ISSN: 2456-2912
NAAS Rating (2025): 4.61
VET 2025; 10(12): 181-188

www.veterinarypaper.com
Received: 06-11-2025
Accepted: 02-12-2025

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Prevalence of *Pseudomonas aeruginosa* with multi-drug resistance and biofilm-forming ability in fresh-water fish

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DOI: https://www.doi.org/10.22271/veterinary.2025.v10.i12c.2813

Abstract
Pseudomonas aeruginosa is a potential public health threat due to their resistant nature to antibiotics. The present study was conducted to detect the prevalence of antibiotic resistance and biofilm genes in *P. aeruginosa* isolated from freshwater fish. A total of fifty (N=50) samples were collected from gills (N=32) and skin (N=18) of freshwater fish. By sample's processing for the isolation of *P. aeruginosa* on *Pseudomonas* isolation agar, 25 samples from gills and 6 samples from skin were phenotypically positive as they produced characteristic green colour pigment and were molecularly confirmed as *P. aeruginosa* using species-specific 16S rRNA primers by Polymerase Chain Reaction (PCR). On performing invitro antibiotic sensitivity test, 100% resistance was observed to aztreonam, ceftriaxone, co-trimoxazole, gentamicin, enrofloxacin, ampicillin, tetracycline and streptomycin. The genetic detection of the Extended Spectrum Beta-Lactamase (ESBL) genes revealed the presence of *bla*$_{OXA}$ (90.3%), *bla*$_{SHV}$ (64.5%) and *bla*$_{TEM}$ (19.3%). Biofilm gene studies targeting *pslA* and *ppyR* genes of *P. aeruginosa* revealed their detection in 80.64% and 96.77% of the isolates, respectively. Thus it is of public health importance as fish is an excellent source of protein and omega -3 fatty acids, proper food safety measures must be ensured for its consumption.

Keywords: *P. aeruginosa*, antibiotic resistance, biofilm, public health, fresh-water fish.

1. Introduction
Developing nations account for 90% of aquaculture production, despite low hygiene standards and inadequate antimicrobial controls (Thuy *et al*., 2011; FAO, 2012; Rico and Van den Brink. 2014) [25, 10, 22]. Antimicrobial drugs are necessary to manage illnesses, minimise economic loss, speed up production (Pham *et al.*, 2015) [18] and meet global demand for fish and shellfish, as wild fisheries are no longer sufficient (Akinbowale *et al*., 2006) [2]. *Pseudomonas aeruginosa* is a major infection in fresh fish, causing significant economic losses and high mortality rates due to its ability to adapt to various habitats, including aquatic environment. *P. aeruginosa* can cause serious lesions in fish, including gill necrosis, haemorrhagic septicaemia, congested kidney, and friable liver, especially when exposed to stress factors and changing environmental conditions such as low oxygen levels and temperature (Ardura *et al*., 2013) [6]. Contaminated fish with enterotoxigenic *Pseudomonas* can induce diarrhoea and skin infections, particularly in immunocompromised consumers including humans thus creating a public health concern (Wong *et al*., 2000) [26]. Development of multi-drug resistance within the bacteria due to the presence of ESBL and biofilm genes makes the therapy aspect difficult. Thus, the current study was undertaken to determine the prevalence of multi-drug resistance and biofilm-forming ability in *P. aeruginosa* isolated from freshwater fish.

2. Materials and Methods
2.1 Collection of samples
Samples were collected from local freshwater fishes using sterile cotton tipped swabs and immediately transferred into normal saline and stored at 4 ℃ until cultured. A total of fifty samples were collected, from the local fish markets of Krishna district of Andhra Pradesh

during the month of August 2023, of which thirty two samples were collected from gills and eighteen samples were collected from the skin.

2.2 Isolation of *P. aeruginosa*

The acquired swab samples were directly inoculated into Brain heart infusion (BHI, Himedia) broth and incubated for 24 h at 37 °C. After evaluating the turbidity, the BHI broth culture was streaked on *Pseudomonas* Isolation Agar (PIA, Himedia). The inoculated plates were incubated at 37 °C for 24 to 48 h. The transparent colonies with green pigment assumed to be *Pseudomonas* spp. based on their shape were further tested using Gram's staining procedure. Presumptive positive colonies were further biochemically characterised using IMViC tests as per Quinn *et al*. (2011) [21].

2.3 Molecular confirmation of *P. aeruginosa* isolates by PCR

2.3.1 Bacterial DNA extraction

For genotypic detection, DNA was extracted from all probable *P. aeruginosa* isolates using a simple boiling and snap chilling procedure. Two mL of overnight grown *P. aeruginosa* culture was added to a micro centrifuge tube and spun for five minutes at 10,000 rpm. The pellet was suspended in 400 µL of nuclease-free water and was placed in a boiling water bath for 10 minutes. The microcentrifuge tube was immediately put on ice. The tube was centrifuged at 8000 rpm for five minutes at 4°C after 20 minutes, and the supernatant was used as a template for numerous PCR assays.

2.3.2 Detection of species-specific 16S rRNA in *P. aeruginosa* isolates by PCR

All presumptively positive *P. aeruginosa* isolates were subjected to PCR to detect the *P. aeruginosa* species-specific *16S rRNA* gene (956 bp), as described by Spilker *et al*. (2004) [24]. In this investigation, characterised and standard *Pseudomonas* spp. were employed as positive controls.

2.3.3 Detection of ESBL genes in *P. aeruginosa* isolates by Multiplex PCR

The positive *P. aeruginosa* isolates that were confirmed by detecting species-specific *16s rRNA* were screened for Multiplex PCR detection of bla_{TEM} (800 bp), bla_{SHV} (713 bp), and bla_{OXA} (564 bp) using Spilker *et al*. (2004) [24] technique. The primer details were stated in table 1.

Table 1: Oligonucleotide primers used for the detection of ESBL genes in *P. aeruginosa* (Spilker *et al*., 2004) [24].

Primer	Target	Nucleotide sequence	Amplicon size (bp)
MultiTSO-T	bla_{TEM}	CAT TTC CGT GTC GCC CTT ATT C	800
		CGT TCA TCC ATA GTT GCC TGA C	
MultiTSO-S	bla_{SHV}	AGC CGC TTG AGC AAA TTA AAC	713
		ATC CCG CAG ATA AAA TCA CCA C	
MultiTSO-O	bla_{OXA}	GGC ACC AGA TTC AAC TTT CAA G	564
		GAC CCC AAG TTT CCT GTA AGT G	

2.3.4 Detection of biofilm genes in *P. aeruginosa*

Oligonucleotide primers required for the detection of genetic determinants of biofilm genes (*ppyR, pslA*) were described in Table 2.

Table 2: Nucleotide sequences and amplicon sizes of biofilm genes (Pournajaf *et al*., 2018) [20]

Species	Target gene	Nucleotide sequence (5'-3')	Amplicon size(bp)
P. aeruginosa	*ppyR*	CGTGATCGCCGCCTATTTCC ACAGCAGACCTCCCAACCG	160 bp
	pslA	TCCCTACCTCAGCAGCAAGC TGTTGTAGCCGTAGCGTTTCTG	656 bp

2.4 Detection of Antibiotic resistance

The antibiotic resistance pattern of *P. aeruginosa* was examined using the Kirby Bauer disc diffusion method (Bauer *et al*., 1966) [6] against 10 different and commonly used antibiotics in veterinary medicine. The susceptibility patterns of *P. aeruginosa* were examined utilising CLSI zone diameter and interpretation break points (CLSI M100-S24 Document, 2020). Table 3 summarises the antimicrobial discs utilised in this study, their concentrations, and the inhibition zone widths used to determine resistance.

Table 3: Antibiotic discs used for ABST of *P. aeruginosa* isolates

S. No	Antimicrobial agent	Symbol	Concentration	Sensitive (mm)	Resistant (mm)
1	Amoxicillin/ Clavulanic acid	AMC	30 µg	≥ 18	≤ 13
2	Ampicillin	AMP	10 µg	≥ 17	≤ 13
3	Aztreonam	AZ	30 µg	≥ 21	≤ 17
4	Ceftriaxone	CTR	30 µg	≥ 23	≤ 19
5	Co-trimoxazole	COT	25 µg	≥ 16	≤ 10
6	Enrofloxacin	EX	10 µg	≥ 18	≤ 14
7	Gentamicin	GEN	10 µg	≥ 15	≤ 12
8	Imipenem	IPM	10 µg	≥ 19	≤ 15
9	Streptomycin	S	10 µg	≥ 15	≤ 11
10	Tetracycline	TE	30 µg	≥ 15	≤ 11

Pseudomonas isolates were initially subcultured in BHI broth and incubated for 24 h at 37 °C. Turbidity was adjusted to 0.5 McFarland units, which corresponds to an approximate cell density of 1.5×10^8 CFU/ml and an absorbance of 0.132 at 600 nm. To create a lawn culture, 200 µL of each inoculum was applied to Mueller Hinton agar (Himedia) using sterile cotton

tipped swabs. Plates were allowed to dry before ten antibiotic discs (five per plate, one in the centre and four in each of the four corners) were placed equidistantly and aseptically using sterile fine forceps. Plates were incubated at 37 ℃ for 18 h under favourable circumstances. The diameter of the inhibition zones was measured to assess antibiotic susceptibility/resistance patterns for each strain.

Results and Discussion

The count of *P. aeruginosa* in fish samples is quite significant as this Gram-negative pathogen is considered as an indicator for food quality as it is responsible for producing food-borne illnesses. (Begum *et al*., 2010; Jay *et al.,* 2006; Shahrokhi *et al*., 2022) [8, 13, 23]. The overall prevalence rate of *P. aeruginosa* in the present study was 62%. Algammal *et al.* 2020 [3] in their studies recorded a prevalence rate of 31.57%, Yagoub. 2009 [27] isolated *Pseudomonas* spp. from 63% of raw fish sold in the markets of Khartoum state of Sudan country and Boss *et al.* 2016 [9] noted a prevalence rate of *P. aeruginosa* from the raw fish and sea food imported to Switzerland to be 27%. The results of the current study were in accordance with Yagoub. 2009 [27]. The difference with the other studies might be due to the variation in the geographical areas from which the samples were collected.

Isolation of *P. aeruginosa* from raw fish is highly significant as *Pseudomonas* spp. could be considered as a pathogenic bacteria for humans and as well as an indicator for food quality. This was in accordance with previously mentioned by Jeyasekaran *et al*. (2006) [14] and Koutsoumanis and Nychas (2000) [16] who identified *Pseudomonas* spp. as a good spoilage index. The highest incidence of *P. aeruginosa* was observed in fresh fish when compared to smoked, frozen, salted or dried fish. Usually, bacteria tend to grow at moist places and other processed fish like smoked, frozen, salted or dried fish harbour limited bacteria as they inhibit the bacterial growth.

3.1 Isolation and characterization of *P. aeruginosa* from fish samples

In the current investigation, 31 (62%) of the 50 fish swab samples taken from both gills and skin included *P. aeruginosa* which was detected by extensive cultural, morphological and biochemical studies. All of the isolated strains produced characteristic cultural growth for *P. aeruginosa* like distinctive, green-pigmented smooth mucoid colonies on PIA. On microscopic examination, pink coloured, short slender Gram-negative rods were observed and biochemically characteristic IMVIC results were read in this manner [---+]. These studies were in co-relation with the findings of Aparmeya. 2013 [5]; Tawab *et al*. 2016 [1], Algammal *et al*. 2020 [3] and Altaee & Aldabbagh. 2022 [4].

3.2 ESBL Profile of *P. aeruginosa* isolates

3.2.1 Detection of genetic determinants of ESBL in *P. aeruginosa* by m-PCR

All 31 positive isolates verified as *P. aeruginosa* by PCR were examined for the presence of ESBL genes using m-PCR. The bla_{SHV}, bla_{OXA}, and bla_{TEM} genes produced PCR products with sizes of 564 bp, 713 bp, and 800 bp, respectively (Figure 1). Out of the 31 isolates, 29 (93.5%) tested positive for at least one of the three ESBL genes bla_{SHV}, bla_{OXA}, and bla_{TEM}. Twenty eight isolates were positive for bla_{OXA}, twenty for bla_{SHV}, and six for bla_{TEM}. Both bla_{TEM} and bla_{OXA} were found in six isolates (19.3%). Both bla_{OXA} and bla_{SHV} were detected in nineteen isolates (61.2%). All three genes (bla_{SHV}, bla_{OXA}, and bla_{TEM}) were identified in five isolates.

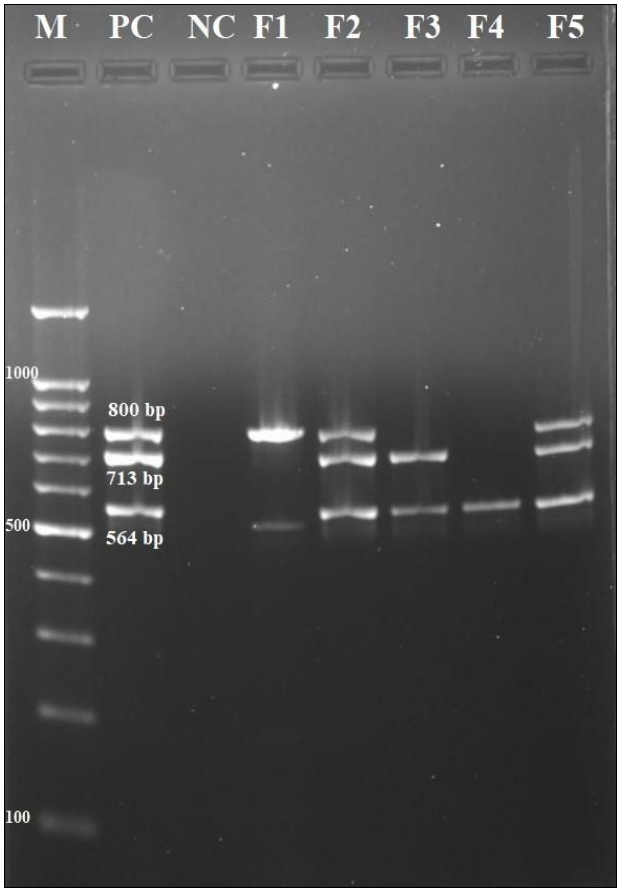

Fig 1: Detection of ESBL genes in *P. aeruginosa* isolates by m-PCR

Extended-spectrum beta-lactamase bacteria were mainly spread from the foods of animal origin as they serve as vectors and aids in transmission (Boss *et al*. 2016) [9]. *Pseudomonas* spp. acts as one of the potential candidates for monitoring antimicrobial resistance. Algammal *et al*. (2020) [3] isolated *P. aeruginosa* and the isolates harbored bla_{TEM}, bla_{CTX-M}, and tetA genes with a prevalence rate of 83.3%, 77.7%, and 75.6%, respectively. Shahrokhi *et al*. (2022) [23] in their studies found out that bla_{TEM}, bla_{CTX-M}, and bla_{SHV} were the most abundant genes that encodes antibiotic resistance for *P. aeruginosa* from fresh fish and different types of processed fish. In our work, we selected only the bla_{SHV}, bla_{OXA}, and bla_{TEM} and were successful in detecting them (Table 5) in the fresh fish in varying percentages. Similarly, other researchers worked with their choice of ESBL' and they also detected them in their studies. The findings in our study differed from other studies mentioned above, the reason may be due to the variations in the geographical conditions and maintenance of hygiene and indiscriminate use of antibiotics in the aquaculture farming.

Table 4: Molecular confirmation of the genetic determinants of antibiotic resistance in *P. aeruginosa* isolates

S. No	Name of the isolate	*bla*$_{TEM}$	*bla*$_{SHV}$	*bla*$_{oxa}$
1	F1	-	-	-
2	F2	+	+	+
3	F3	-	+	+
4	F4	-	-	-
5	F5	-	-	+
6	F6	-	-	-
7	F7	-	+	+
8	F8	-	+	+
9	F9	-	+	+
10	F11	-	+	+
11	F12	-	+	-
12	F13	-	-	-
13	F17	-	+	+
14	F18	-	-	-
15	F19	-	-	-
16	F21	-	-	+
17	F23	-	-	+
18	F24	-	-	+
19	F25	-	+	+
20	F27	+	-	+
21	F29	+	+	+
22	F30	-	+	+
23	F31	+	+	+
24	F32	-	+	+
25	F33	-	-	+
26	F34	+	+	+
27	F35	-	-	+
28	F37	-	+	+
29	F38	-	-	-
30	F39	-	-	+
31	F40	-	+	+
32	F41	-	+	+
33	F43	-	+	+
34	F44	-	-	-
35	F45	-	+	+
36	F46	-	-	+
37	F47	-	+	+
38	F48	-	-	-

3.3 Detection of genetic determinants of biofilm formation in *P. aeruginosa* by PCR

The 31 positive *P. aeruginosa* isolates were further assessed for presence of biofilm genes (*ppyR, pslA)*. Out of the 31 isolates, *ppyR* gene with amplicon size of 160 bp (Fig. 2) was detected in 30 (96.77%) isolates whereas *pslA* gene was detected in 25 (80.64%) isolates with amplicon size of 656 bp (Figure 3; Table 4). The presence of *ppyR* gene alone was noticed in five isolates whereas *pslA* gene alone was detected in none of the isolates. Both the genes (*ppyR, pslA)* were noticed in 25 isolates. Neither of the two genes (*ppyR, pslA)* were found in one isolate.

Table 5: Molecular confirmation and biofilm formation of *P. aeruginosa* isolates

S. No	Name of the isolate	*16S rRNA*	*pslA* gene	*ppyR* gene
1	F1	-	-	-
2	F2	+	+	+
3	F3	+	+	+
4	F4	-	-	-
5	F5	+	+	+
6	F6	+	-	-
7	F7	+	+	+
8	F8	+	+	+
9	F9	+	+	+
10	F11	+	+	+
11	F12	+	+	+
12	F13	-	-	-
13	F17	+	-	+
14	F18	-	-	-
15	F19	-	-	-
16	F21	+	-	+
17	F23	+	+	+

18	F24	+	+	+
19	F25	+	+	+
20	F27	+	+	+
21	F29	+	+	+
22	F30	+	+	+
23	F31	+	+	+
24	F32	+	+	+
25	F33	+	+	+
26	F34	+	+	+
27	F35	+	-	+
28	F37	+	+	+
29	F38	-	-	-
30	F39	+	-	+
31	F40	+	+	+
32	F41	+	+	+
33	F43	+	-	+
34	F44	-	-	-
35	F45	+	+	+
36	F46	+	+	+
37	F47	+	+	+
38	F48	+	+	+

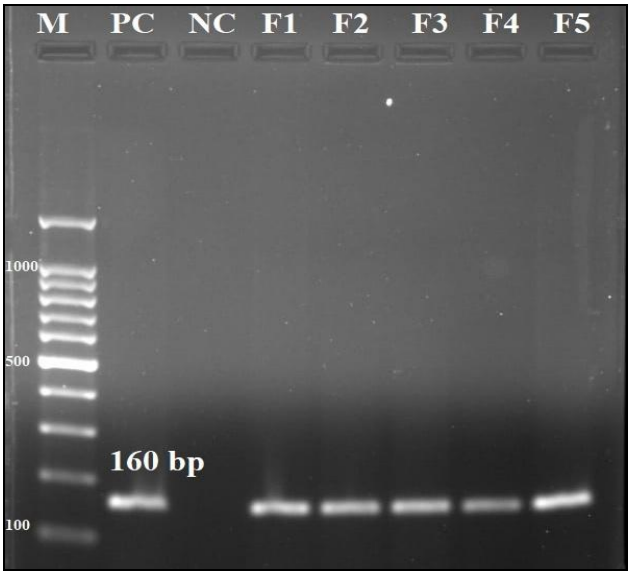

Fig 2: Detection of biofilm gene, *ppyR* in *P. aeruginosa* isolates by PCR test

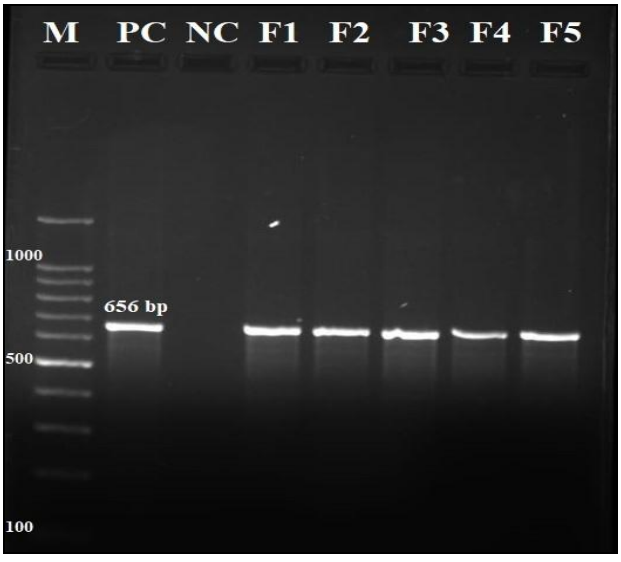

Fig 3: Detection of biofilm gene, *pslA* in *P. aeruginosa* isolates by PCR test

The reports on the prevalence of these genes in fish were so limited. Many published studies in other species reported the prevalence of *ppyR and pslA* genes ranging from 94-99% and 60.8-89.5% respectively which is following the current study results which were within the same range (Ghadaksaz *et al.*, 2015; Pournajaf *et al.* 2018; Ocak and Turkyilmaz 2022; Plokarz *et al.*, 2022) [11, 20, 17, 19]. The higher prevalence of biofilm genes (*ppyR and pslA*) enables the organism to be drug resistant as biofilm acts as an effective barrier against antibiotics and leads to chronic infections whose treatment would be a highly challenging task. Shahrokhi *et al*. (2022) [23] also worked on virulence genes present in *P. aeruginosa* and was able to detect algD, algU, lasB, toxA, exoS, exoT, and apr.

3.4 Antibiotic sensitivity test (ABST) for *P. aeruginosa*

Antibiotic sensitivity test was performed for all the 31 *P. aeruginosa* confirmed isolates using Kirby-Bauer disc diffusion test on Muller Hinton agar using a panel of 10 antibiotics. On performing invitro antibiotic sensitivity test, 100% resistance was observed to aztreonam, ceftriaxone, co-trimoxazole, gentamicin, enrofloxacin, ampicillin, tetracycline and streptomycin. In the selected panel of antibiotics, sensitivity was noticed only to two antibiotics i.e., amoxiclav (16.21% sensitive) and imipenem (58.06% sensitive) by the positive isolates. The details of the resistance exhibited by the positive isolates to different antibiotics was mentioned in Table 6 & Figure 4. All the 31 (100%) isolates were found to be MDR and XDR. Tawab *et al*. (2016) [1] in his studies revealed sensitivity to gentamicin; enrofloxacin; norfloxacin; ciprofloxacin and florphenicol and resistant for cefotaxime; erythromycin; amoxicillin; methicillin; oxacillin and ampicillin. ABST results slightly differ from the present studies as all the isolates were ESBL's producers thus they were MDR and XDR. Almost 90% of the aquaculture products raised in developing countries have poor standards for hygiene and do not follow the regulations of antibiotic usage (Pham *et al*. 2015) [18]. The monitoring of antimicrobial resistance is very much necessary in aquafoods as the transmission of resistant genes to humans via the consumption of sea food is alarmingly increasing and it is of public health significance (Gorgani *et al*. 2009, Boss *et al*. 2019) [12, 9].

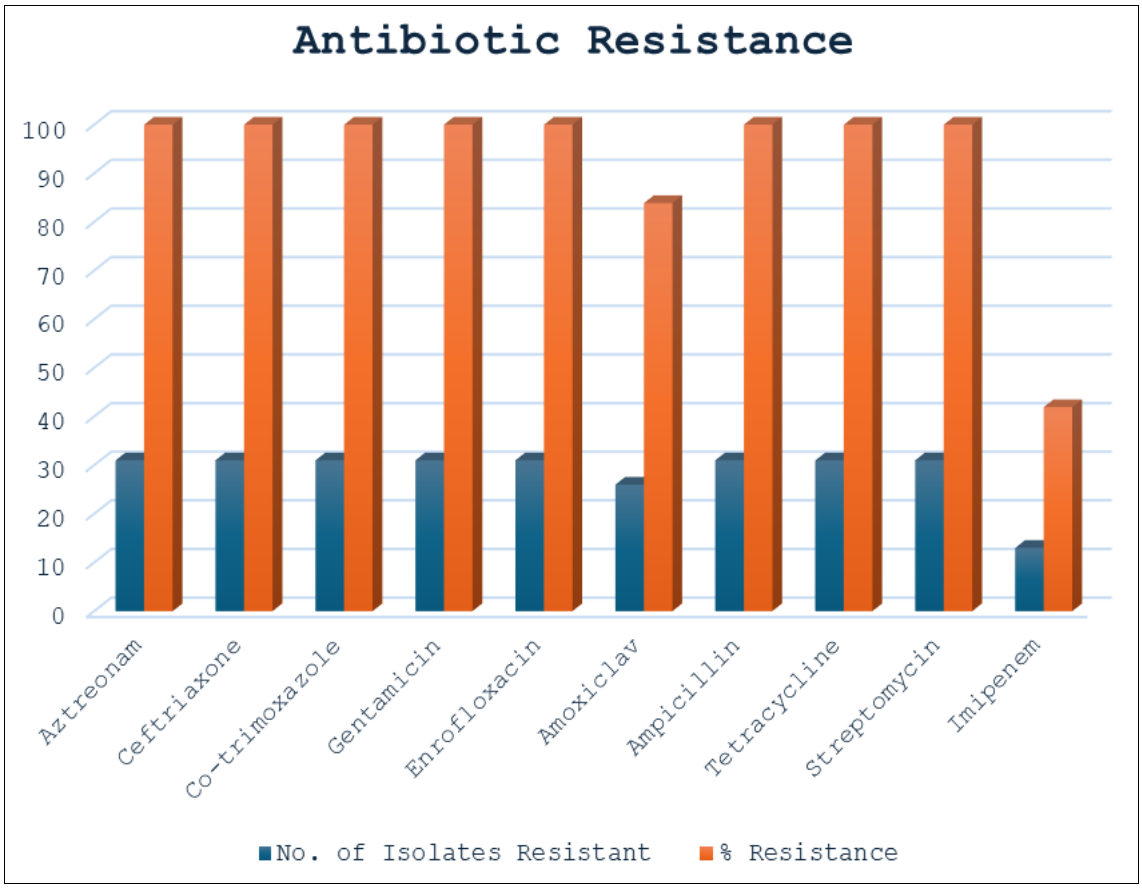

Fig 4: Resistance exhibited by different fish isolates towards each antibiotic

Table 6: Antibiotic sensitivity pattern (ABST) and Multiple Antibiotic Resistance (MAR) index of *P. aeruginosa*

Sample No	Aztreonam	Ceftriaxone	Co-trimoxazole	Gentamicin	Enrofloxacin	Amoxiclav	Ampicillin	Tetracycline	Streptomycin	Imipenem	MAR Index for 10
F-2	R	R	R	R	R	R	R	R	R	R	1.0
F-3	R	R	R	R	R	R	R	R	R	S	0.9
F-5	R	R	R	R	R	S	R	R	R	S	0.8
F-6	R	R	R	R	R	R	R	R	R	S	0.9
F-7	R	R	R	R	R	R	R	R	R	S	0.9
F-8	R	R	R	R	R	R	R	R	R	R	1.0
F-9	R	R	R	R	R	R	R	R	R	R	1.0
F-11	R	R	R	R	R	R	R	R	R	S	0.9
F-12	R	R	R	R	R	R	R	R	R	S	0.9
F-17	R	R	R	R	R	R	R	R	R	R	1.0
F-21	R	R	R	R	R	R	R	R	R	R	1.0
F-23	R	R	R	R	R	R	R	R	R	S	0.9
F-24	R	R	R	R	R	R	R	R	R	S	0.9
F-25	R	R	R	R	R	R	R	R	R	R	1.0
F-27	R	R	R	R	R	R	R	R	R	S	0.9
F-29	R	R	R	R	R	R	R	R	R	S	0.9
F-30	R	R	R	R	R	R	R	R	R	S	0.9
F-31	R	R	R	R	R	R	R	R	R	S	0.9
F-32	R	R	R	R	R	S	R	R	R	S	0.8
F-33	R	R	R	R	R	R	R	R	R	R	1.0
F-34	R	R	R	R	R	R	R	R	R	R	1.0
F-35	R	R	R	R	R	S	R	R	R	S	0.8
F-37	R	R	R	R	R	R	R	R	R	R	1.0
F-39	R	R	R	R	R	S	R	R	R	R	0.9
F-40	R	R	R	R	R	R	R	R	R	S	0.9
F-41	R	R	R	R	R	R	R	R	R	S	0.9
F-43	R	R	R	R	R	R	R	R	R	R	1.0
F-45	R	R	R	R	R	S	R	R	R	S	0.8
F-46	R	R	R	R	R	R	R	R	R	R	1.0
F-47	R	R	R	R	R	R	R	R	R	S	0.9
F-48	R	R	R	R	R	R	R	R	R	R	1.0

Conclusion

From the above studies it was noted that resistant *P. aeruginosa* was prevalent in the fresh fish, the microorganism throws an alarm for humans, hence it is necessary to take precautions to minimize the pathogen. As aquaculture creates a suitable environment for the growth of microbes, good hygienic practices should be maintained from fishing to transportation so that it could decrease the contamination by *P. aeruginosa* to some extent.

Acknowledgement

The authors are grateful to Sri Venkateswara Veterinary University, Tirupati for providing the grants to carry out the work. We are also thankful to the Department of Veterinary Microbiology, NTR College of Veterinary Science, Gannavaram for providing the necessary facilities in the research laboratory to perform the work.

Author's Contribution

Not available

Conflict of Interest

Not available

Financial Support

Not available

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How to Cite This Article
Swathi G, Swaroop T, Kumar PK, Kumari DG, Kumar AP. Prevalence of *Pseudomonas aeruginosa* with multi-drug resistance and biofilm-forming ability in fresh-water fish. International Journal of Veterinary Sciences and Animal Husbandry. 2025;10(12):181-188.