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Assessment of Antimicrobial Resistance (AMR) in *Aeromonas hydrophila* Isolated from Freshwater Rohu (*Labeo rohita*) in Ganjam District, Odisha

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ABSTRACT: Swab samples from the gills of live rohu (*Labeo rohita*) were collected in TSB from various aquaculture farms and reservoirs in the Ganjam district of Odisha and examined for the presence of *Aeromonas hydrophila* species. The isolates which had shown growth on RS agar or Cetrimide agar were furthered studied by doing gram staining, biochemical tests and observation of their morphology under compound microscope. Bacteria isolates that tested positive for catalase, oxidase, indole, MR, VP, and produced H_2S were identified as potential *A. hydrophila*. In contrast, isolates that tested positive for oxidase but negative for indole, MR, VP, and did not produce H_2S were identified as potential *P. aeruginosa* species. The antimicrobial resistance (AMR) of these isolates was evaluated using the disk diffusion method with antibiotics such as Chloramphenicol (C), Tetracycline (TE), Ciprofloxacin (CIP), Gentamicin (GEN), and Cefotaxime (CTX). The inhibited zone was measured to govern the sensitivity or resistance of the isolates. *A. hydrophila* isolates from different regions of the Ganjam district exhibited complete resistance (100%) to Ciprofloxacin (CIP) and Chloramphenicol (C), while showing complete susceptibility (100%) to Gentamicin (GEN), Tetracycline (TE), and Cefotaxime (CTX).

Keywords: *A. hydrophila*, Biochemical test, Antibiotics, AMR, Rohu.

INTRODUCTION

The rise of antimicrobial resistance (AMR) has become a critical global issue due to the emergence and rapid spread of resistant microbial strains among humans and animals. The World Health Organization (WHO, 2014) has identified AMR as one of the most serious threats to public health in the 21st century. For decades, antimicrobial drugs have played a crucial role in human and veterinary medicine, significantly improving health outcomes. However, the increasing resistance to these drugs now poses a substantial risk to both aquaculture and public health.

The excessive use of antimicrobials in both medical and agricultural settings has led to the selection of resistant bacterial strains, which increases the likelihood of horizontal gene transfer to human pathogens. This evolutionary pressure allows resistant bacteria to thrive and spread, raising the probability of infections that are difficult to treat. If left unchecked, AMR could surpass cancer as the leading cause of death by 2050, with an estimated 10 million premature deaths annually (Das *et al*., 2020). The misuse and overuse of antibiotics in healthcare, livestock, and aquaculture have accelerated this crisis, as antibiotic residues and resistant bacteria easily enter the environment, affecting both ecosystems and human health.

In the US, antibiotic-resistant infections account for over 20 lakh cases and approximately 23,000 mortality annually (Centers for Disease Control and Prevention, 2017). In India, over 58,000 newborn deaths in a single year have been linked to infections caused by resistant bacteria (Laxminarayan *et al*., 2013; CDDEP, 2016). The expansion of aquaculture has increased the occurrence of opportunistic pathogens and disease outbreaks, resulting in significant production losses (Asche *et al*., 2009; Mishra *et al*., 2015; Nayak *et al*., 2007). To mitigate these losses, antibiotics are often administered indiscriminately at sub-therapeutic doses for disease prevention and treatment (Cabello, 2006). Many of these antibiotics are broad-spectrum, which enhances the risk of resistance development among environmental and pathogenic bacteria (Thanner *et al*., 2016). This, in turn, increases the adaptive capacity of microbes to survive in these environments (Zampieri *et al*., 2017).

The extensive usage of antimicrobial representatives in aquaculture not only weakens the immune response of aquatic organisms but also disturbs the natural microbial balance in aquatic ecosystems. The presence of antibiotic-resistant bacteria in fish farming environments creates a reservoir of resistance genes, which can be transferred to human pathogens, thereby complicating treatment options and increasing public health risks. This trend threatens both the quality of fish production and the safety of human health.

The development of antimicrobial resistance is inevitable once a new antibiotic is introduced. Initial resistance rates to newly developed drugs are typically

around 1%, but widespread and prolonged use often leads to a rapid increase in resistance. Within 8 to 12 years of introducing a new antibiotic, resistance to multiple drugs typically becomes prevalent, leaving few effective treatment options.

To address the growing threat of AMR in aquaculture and beyond, stricter regulations on antibiotic use, increased public awareness, and improved monitoring and reporting systems are essential. Encouraging the development of alternative disease control methods, such as probiotics, vaccines, and improved biosecurity measures, may help reduce the reliance on antibiotics and slow the spread of resistance. Strengthening international cooperation and investing in research on antimicrobial resistance are key steps toward safeguarding human and animal health in the face of this global challenge.

Farmers are using Antibiotic in feed for health management of fish, but they should know about AMR. So this AMR study of the most common pathogen of fish *Aerpmonas hydrophilla* will definitely helpful for them.

MATERIALS AND METHODS

This study aimed to assess the antibiotic resistance of bacteria isolated from freshwater rohu (*Labeo rohita*) against selected antibiotics, including Chloramphenicol (C), Tetracycline (TE), Ciprofloxacin (CIP), Gentamicin (GEN), and Cefotaxime (CTX) in the Ganjam district of Odisha. Live fish samples were collected from various aquaculture farms and a reservoir in Ganjam for investigating the presence of *Aeromonas hydrophila*. All fish samples were processed on the same day. Gill swab samples were collected in TSB at the pond site and transported to the Department of Aquatic Animal Health Management, College of Fisheries (Odisha University of Agriculture and Technology), Rangailunda, Berhampur-7, Odisha, for analysis. The samples were obtained from Venktesh Farm, Humari Fish Farm, Sanot Patro Farm, Sahadev Sahu Farm, and Bhanjanagar Reservoir.

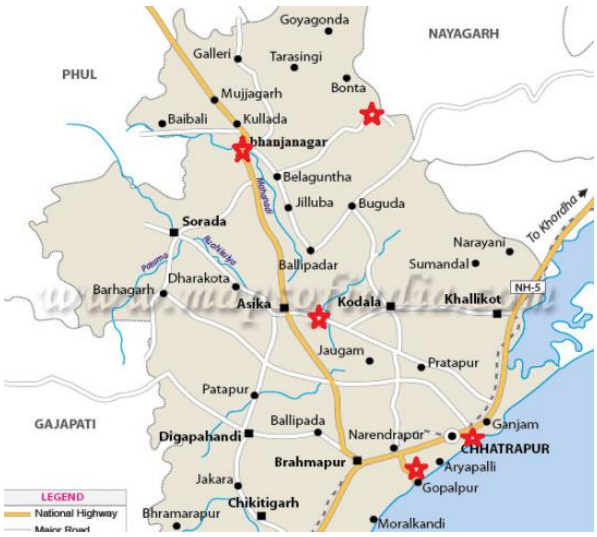

Fig. 1. Sampling site of Ganjam district.

Isolation and phenotypic characterization of bacterial isolates. The samples were processed in various microbiological media for isolation of bacterial using the standard microbiological methods and standard Operating Procedure (NBFGR, 2018). Tissue samples in enrichment medium containing Tryptic Soya Broth (M011, HiMedia) were further processed for isolation of *Aeromonas hydrophila* species and *Pseudomonas aeruginosa* species in fish samples following the protocols. The pure culture of each of isolates were obtained following standard streaking procedure and kept for phenotypic characterization of isolates.

Aeromonas hydrophila species was phenotypically characterized for their presumptive identification, using a battery of biochemical tests like cell morphology, Gram's reaction, colony morphology, oxidase test, catalase test, nitrate test, sugar fermentation tests, Indole test, Citrate test, Methyl red test, Voges-Proskauer test and gas production from glucose (Khuntia, 2011).

Analysis of the observation. The microorganism isolated from the fish sample with the help of enrichment media and selective media were assessed using the growth characteristics of the colonies on the media and different confirmative biochemical tests as mentioned earlier.

Antibiotic Susceptibility Test (AST) by Bauer Kirley Disc Diffusion Technique. In the present study, Kirby-Bauer the disk-diffusion test was carried out for AST analysis of all isolates. The Kirby-Bauer the disk-diffusion test (Bauer *et al*., 1966) based CLSI method was used for antibiotic susceptibility testing all bacterial isolates following Standard operating protocol (SOP) as developed by NBFGR (NBFGR, 2018). For this test the culture organism was uniformly and aseptically inoculated in Mueller-Hinton agar. Different antibiotic discs (HiMedia), which were impregnated with a specific Concentration of a particular antibiotic were laid on the medium. The plates were incubated at 35°C for 24-48 hrs. The "zone of inhibition" was then documented for further assessment.

Inoculation of Plates. A total of 100 µl of each bacterial isolate suspension was carefully pipetted onto the surface of the agar plates using a micropipette. The suspension was then uniformly spread over the medium using a glass spreader to ensure even distribution.

Preparation and Application of Antibiotic Discs. Antibiotic discs of uniform size and shape were prepared by punching out discs from Whatman filter paper no. 1 and sterilizing them. Under sterile conditions, the discs were carefully placed onto the agar plates using sterile forceps, ensuring that they were not positioned too close to each other. After positioning, the discs were gently pressed with sterile forceps to establish firm contact with the agar surface. A control plate without antibiotics was also prepared for each isolate. All steps were conducted in duplicate (Bauer *et al*., 1966).

Measurement of the Inhibition Zone. The diameter of the inhibition zone was estimated by a ruler held against the back of the inverted plate over a dark, non-reflective background. The clear margin indicating the edge of bacterial growth inhibition was measured to determine the zone size. Any bacterial growth within the inhibition zone was considered an indicator of resistance. The isolates were classified as per the CLSI guidelines (2010).

RESULT AND DISCUSSION

Bacteria isolates were cultured in TSA agar, RS agar, Cetrimide agar and EMB agar and their colony characteristic are observed. The isolates which had shown growth on RS agar or Cetrimide agar were furthered studied by doing gram staining, biochemical tests and observation of their morphology under compound microscope.

Biochemical test: Biochemical test was done for bacteria isolates which is presented in Table 4. The Biochemical characteristics of *Aeromonas hydrophilla* has been presented in the Table 1. Coding of sample collection from different sites are presented in the Table 2. Results of Oxidation-Fermentation test of bacterial isolates are presented in Table 4. All the bacteria isolate we have examined for oxidation-fermentation test all had change colour from purple to yellow except in case of VFG 10 and BRR 3 which did not change their colour to yellow in anaerobic tube, which showed that these two isolates are oxidative in nature. While rests are fermentative in nature. Results of catalase test of bacterial isolates are presented in Table 4. If bubbling/foaming occurs *i.e.* taken as catalase positive and if no bubble produced then *i.e.* catalase negative.

From the gram staining and biochemical data, we have assumed JFG 2, VFG 7, VFG 8, BRR 4, BRR 5 , PFG 3, PFG 5 and PFG 6 isolates are *Aeromonas hydrophila* and their sampling site is presented in Table 3. AST analysis of JFG 2, VFG 7, VFG 8, BRR 4, BRR 5 PFG 3, PFG 5 and PFG 6 (presumed *A. hydrophila*) isolates by disc diffusion method are presented in Table 5.

Table 1: Observation of biochemical test of *Aeromonas hydrophila.*

Test	Observation
Gram Staining	Gram-negative
Shape	Rod
Oxidase	+ve
Citrate	+ve
Catalase	+ve
Indole	+ve
Methyl red	+ve
Voges Proskauer	+ve
Nitrate reduction	+ve
TSIA (Triple Sugar Iron Agar)	Acid but/alkaline slant (Y/R)
OF (Oxidative-Fermentative)	Fermentive

Table 2: Coding of bacterial samples used for identification and characterization.

Sr. No.	Sample Codes	Location
1.	VFG	Gill sample of rohu collected from Venktesh farm
2.	JFG	Gill sample of rohu collected from Jaganathprasad
3.	BRR	Gill sample of rohu collected from Bhanjanagar reservoir
4.	PFG	Gill sample of rohu collected from Pitala, Katu

Table 3: *A. hydrophila* collection site.

Bacterial isolates	Sample site	Presumed organism
JFG2	Jaganathprasd	*Aeromonas hydrophila*
VFG7	Rangailunda	*Aeromonas hydrophila*
VFG8	Rangailunda	*Aeromonas hydrophila*
VFG10	Rangailunda	*Absent*
BRR3	Bhanjanagar reservoir	*Absent*
BRR4	Bhanjanagar reservoir	*Aeromonas hydrophila*
BRR5	Bhanjanagar reservoir	*Aeromonas hydrophila*
PFG3	Pitala	*Aeromonas hydrophila*
PFG5	Pitala	*Aeromonas hydrophila*
PFG6	Pitala	*Aeromonas hydrophila*

Table 4: Biochemical Test.

Sr. No.	Isolate	Gram staining	Methyl red	VP Test	Indole test	Oxidase test	Citrate utilization test	Nitrate reduction test	Oxidation-Fermentation test	Catalase test
1.	JFG2	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Fermentative	Positive
2.	VFG7	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Fermentative	Positive
3.	VFG8	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Fermentative	Positive
4.	VFG10	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Oxidative	Positive
5.	BRR3	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Oxidative	Positive
6.	BRR4	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Fermentative	Positive
7.	BRR5	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Fermentative	Positive
8.	PFG3	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Fermentative	Positive
9.	PFG5	Negative	Positive	Positive	Positive	Positive	Positive	Negative	Fermentative	Positive
10.	PFG6	Negative	Positive	Positive	Positive	Positive	Positive	Negative	Fermentative	Positive

Table 5: Zone of Inhibition of different Antibiotics with different concentrations for *Aeromonas hydrophilla.*

Antibiotic	Concentration (µ g)	Characteristics	Zone of Inhibition in mm of Different sites							
			JFG 2	VFG7	VFG8	BRR4	BRR5	PFG3	PFG5	PFG6
Ciprofoxacin-CIP	5	R	13±0.26	10±0.23	11±0.54	14±0.36	09±0.28	10±0.12	12±0.43	13±0.38
Gentamycin-GEN	10	S	14± 0.61 (I)	18±0.16	20±0.59	21±0.72	20±0.86	18±0.33	17±0.47	19±0.29
Tetracycline-TE	30	S	19±0.28	13±0.14 (I)	17±0.35	18±0.54	22±0.46	21±0.68	17±0.59	16±0.56
Chloramphenicol-C	30	R	09±0.25	07±0.28	11±0.15	06±0.34	10±0.46	11±0.53	8±0.58	10±0.62
Cefotaxime-CTX	30S	S	16±0.12	15±0.54	17±0.95	19±0.27	20±0.48	16±0.50	18±0.44	19±0.32

Note : Data are presented as mean ± SE (r=3), R = Resistant , S = Susceptible and I= Intermediate

Anyanwu *et al*. (2014) found that *Aeromonas* sp. shows antibiotic resistance with the following proportion of the samples: 86.5 %: Ampicillin, 100%: Oxacillin, 89.2 %: Amoxicillin, 86.5%: Augmentin, 8.1 %: Cefuroxime, 2.7%: Ceftriaxone, 10.8 %: Chloramphenicol, 13.5 %: Cotrimoxazole, 21.6 %: Erythromycin, 45.9%: Nalidixic acid, 5.4%: Ofloxacin, 2.7 %: Ciprofloxacin, 78.3%: Tetracycline, 10.8%: Azithromycin.

Dias *et al*. (2012) found that *Aeromonas* sp. shows 100% resistance to Ampicillin and Amoxyclav, 16% resistance to Amikacin, while showing 100% susceptibility towards Ceftazidime and Cefotaxime. All the pathogenic pseudomonads and aeromonads confirmed sensitivity towards Oxytetracyclin, Nalidixic acid and Chloramphenicol (Hatha *et al*., 2005).

Here it is found that *A. hydrophilla* was 100% resistant to Ampicillin and Amoxyclav which is the same result found in different region of India. *A. hydrophilla* was 100% susceptible towards Tetracycline, Ceftazidime, Amikacin, Gentamicin, Cefotaxime and Co-trimoxazole. It indicates that there is no contamination of above antibiotics in aquaculture environment. In this work it was found that resistance in case of ciprofloxacin and nalidixic acid was only in Pitala farm (38%) but not in other part of Ganjam district. *P. aeruginosa* showed 100% resistance towards Ampicillin, Amoxyclav and Ciprofloxacin.

Regarding chloramphenicol resistance, approximately 4% of *A. hydrophila* strains isolated from fish were reported to be resistant to this antibiotic, while resistance to nalidixic acid was found in 16% of the strains (Vivekanandhan *et al*., 2002). However, the present study found no evidence of chloramphenicol resistance among *A. hydrophila* isolates. Previous reports have indicated that resistance to chloramphenicol in *A. hydrophila* can be as low as 20% (Hatha *et al*., 2005), whereas Vivekanandhan *et al*. (2002) reported a 4% resistance rate among fish isolates. Interestingly, the present findings revealed that 37% of the strains exhibited resistance to nalidixic acid, which is consistent with earlier research.

Accoroding to Chandravanshi *et al*. (2020), *A. hydrophila* was 100% resistant to Penicillin class of antibiotics *i.e.* Amoxyclav, Ampicillin and Penicillin. Here it was found that *A.hydrophilla* is 100% resistance to Penicillin, Ampicillin and Amoxyclav. Also, Dias *et al*. (2012) found that *Aeromonas* sp. isolated from ornamental fish shows 100% resistance to Ampicillin and Amoxyclav. Several studies have reported complete resistance (100%) of *A. hydrophila* to Penicillin-class antibiotics such as Penicillin, Ampicillin, and Amoxyclav, which aligns with the verdicts of the current study.

Reports from various regions have documented different levels of resistance of *A. hydrophila* isolates to Ciprofloxacin, with Samal *et al*. (2014) reporting 30.8% resistance in Odisha and Andhra Pradesh (India), Sarder *et al*. (2016) recording 6.25% resistance in Dhaka (Bangladesh), and Yang *et al*. (2017) noting 31.75% resistance in South China. In this study, 37.5% of *A. hydrophila* isolates showed resistance to Ciprofloxacin.

The *A. hydrophila* isolates from various locations in Ganjam district exhibited complete resistance (100%) to both Ciprofloxacin (CIP) and Chloramphenicol (C), while showing full sensitivity (100%) to Gentamicin (GEN), Tetracycline (TE), and Cefotaxime (CTX).

CONCLUSIONS

It gives an important message to the society that the fishes contaminated with these bacteria from the respective farm if come in contact with human being these bacteria also show the antibiotic resistance to these particular antibiotics. Some antibiotics are susceptible to these bacteria, so further research may be needed for their use in Aquaculture practices. Again some antibiotics are banned still some fish farm of Ganjam district are using this, so information shall be given to them not to use that. Further AMR study is necessary for other pathogen also in aquafarming sector.

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