high sensitivity of norfloxacin against the isolates is favourable, continued monitoring for fluoroquinolone resistance in *Pseudomonas* is essential due to reports of increased resistance (12).

Density plots (Fig. 2) indicated that most zone sizes were consistent for most drugs. Single peaks, indicating consistency in sensitivity, were observed for ceftazidime, aztreonam, cefepime, piperacillin-tazobactam, and ciprofloxacin. In contrast, relatively flat curves with multiple peaks were observed for polymyxin-B, tobramycin, gentamicin, ofloxacin, and levofloxacin, indicating variation in zone sizes between different isolates. Drugs with lower median zones showed wider variation than those with larger zone diameters. (Fig. 2) . Among the sensitive drugs, variation in zone diameter occurred for tobramycin, gentamicin, ofloxacin, levofloxacin, imipenem, and norfloxacin.



**Fig.2. Stacked density plot showing the diameter of zone of inhibition against different antibiotics by the isolates. X-axis indicates the zone diameter in mm.**

Polymyxin-B showed poor zone size and variation in sensitivity, may be attributed to its large molecular size and poor diffusion in Mueller-Hinton agar (MHA). Polymyxin B is used in conjunction with other antibiotics for treating *Pseudomonas*. Cases of multidrug-resistant *Pseudomonas* infections have been treated with polymyxin B in combination with netilmicin (13). Resistance to polymyxin B is associated with mutations and modifications in the O antigen of the bacteria(14).Ceftriaxone-tazobactam was identified as a better choice over ceftriaxone-sulbactam when comparing the zone sizes of both drugs(15). However, ceftriaxone-tazobactam was found to be sensitive to only six out of nineteen *Pseudomonas* isolates in one study(16). Among the fluoroquinolones tested, levofloxacin was the least effective. Differences in zone sizes among fluoroquinolones were reported in staphylococci(17), but not observed in *Pseudomonas*(18).

The moderate interquartile deviation (IQD) of tobramycin, gentamicin, ofloxacin, ceftazidime, and levofloxacin indicates variation in response to these antimicrobials. Aminoglycosides like gentamicin and tobramycin are widely used in treating *Pseudomonas* infections. These drugs exert bactericidal action by binding to the A site of the 16S rRNA and inhibiting protein synthesis(19). Although newer beta-lactams have reduced dependence on aminoglycosides, renewed interest in aminoglycosides has emerged due to resistance development in newer strains of *Pseudomonas*(20). The findings of the present study align with the opinions of(21).

The distribution of zone diameters was positively skewed for gentamicin, ceftriaxone-tazobactam, levofloxacin, ceftazidime, ciprofloxacin, and ofloxacin, while the distribution for the remaining drugs skewed negatively. Imipenem had the maximum negative skewness, and ofloxacin had the maximum positive skewness. Most drugs exhibited a platykurtic distribution, with gentamicin having the lowest kurtosis value. Imipenem exhibited a kurtosis value of more than three. The Shapiro-Wilk test revealed that the data were normally distributed for most antibiotics, except for polymyxin-B and imipenem. One-way ANOVA of the normally distributed drugs revealed an F-value of 10.2 ( $p < 0.01$ ), indicating significant differences in zone sizes among different antibiotics. Tukey's Honest Significant Differences Test showed that the zone sizes of ciprofloxacin differed significantly ( $p = 0.05$ ) from ceftriaxone-tazobactam, gentamicin, levofloxacin, ofloxacin, tobramycin, and ceftazidime. The zone size of ceftriaxone-tazobactam differed significantly ( $p = 0.05$ ) from cefepime, norfloxacin, and piperacillin-tazobactam. The zone size of norfloxacin differed significantly from tobramycin ( $p = 0.05$ ). The Kruskal-Wallis test, applied to compare the zone sizes of polymyxin-B and imipenem (which did not follow a normal distribution), revealed a significant statistical difference ( $p < 0.01$ ) between the zone sizes of these two drugs.

. Positive skewness indicates that more isolates had zone sizes smaller than the mean zone diameter, with fewer isolates having much larger zone sizes. *Pseudomonas* are well known for their genetic plasticity and ability to acquire resistance from their environment(22). Positive skewness of fluoroquinolones indicates the presence of resistance mechanisms like efflux pumps working in the bacterial isolates, leading to the narrowing of many zones (23). Conversely, a negative skew indicates that the majority of isolates had zone sizes larger than the median, and the mean zone size is less than the median due to a few isolates with significantly larger zone sizes. It is interesting to note that ceftriaxone tazobactam had a positive skew, while ceftazidime and piperacillin had a negative skew, indicating the superiority of ceftazidime and piperacillin-tazobactam over ceftriaxone tazobactam. Since both piperacillin-tazobactam and ceftriaxone tazobactam had variation in zone size, inhibition of tazobactam (24) by *Pseudomonas* may be ruled out. Increased sensitivity to newer antibiotics like piperacillin and fourth-generation cephalosporin like ceftazidime suggests that *pseudomonas* may be gaining resistance to an older third-generation cephalosporin (25). Most drugs exhibited a platykurtic distribution, indicating wider dispersion of zone diameters compared to the median zone, showing increased variability among the isolates in response to the antimicrobials. One-way ANOVA and post-hoc tests showed that ciprofloxacin exhibited superior efficacy against *Pseudomonas* compared to ceftriaxone-tazobactam, gentamicin, levofloxacin, ofloxacin, tobramycin, and ceftazidime. Cefepime was more effective than ceftriaxone-tazobactam. These findings are in agreement with existing literature (26).

### **3.3 BIOCIDES TOXICITY**

A comparison of the MIC of chlorhexidine and cetyl trimethyl ammonium bromide revealed that the MIC of CTAB was greater than chlorhexidine. The mean MIC for chlorhexidine was 0.007 µg/mL, whereas the average MIC for CTAB was 208.3 µg/mL. Isolates Kit and 9 had the lowest MIC against chlorhexidine, while isolates Kitten, 10, and 865 had the highest MIC of 0.0125 µg/mL. Isolates M1, Kitten, and 10 had an MIC of 125 µg/mL against CTAB, while all other isolates had an MIC of 250 µg/mL.

The Shapiro-Wilk test for normality and QQ plot analysis revealed that the MIC data were not normally distributed. Brunner-Munzel test with full permutation revealed that MIC values between groups varied significantly ( $p < 0.01$ ). The test indicated that the MIC of chlorhexidine was significantly lower than that of CTAB.

Pearson's correlation test revealed a moderate negative correlation ( $r = -0.539$ ) between the MIC values for CTAB and chlorhexidine, but the data were statistically insignificant ( $p > 0.05$ ). These findings suggest a wide variability in biocide tolerance among *Pseudomonas* isolates from otitis, indicating that tolerance to one biocide is independent of the other. The Brunner-Munzel test was chosen over the Mann-Whitney U test for analyzing the minimum inhibitory concentration (MIC) because the Brunner-Munzel test does not require the assumption of similar distribution shapes and is more robust to differences in variability between groups. This ensures a better and more valid comparison of MIC values between groups. The increased tolerance to CTAB in *Pseudomonas* has been reported to be caused by the activity of QAC efflux pumps (27).

#### **4. CONCLUSION**

The study isolated *Pseudomonas* bacteria from cases of canine otitis and profiled their antibiotic sensitivity pattern. The tolerance of the isolates against two commonly used biocides- Chlorhexidine and CTAB was also tested. Most of the isolates were sensitive to the antibacterials. Ciprofloxacin exhibited a superior and consistent response compared to other antibiotics. Overall, the antimicrobials had a consistent zone diameter. Polymixin and ceftriaxone-tazobactam had smaller zone sizes. Chlorhexidine was more efficient than CTAB in eliminating *Pseudomonas*, as chlorhexidine had a significantly lower MIC than CTAB.

#### **DISCLAIMER (ARTIFICIAL INTELLIGENCE)**

Author(s) hereby declare that generative AI technologies such as Large Language Models, etc have been used during editing of manuscripts. This include ChatGPT and Grammarly AI for reviewing and editing the text of manuscript.

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