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Research Article

PREVALENCE AND ANTIBIOTIC RESISTANCE PATTERN IN *PSEUDOMONAS AERUGINOSA* ISOLATES FROM CHRONIC OTITIS AND PYODERMA SAMPLES OF DOGS IN ANDHRA PRADESH, INDIA

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ABSTRACT: *Pseudomonas aeruginosa* is an ESKAPE bacterial pathogen known to be the reason for causing recurrent otitis and suppurative skin infections in dogs. The current study was undertaken to detect the prevalence of antibiotic-resistant *P. aeruginosa* isolates from chronic otitis and pyoderma samples of dogs. A total of ninety-seven (n=97) samples were collected, from otitis cases (n=45) and pyoderma cases (n=52). Preliminarily, cultural, and biochemical tests were carried out for the isolation of *P. aeruginosa*, and molecular tests like Polymerase Chain Reaction (PCR) using species-specific 16S rRNA primers that yielded a specific PCR product size of 956bp were utilized as a confirmative test in detecting 35(36.08%) isolates of *P. aeruginosa* from suspected cases. An antibiotic sensitivity test was performed on all 35 positive *P. aeruginosa* isolates by Kirby-Bauer disc diffusion method using a panel of 10 different antibiotics. The results revealed that *P. aeruginosa* has the highest resistance to tetracycline (97.14%) and the highest sensitivity to imipenem (88.58%). The Multiple Antibiotic Resistance (MAR) index of all the positive isolates ranged between 0.5-1. All 35 (100%) positive *P. aeruginosa* isolates were found to be multi-drug resistant (MDR) and 30 (85.71%) isolates were extensively drug-resistant (XDR). In conclusion, this study revealed a high prevalence of MDR and XDR strains of *P. aeruginosa* in the collected dog samples.

Keywords: *Pseudomonas aeruginosa*, Dogs, Otitis, Pyoderma, Antibiotic sensitivity test.

INTRODUCTION

Pseudomonas aeruginosa, a superbug is a threatful pathogen to both humans and animals as it is considered a multi-drug resistant bacterium [1] owing to its resistance to almost all commonly used antibiotics. Acquiring drug resistance by a bacterium is multifactorial and is attributed to the misuse of antibiotics, inappropriate dosage as well as the ability of the bacterium to develop various fighting mechanisms to surpass the effects of antibiotics [2]. The other probable reason for the resistance of *P. aeruginosa* was due to low membrane permeability attributed to intrinsic resistance and synthesis of AmpC beta-lactamase enzymes [3]. In dogs, *P. aeruginosa* is responsible for 20% of infections which mainly include otitis, skin and wound infections, urinary tract infections, and ulcerative keratitis. *P. aeruginosa* in recent years has been unresponsive to commonly used antibiotics like tetracyclines, fluoroquinolones, and aminoglycosides [4]. *P. aeruginosa* being a resistant pathogen, along with the other virulence factors leads to severe acute and chronic infections in dogs [5].

Antimicrobial resistance is an emerging worldwide concern as it has become a huge challenge nowadays. Thus, the present study was taken up to find out the prevalence and assess the resistance of *P. aeruginosa* isolates of chronic otitis and pyoderma in dogs to the antibiotics commonly used for their treatment. Veterinary practitioners should consider these findings while treating the dogs against *P. aeruginosa* infections as regular monitoring of the antibiotics used can evaluate the consequences of antimicrobial resistance

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and thus give a ray of hope that antibiotics save us from microbes rather than being a cause of death [6].

MATERIALS AND METHODS

Samples were collected from dogs presented with different clinical conditions like otitis and pyoderma. The samples were collected from different locations such as the Veterinary Clinical Complex of NTR College of Veterinary Science in Gannavaram, Veterinary Super Speciality Hospital - Vijayawada (VSSH), Prathyusha Pet Clinic in Vijayawada, Veterinary Polyclinics (VPCs) of Kakinada, Rajamahendravaram and Guntur during the period from 20th March 2023 to last week of April 2023 and from mid-June to last week of September 2023. The samples were collected using sterile, cotton-tipped swabs from the external auditory canal and deeply seated skin infections and were immediately transferred into sterile normal saline and then capped [7]. Collected samples were transported on ice and immediately stored at 4°C for a minimum of 24h until cultured/ enriched. The sample details along with their rate of isolation are presented in Table 1.

Isolation and characterization of *Pseudomonas* species

Ear swabs were inoculated directly in Brain Heart Infusion (BHI, HiMedia) broth which acts as an enrichment medium, and incubated at 37°C for 24h. After observing the turbidity, the BHI broth culture was streaked on *Pseudomonas* Isolation Agar (PIA, HiMedia). The PIA plates were incubated at 37°C for 24-48h. The translucent colonies with green pigment were presumed to be *Pseudomonas* spp. by their morphology and were further examined by employing Gram's staining method. Presumptive positive colonies were then subjected to biochemical tests like indole, Methyl Red-Voges Proskauer (MR-VP), catalase, oxidase, and citrate tests [8].

Molecular characterization of *P. aeruginosa* by PCR

Template preparation by boiling and snap chilling method

In a microcentrifuge tube, two mL of overnight grown *P. aeruginosa* broth culture was placed and spun at 10,000 rpm for five minutes. The pellet was suspended in 400 µL of nuclease-free water and cooked in a boiling water bath for 10 minutes. The microcentrifuge tube was immediately placed on ice. The tube was centrifuged at 8000 rpm for five minutes at 4°C after 20 minutes, and the supernatant was employed as a template for several PCR experiments [9].

Molecular confirmation of *P. aeruginosa* isolates

Molecular confirmation of *P. aeruginosa* isolates was done by PCR using oligonucleotide primers targeting species-specific genes (Table 2). The details of the standardized thermal cycling conditions are mentioned in Table 3.

Detection of antibiotic resistance by Kirby Bauer disc diffusion method

The antibiotic resistance pattern of *P. aeruginosa* isolates was investigated using the Kirby Bauer disc diffusion method [10] against ten distinct and regularly used antibiotics in Veterinary clinics. *P. aeruginosa* susceptibility pattern was investigated using zone diameter and interpretation breakpoints from CLSI recommendations (CLSI M100-S24 document, 2020). The details of the antibiotic discs (HiMedia) used in the present study were Amoxicillin/Clavulanic acid (AMC)-30 µg, Ampicillin (AMP)-10 µg, Aztreonam (AZ)-30 µg, Ceftriaxone (CTR)-30 µg, Co-trimoxazole (COT)-25 µg, Enrofloxacin (EX)-10 µg, Gentamicin (GEN)-10 µg, Imipenem (IPM)-10 µg, Streptomycin (S)-10 µg and Tetracycline (TE)-30 µg.

Pseudomonas isolates were first subcultured in BHI broth and incubated for 24h at 37°C. The turbidity was adjusted to 0.5 McFarland units [corresponding to an approximate cell density of 1.5×10^8 CFU/mL of the bacterial suspension and absorbance of 0.132 at 600 nm]. To obtain a lawn culture, 200 µL of each inoculum was plated on Mueller Hinton agar (MHA, HiMedia) using a cotton-tipped sterile swab. Plates were allowed to dry before ten antibiotic discs, five discs per plate, one in the centre and four on each of the four corners were placed equidistantly with the help of sterile fine forceps. The plates were incubated at 37°C for 18h under favourable conditions, and the diameter of the inhibition zones was quantified to determine the antibiotic susceptibility/resistance patterns for each strain.

Determination of Multiple Antibiotic Resistance (MAR) index

MAR indexing has been demonstrated to be a cost-effective and dependable method of tracing bacterial sources. The MAR index is calculated as the ratio of antibiotics to which an organism is resistant to the total number of antibiotics to which the organism has been exposed [11].

MAR index = a/b

Where 'a' = number of antibiotics to which the isolate shows resistance,

'b' = number of antibiotics to which the isolate was exposed.

RESULTS AND DISCUSSION

Of the ninety-seven samples enriched using BHI broth, 35 (36.08%) samples were presumptively positive as they predominantly produced characteristic green pigmentation on PIA (Fig. 1) and exhibited fluorescence under UV light. On Gram's staining, Gram-negative rods were observed in 18 samples of otitis swabs and 17 samples of pyoderma swabs. It indicates that 36.08% of the samples collected were positive for *P. aeruginosa*, which was lower when compared with the earlier published reports on the prevalence of *P. aeruginosa* collected from different kinds of samples [12, 13].

The percentage of *P. aeruginosa* isolated from otitis was 40% in the present study and several other researchers isolated *P. aeruginosa* from canine otitis cases with higher [14] and lower isolation rates [15] when compared with the present study report. The prevalence rate of *P. aeruginosa* from the pyoderma samples was 32.6%. The isolation rate from the canine pyoderma samples of the present study was found to be much higher than the reports of several other workers [16, 17, 18].

The current study results indicated a similar isolation rate to the earlier report [19]. The results of the present study were slightly higher compared to the isolation rate of *P. aeruginosa* from dog wound infections in the previous report [20]. The variation in the prevalence rates depends on many factors which may be categorized as geographical locations, the stage of the disease at which the sample was collected, the use of antibiotics to treat the infections, etc. [21].

The biochemical profile revealed that all 35 samples were positive for catalase, oxidase, and citrate tests and negative for indole and MR-VP tests. The results were in correlation with other research reports [22, 23, 24].

Table 1. Details of samples collected along with the isolation rate of *P. aeruginosa* from different places.

Place	Type of sample					
	Otitis swabs (E)		Pyoderma swabs (S)		Total swabs (E+S)	
	Collected	Positive isolates	Collected	Positive isolates	Collected	Positive isolates
VCC, NTR CVSc Gannavaram	10	06	06	02	16	08
VSSH, Vijayawada	06	03	06	02	12	05
Prathyusha pet clinic, Vijayawada	03	03	0	0	03	03
VPC, Rajamahendravaram	12	03	33	10	45	13
VPC, Kakinada	12	01	04	0	16	01
VPC, Guntur	02	02	03	03	05	05
Total	45	18	52	17	97	35

Table 2. Species specific oligonucleotide primers for *P. aeruginosa* [30].

	Target gene	Nucleotide sequence (5'-3')	Amplicon size (bp)
P. aeruginosa	16S rRNA	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	956

Table 3. Standardized thermal cycling conditions for *P. aeruginosa* [30].

	Standardized conditions		
Steps	Temperature	Duration	No. of cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	45 sec	
Annealing	55°C	45 sec	30
Extension	72°C	60 sec	
Final extension	72°C	5 min	1

PCR test for confirmation of the *P. aeruginosa* isolates

All the 35 isolates that were culturally and biochemically confirmed as *P. aeruginosa* were subjected to species-specific confirmation by PCR using 16S rRNA primers that yielded a specific PCR product of

Table 4. The pattern of antibiotic resistance exhibited by *P. aeruginosa* isolates obtained from otitis and pyoderma cases.

S.No.	Name of the Antibiotic	Total isolates resistant (%)	Resistant from Otitis isolates (%)	Resistant from Pyoderma isolates (%)
1	Amoxiclav	28(80%)	16 (88.8%)	12 (70.5%)
2	Ampicillin	33(94.2%)	17 (94.4%)	16 (94.1%)
3	Aztreonam	32 (91.4%)	17 (94.4%)	15 (88.2%)
4	Ceftriaxone	23(65.7%)	17 (94.4%)	6 (35.2%)
5	Cotrimoxazole	31(88.5%)	15 (83.3%)	16 (94.1%)
6	Enrofloxacin	32(91.4%)	17 (94.4%)	15 (88.2%)
7	Gentamicin	26(74.2%)	15 (83.3%)	11 (64.7%)
8	Imipenem	4(11.4%)	2 (11.1%)	2 (11.7%)
9	Streptomycin	32(91.4%)	15 (83.3%)	17 (100%)
10	Tetracycline	34(97.1%)	17 (94.4%)	17 (100%)

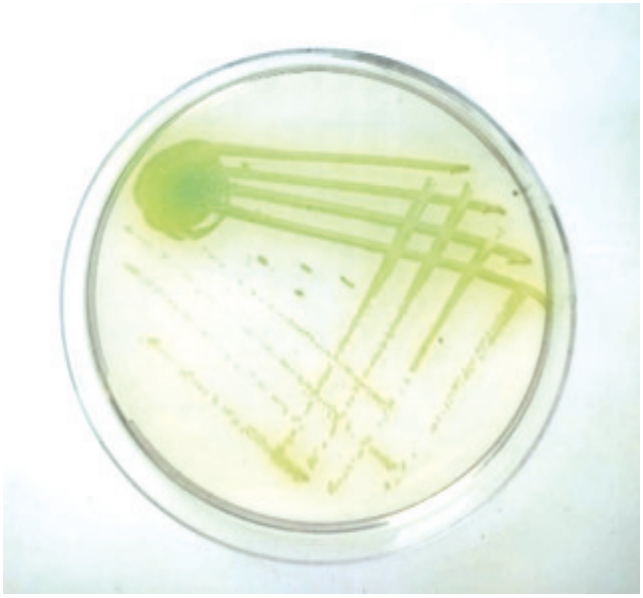

Fig. 1. *P. aeruginosa* producing characteristic green pigmentation on PIA.

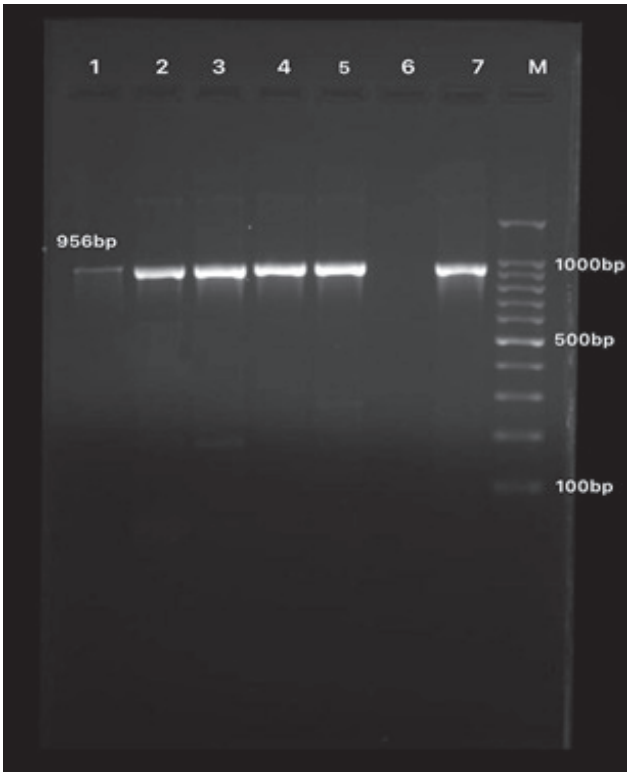

Fig. 2. PCR product of 16S rRNA for detecting the *P. aeruginosa* isolates from dogs. [Lane 1: O27 isolate; 2: O34 isolate; 3: O38 isolate; 4: W1 isolate; 5: W13 isolate; 6: Negative control; 7: Positive control; M: Marker of 1100 bp].

956 bp (Fig. 2). Subsequently, all the 35 (36.08%) presumptively positive samples out of the total 97 samples collected are confirmed as *P. aeruginosa* by PCR. A similar observation was also noticed in the earlier studies [25].

Antibiotic sensitivity test

There are several complex mechanisms involved in determining the antibiotic sensitivity pattern of *P. aeruginosa* which include mutations, low permeability of the bacterial cell wall, enzyme inactivation, efflux pumps, etc. [12]. The results of the antibiotic sensitivity test revealed highest resistance to tetracycline (97.14%), followed by ampicillin (94.28%), aztreonam, streptomycin and enrofloxacin (91.42% each), co-trimoxazole (88.57%), amoxiclav (80%),

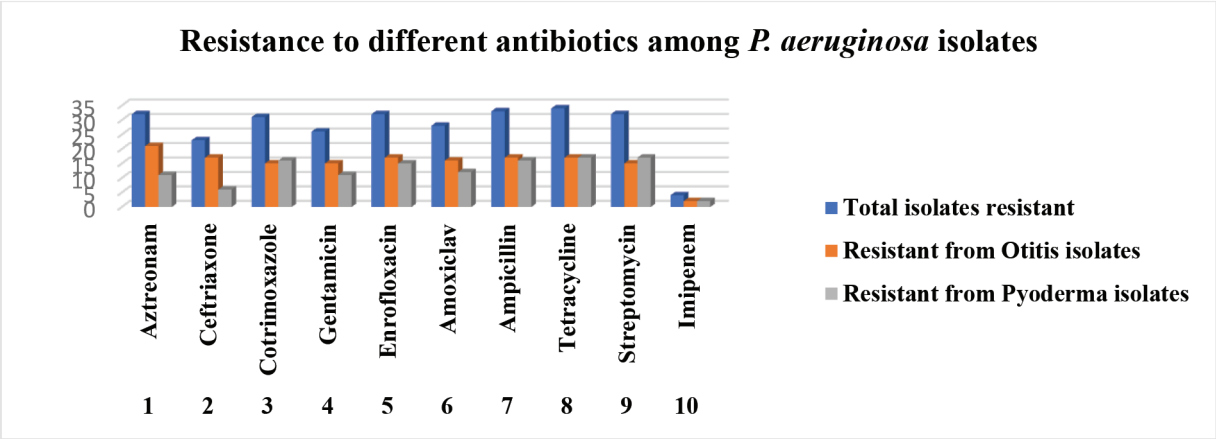

Fig. 3. Comparison of antibiotic resistance pattern among otitis and pyoderma *P. aeruginosa* isolates of dogs.

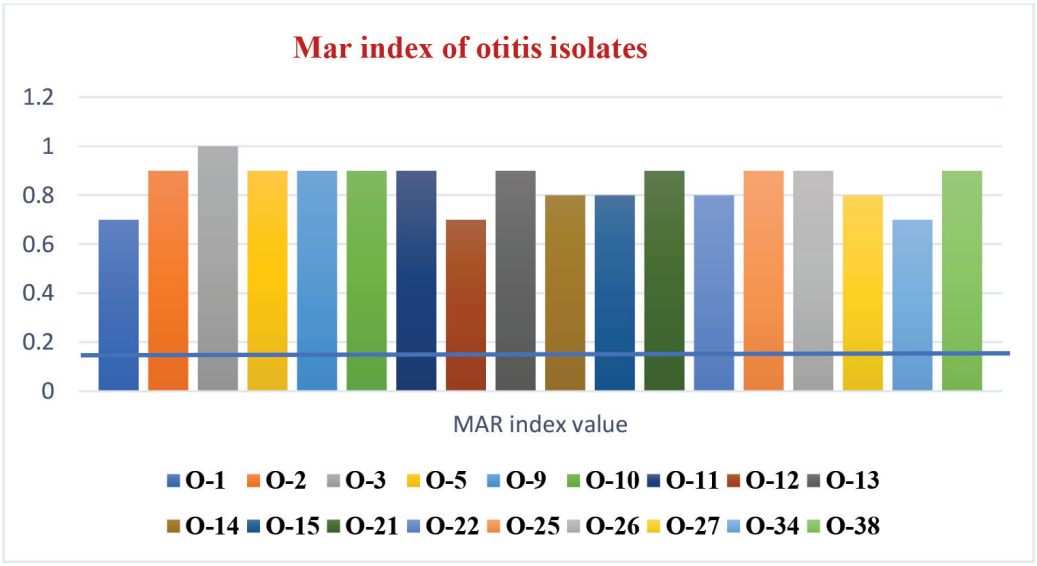

Fig. 4. MAR index values of *P. aeruginosa* isolates from otitis.

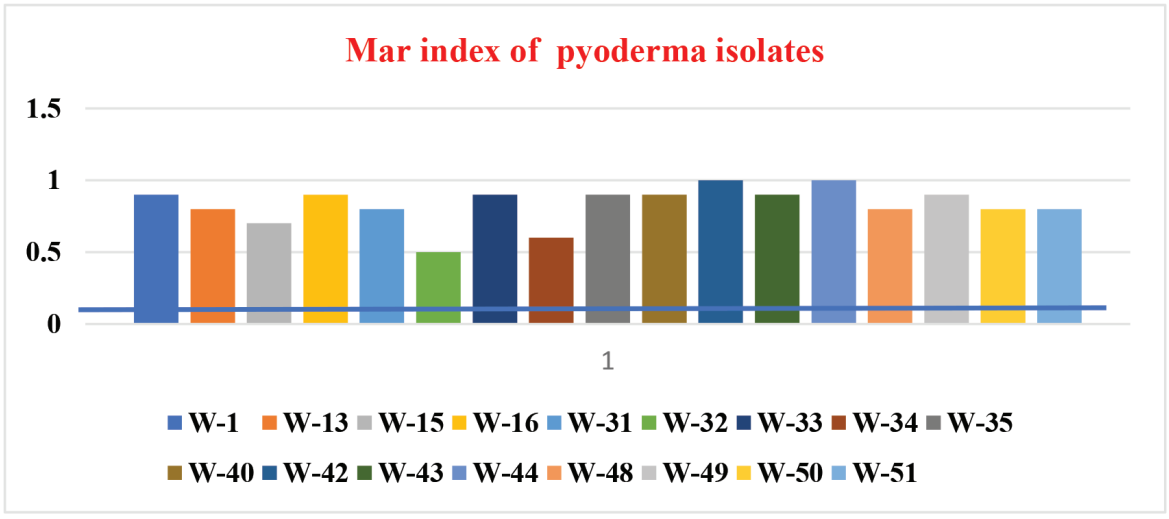

Fig. 5. MAR index values of *P. aeruginosa* isolates from pyoderma isolates.

gentamicin (74.28%), ceftriaxone (65.71%) whereas lowest resistance was observed to imipenem (11.42%). The details of the antibiotic resistance pattern of *P. aeruginosa* isolates to different antibiotics was mentioned in Table 4 and Fig. 3. The antibiotic-sensitive patterns of *P. aeruginosa* vary depending on the dosage and prolific usage of antimicrobials, the inherent resistance capacity, and the biofilm-forming ability of the bacteria.

Due to the difference in the type of antibiotic used more frequently, significant difference in the resistance pattern was noticed in various studies conducted at different regions. In a study conducted on *P. aeruginosa* strains from dogs and cats to assess antibiotic resistance, the results indicated lower resistance to imipenem (14%), and higher resistance of 83% was conferred to enrofloxacin [5] which was similar with a slight deviation to that of the current study results which indicated 11.42% resistance to imipenem and 91.42% resistance to enrofloxacin. The findings in the current study reveal that imipenem drug would be the best choice to treat the beta lactam-resistant *P. aeruginosa* in canine infections as the sensitivity is quite high when compared to other antimicrobials and the drug is also supported by other researchers [5].

The earlier experiment on the antibiotic susceptibility profile of 58 *P. aeruginosa* isolates indicated 51.72% resistance to aztreonam and 37.93% resistance to gentamicin [18] which exhibited significant variation compared to the antibiotic sensitivity results of the present study. The present study results were quite contrary to the other report [25] where only 9.61% and 21.15% resistance to enrofloxacin and gentamicin respectively were detected. This might be due to the usage of certain antibiotics for a prolonged period to treat chronic infections.

P. aeruginosa being a "multidrug-resistant (MDR) pathogen" has become the reason for the high fatality rate though it is an opportunistic organism. The results of the current study indicated that all 35 (100%) *P. aeruginosa* isolates were MDR as they are resistant to more than one class of antimicrobial drugs and 30 (85.71%) of the positive isolates were extensively drug-resistant (XDR) as they are susceptible only to one or two classes of antimicrobials used in this study.

The other similar study on twenty-three *P. aeruginosa* isolates to identify the MDR and XDR isolates noticed MDR in 47.8% and XDR in 34.7% [6] which was lower compared to the results of the present study but their result was also supported by other workers who also noticed MDR in 34.8%- 49% of *P. aeruginosa* isolates [26, 27].

The previous results on the prevalence of multi-drug resistance indicated 97.56% of the positive isolates to be MDR [28] which was similar to that of the current study. The published report on 143 *P. aeruginosa* isolates regarding antimicrobial susceptibility revealed only 8.4% of the isolates to be MDR [29] which showed a significant difference from the results of the current study. The significantly higher percentage of MDR in the current study might be due to the selection of samples from chronic cases of otitis and pyoderma.

Determination of the MAR index

The results of the MAR index for all the isolates were calculated and presented in Fig. 4 and 5. None of the positive isolates showed a MAR index below 0.2 and all the positive samples (100%) showed a MAR index >0.2. The isolates exhibiting intermediate resistance patterns were also considered as "resistant" for calculating the MAR index.

A good risk assessment tool to detect antibiotic resistance is the MAR index. A MAR index value of 0.2 is used to differentiate between low and high antibiotic resistance. In this study, the MAR index value of all the 35 positive isolates ranged between 0.5 to 1 which clearly suggests it to be due to high antibiotic use and selective pressure. This result was strongly supported and was in good correlation with the MAR index results of the other similar study conducted where all the 55 *P. aeruginosa* isolates had MAR indices >0.2 [25].

CONCLUSION

The present study brings insight into the prevalence of multidrug and extensively drug-resistant *P. aeruginosa* isolates in otitis and pyoderma cases of dogs. Further detailed characterization has to be taken up to assess the resistance factors like extended-spectrum beta-lactamases and biofilm production. The usage of antibiotics especially in the treatment of chronic infections needs to be well studied in terms of culture and sensitivity patterns for appropriate therapeutic knowledge, for good prognosis of the clinical cases and to avoid the evolution of further resistant bacterial pathogens as well.

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