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: We collecteds~~S~~amples ~~were collected~~ aseptically. Antibiogram was done by disc diffusion test. The broth microdilution method was applied for biocide tolerance against chlorhexidine (CHX) and CetylTrimethyl ammonium ~~bromide~~bromide(CTAB).

Place and Duration of Study: The study period was from March 2023 to March 2024. Samples (n=??) were 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 laboratory.

Methodology: ~~*Pseudomonas*~~ *P.aeruginosa*~~were~~ was identified based on colony characteristics and biochemical tests. Antibiotic sensitivity was estimated by a disc diffusion ~~testin~~ on Muller Hinton Agar. Broth microdilution assay for the minimum inhibitory concentration (MIC) of biocides was done in 250 ul~~microtitre~~ 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*~~ *P.aeruginosa* were obtained. Ciprofloxacin was most effective against most isolates. The difference was statistically significant (P<0.05). 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

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statistically significant (P<0.05) lower MIC than for CTAB.

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

Keywords:~~Pseudomonas aeruginosa,Biocide tolerance,~~ antibiogram, Biocide tolerance, ~~ciprofloxacin,~~ Chlorhexidine, ciprofloxacin.Pseudomonas aeruginosa.

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(P.)* species are often implicated. ~~*Pseudomonas*~~*P.* 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*~~ *P.* 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 *P.*~~Pseudomonas~~ is ~~thus~~ imperative for better antimicrobial stewardship and informed decision-making in the treatment of canine otitis.

Biocides are chemical~~ agent~~s 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 *P.*~~Pseudomonas~~, particularly from veterinary sources.

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

2. MATERIAL AND METHODS

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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~~ 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°~~o~~c for 24 h ~~for colonies to form~~. ~~*Pseudomonas*~~ *P. aeruginosa* ~~were~~ was 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~~following 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~~ millimeters using ?????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

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The biocide tolerance of the isolates was evaluated using chlorhexidine(CHX) and cetyltrimethyl 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 µL of the required drug solution was added to each well. For wells 2 to 12, 50 µL of sterile cation-adjusted Mueller Hinton Broth (MHB) was added. A serial dilution was then performed by transferring 50 µL from the first well to the second well and continuing this process up to the 11th well, discarding 50 µL 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 µL 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 µL 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 µg/mL and 250 µg/mL, respectively.

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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.

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

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3. RESULTS AND DISCUSSION

3.1 ISOLATION AND IDENTIFICATION OF PSEUDOMONAS

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

~~*Pseudomonas*~~ *P. 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*~~ *P.* forms large, flat, spreading colonies with a greenish-blue hue and serrated edges. These colonies are known for their characteristic fruity odo~~u~~r (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. *P.* ~~Pseudomonas~~ are oxidase-positive and fermentation-negative. They produce pale colonies on MacConkey agar, as they cannot ferment lactose (8). ~~*Pseudomonas*~~ *P. aeruginosa* typically produces pyoverdin, 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*~~ *P. aeruginosa*.

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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~~CIT90 and ~~polymyxin-~~PB300, ~~while~~ COPM7~~was resistant~~ to ~~ofloxacin~~ OF5and ~~aztreonam~~AT30, ~~Isolates~~ COPM4 and COPM5~~showed resistance~~ to ~~the highest number of antibiotics, being resistant to levofloxacin~~LE5, ~~polymyxin-~~PB300, ~~ofloxacin~~OF5, and CIT90~~ceftriaxone-tazobactam. Additionally~~,COPM4 ~~was resistant~~ to ~~tobramycin~~TOB10, and ~~isolate~~ COPM5 to ~~aztreonam~~AT30. (TABLE 1). Only four of the thirteen antibiotics tested were effective against

all isolates: ~~gentamicin~~GEN10, ~~piperacillin-tazobactam~~PIT100/10, ~~cefepime~~CPM30, and ~~norfloxacin~~NIX10. Eight isolates were sensitive to ~~imipenem~~IPM10, NIX10~~ciprofloxacin~~, and ~~ceftazidime (CAZ)~~CAZ30.

Table 1: Results of the disc diffusion test

Sample	*PIT 100 /10*	*AT 30*	*CPM 30*	*IPM 10*	*NIX 10*	*CIP 5*	*CAZ 30*	*GEN 10*	*CIT 90*	*TOB 10*	*OF 5*	*PB 300*	*LE 5*	*Sensitivity index*
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

P.~~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 exis~~iting literet~~ing literature (10), ~~who~~ which reported that clinical samples of ~~*Pseudomonas*~~ *P.* are more sensitive to antimicrobials than environmental isolates.

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Among the thirteen drugs tested, nine had a median zone diameter greater than the CLSI cutoff limit for sensitivity. P~~olymyxin~~ B300, ~~ceftriaxone-tazobactam~~CIT90, and ~~levofloxacin~~ LE5had median zone diameters below the CLSI cutoffs. The smallest median zone diameters were observed for ~~polymyxin~~ PB300 and CIT90~~ceftriaxone-tazobactam~~, with only two and three isolates being sensitive, respectively. The median diameter of the zone of inhibition was moderate for ~~tobramycin~~TOB10, ~~gentamicin~~GEN10, ~~ofloxacin~~OF5, ~~ceftazidime~~CAZ30, and ~~levofloxacin~~LE5. The interquartile deviation (IQD) was also moderate for these antibiotics, with levofloxacin showing a wider deviation. ~~Aztreonam~~AT30, ~~imipenem~~IPM10, ~~cefepime~~CPM30, ~~piperacillin-tazobactam~~PIT100/10, and ciprofloxacin had higher median zone diameters, and the IQD was least for ~~norfloxacin~~NIX10, ciprofloxacin,

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and ~~imipenem~~IPM10. Compared to other drugs with higher median diameters, ~~norfloxacin~~ NIX10exhibited 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).

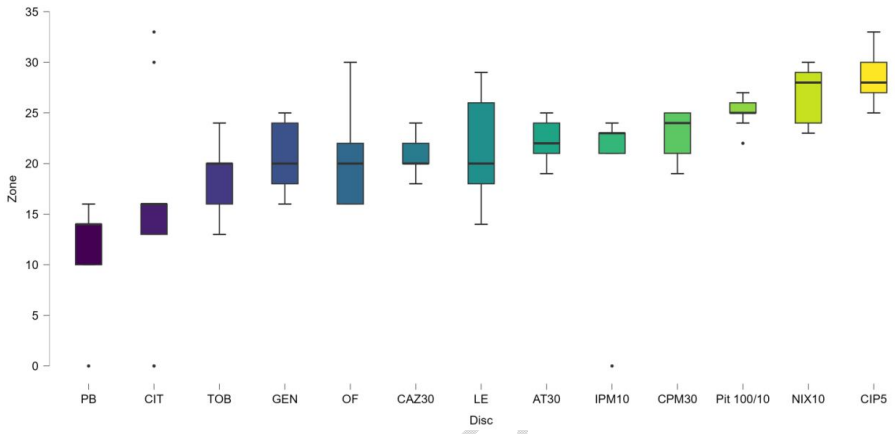

Fig.1.Box and whiskers plot showing the diameter of the zone of inhibition against different antibiotics by the isolates. The Y axis indicates the zone diameter in mm.

The first line of treatment for *~~Pseudomonas~~P.* 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 *P.*~~Pseudomonas~~, continued to show high efficacy(11). Fluoroquinolones are also effective against *P.*~~Pseudomonas~~ by inhibiting bacterial DNA gyrase. Although the high sensitivity of ~~norfloxacin~~ NIX10against the isolates is favo~~u~~rable, continued monitoring for fluoroquinolone resistance in *P.*~~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~~CAZ30, ~~aztreonam~~AT30, ~~cefepime~~CPM30, ~~piperacillin-tazobactam~~PIT100/10, and ciprofloxacin. In contrast, relatively flat curves with multiple peaks were observed for ~~polymyxin-~~PB300, ~~tobramycin~~TOB10, ~~gentamicin~~GEN10, ~~ofloxacin~~OF5, and ~~levofloxacin~~LE5, 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,

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variation in zone diameter occurred for ~~tobramycin~~TOB10, ~~gentamicin~~GEN10, ~~ofloxacin~~OF5, ~~levofloxacin~~LE5, ~~imipenem~~IPM10, and ~~norfloxacin~~NIX10.

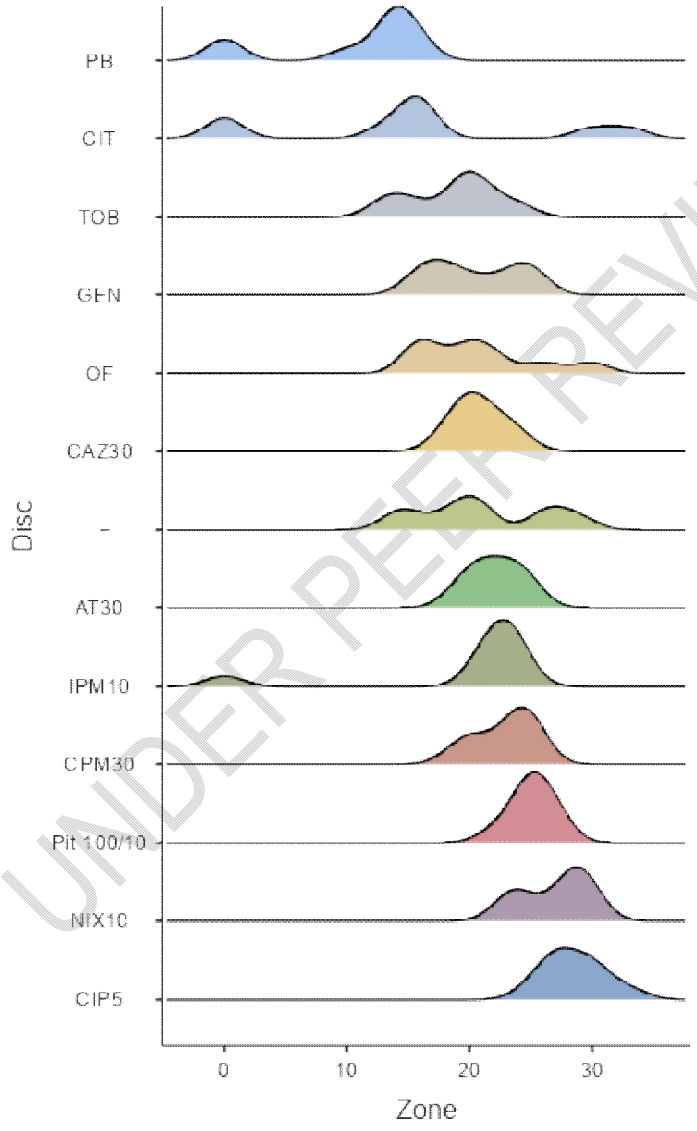

Fig.2.Stacked density plot showing the diameter of zone of inhibition against different antibiotics by the isolates. The X-axis indicates the zone diameter in mm.

~~Polymyxin-~~PB300 showed poor zone size and variation in sensitivity, which may be attributed to its large molecular size and poor diffusion in Mueller-Hinton agar (MHA). ~~Polymyxin-~~PB300 is used in conjunction with other antibiotics for treating *P.*~~Pseudomonas~~. Cases of multidrug-resistant *P.*~~Pseudomonas~~ infections have been treated with polymyxin B in combination with netilmicin (13). Resistance to ~~polymyxin-~~PB300 is associated with mutations and modifications in the O antigen of the bacteria(14).~~Ceftriaxone-tazobactam~~CIT90 was identified as a better choice over ceftriaxone-sulbactam when comparing the zone sizes of both drugs(15). However, ~~ceftriaxone-tazobactam~~CIT90 was found to be sensitive to only six out of nineteen *P.*~~Pseudomonas~~ isolates in one study(16). Among the fluoroquinolones tested, ~~levofloxacin-~~LE5was the least effective. Differences in zone sizes among fluoroquinolones were reported in ~~*staphylococci*~~*Staphylococci*(17), but not observed in *P.*~~Pseudomonas~~(18).

The moderate interquartile deviation (IQD) of TOB10~~tobramycin~~, ~~gentamicin~~GEN10, ~~ofloxacin~~OF5, ~~ceftazidime~~CAZ30, and ~~levofloxacin-~~LE5indicates variation in response to these antimicrobials. Aminoglycosides like ~~gentamicin-~~GEN10and ~~tobramycin-~~TOB10are 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 *P.*~~Pseudomonas~~(20). The findings of the present study align with the opinions of(21).

The distribution of zone diameters was positively skewed for ~~gentamicin~~GEN10, ~~ceftriaxone-tazobactam~~CIT90, ~~levofloxacin~~LE5, ~~ceftazidime~~CAZ30, ciprofloxacin, and ~~ofloxacin~~OF5, 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-~~PB300 and ~~imipenem~~IPM10. 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

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differed significantly (p = 0.05) from ceftriaxone-tazobactamCIT90, gentamicinGEN10, levofloxacinLE5, ofloxacinOF5, tobramycinTOB10, and ceftazidimeCAZ30. The zone size of ceftriaxone-tazobactamCIT90 differed significantly (p = 0.05) from cefepimeCPM30, norfloxacinNIX10, and piperacillin-tazobactamPIT100/10. The zone size of norfloxacin differed significantly from tobramycin TOB10(p = 0.05). The Kruskal-Wallis test, applied to compare the zone sizes of polymyxin PB300 and imipenem IPM10(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. *P.*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-tazobactamCIT90 had a positive skew, while ceftazidime CAZ30and piperacillinPIT100/10had a negative skew, indicating the superiority of ceftazidime CAZ30and piperacillin-tazobactamPIT100/10 over ceftriaxone-tazobactamCIT90. Since both piperacillin-tazobactamPIT100/10 and ceftriaxone-tazobactamCIT90 had variations in zone size, inhibition of tazobactam(24) by *P.*Pseudomonas may be ruled out. Increased sensitivity to newer antibiotics like piperacillin and fourth-generation cephalosporin like ceftazidime CAZ30suggests that *P.*pseudomonas may be gaining resistance to an older third-generation cephalosporin (25). Most drugs exhibited a platykurtic distribution, indicating a 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 *P.*Pseudomonas compared to ceftriaxone-tazobactamCIT90, gentamicinGEN10, levofloxacinLE5, ofloxacinOF5, tobramycinTOB10, and ceftazidimeCAZ30. Cefepime was more effective than ceftriaxone-tazobactamCIT90. These findings are in agreement with existing literature (26).

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3.3 BIOCIDE TOLERANCE

A comparison of the MIC of chlorhexidine CHXand cetyl trimethyl ammonium bromideCTAB revealed that the MIC of CTAB was greater than chlorhexidineCHX. 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 chlorhexidineCHX, 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~~CHX, but the data were statistically insignificant ~~($p > 0.05$)~~. These findings suggest a wide variability in biocide tolerance among *P.*~~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 *P.*~~Pseudomonas~~ has been reported to be caused by the activity of QAC efflux pumps (27).

4. CONCLUSION

The study isolated *P.*~~Pseudomonas~~ bacteria from cases of canine otitis and profiled their antibiotic sensitivity pattern. The tolerance of the isolates against two commonly used biocides- ~~Chlorhexidine~~ CHXand 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~~ CHXwas more efficient than CTAB in eliminating *P.*~~Pseudomonas~~, as ~~chlorhexidine~~ CHXhad a significantly lower MIC than CTAB.

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DISCLAIMER (ARTIFICIAL INTELLIGENCE)

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

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Comment [es1]: MUST BE UPDATED as 33.3% (9 out of 27) of the listed references were published in the past five years. The percentage has to increase to at least 35-40%. Old references and lack of updates indicate that the study is no longer a point of interest

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