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Short Communication

Co-infection of multidrug-resistant *Aeromonas hydrophila* and *A. veronii* in pangas (*Pangasianodon hypophthalmus*) reared in cage culture units of Sarodha Reservoir, Kawardha, Chhattisgarh, India– An outbreak report

M. I. R. Khan[1*] and K. Panda[1]

[1]Department of Aquatic Environment and Health Management, Late Shri Punaram Nishad College of Fisheries, Dau Shri Vasudev Chandrakar Kamdhenu Vishwavidyalaya, Kawardha- 491 995, Chhattisgarh, India

Abstract

During the winter season in January 2022, an outbreak of a disease with moderate mortality was reported in pangas fingerlings (*Pangasianodon hypophthalmus*) that were farmed in cages at Kawardha, Chhattisgarh, India. Morbid pangas had reddish skin and dropsy, which are typical lesions associated with motile aeromonad septicaemia (MAS). Upon initial screening with an Aeromonas-isolation medium, two multidrug-resistant *Aeromonas* strains were found to have infected the pangas. Later, the isolates were subjected to phenotypic characterization and antibiotic sensitivity tests followed by molecular characterization. The universal 16S rRNA gene analyses revealed the isolates *Aeromonas hydrophila* and *A. veronii* with up to 99% homology. The antibiogram revealed the sensitivity where *A. hydrophila* and *A. veronii* were resistant to six and seven antibiotics out of eleven tested antibiotics, respectively. Moreover, the present study also highlights an association between outbreaks of virulent pathogens and poor water quality. The reported mortality hints towards the co-infection of two common pathogens from the aquatic environment. However, further investigation is warranted to understand the pathogenesis.

Keywords: 16S rRNA gene, Antibiogram, Mortality, Motile aeromonad septicaemia (MAS), Virulent

Highlights

- Simultaneous infection of two opportunistic pathogens was reported in cage-reared pangas.
- 16S rRNA gene analysis revealed isolates as *Aeromonas hydrophila* and *A. veronii*.
- Isolates exhibited differential sensitivity against eleven antibiotics of six classes.

Globally, the aquaculture sector serves as a key source of income and nutrition for a large population including India (Ekpenyong *et al*., 2022). Where varieties of aquatic organisms are reared over diverse culture system with organisms ranging from finfish to shellfish in freshwater or marine culture environments. Pangas (*Pangasianodon hypophthalmus*) has been considered a potential species for aquaculture because of its fast growth. As a consequence of successful seed production technology and recent development in scientific farming practices, there has been a significant rise in cage farming practices throughout India. However, several pathogenic microorganisms threaten the sector, such as bacteria, fungi, viruses, parasites, etc. Among all these agents, bacterial pathogens can endure well in diverse ecosystems with a broad host range and ultimately impede aquaculture productivity (Pridgeon and Klesius, 2012).

Bacterial infections, caused by members of *Aeromonas* spp., are among the most frequent and troublesome fish diseases. The widespread distribution, survival capabilities in unfavourable conditions and intensive culture practices-induced stress are some predisposing factors that play a crucial role in *Aeromonas* infection (Pridgeon and Klesius, 2012). Motile aeromonad septicaemia (MAS) is a common

*Corresponding Author, E-mail: idrish.raja.khan@gmail.com

disease caused by *Aeromonas* spp.; these are opportunistic pathogens that cause haemorrhagic septicaemia and ulcerative lesion in various aquatic and terrestrial organisms (Austin and Austin, 1993). Bacterial infections caused by members of the genus *Aeromonas* have a relatively high resistance to chemotherapeutics, including antibiotics, which led to *Aeromonas* spp. as a common and difficult pathogen to tackle (Saavedra *et al*., 2004). Infections by aeromonads (genus *Aeromonas* and family Aeromonadaceae) have been referred to by various names, including motile aeromonad septicemia (MAS), motile aeromonad infection (MAI), red pest or red sore (Camus *et al*., 1998).

In recent times co-infection of multiple pathogens has become familiar and very often causes extensive economic losses (Kotob *et al*., 2016). Whether acting alone or through co-infection, the motile aeromonads can cause significant financial loss, where mortalities can go up to 100 per cent (Camus *et al*., 1998). Among known members of the genus *Aeromonas*, motile aeromonads such as *A. hydrophila* and *A. veronii* are well-known fish pathogens. Since *Aeromonas* spp. are well identified for its wide host range ranging from amphibians to humankind (zoonotic potential), such opportunistic pathogens can possess a significant threat as co-infecting agents across the environment (Chandrarathna *et al*., 2018). Synergistic infection often influences the virulence capacity of pathogens and frequently leads to rampant mortality (Chandrarathna *et al*., 2018).

In the present report, mortality was observed in pangas (mean length: 11.4±1.5 cm; mean weight: 17.5±1.5 g) reared in 6m×4m×4m high-density polyethene (HDPE) cages during the winter season at Kawardha, Chhattisgarh, India (latitude: 22°0′32.2236″ N; longitude 81°13′27.64″ E). Before the onset of infection, fish were fed a commercial diet at 4-5% of biomass daily. Water quality, including dissolved oxygen (DO), unionized ammonia, and temperature of the cage water, were measured at regular intervals using portable instruments or a kit-based method. Moribund fish samples were collected and diagnosed for pathogens through different microbiological and molecular practices. Data obtained from the present experiment were subjected to analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) IBM version 21 (SPSS Inc., Chicago IL, USA). Differences in the means were compared using Duncan's multiple range test, and significance was confirmed at $P = 0.05$.

After the proper observation and record of the gross lesions and clinical history, moribund fish (n = 13 from each 96 m^3 cage) were immediately transported to the laboratory. Bacterial isolates were transferred aseptically from blood, kidney and ascitic fluid on nutrient agar (HiMedia, Mumbai), followed by 24 h incubation at 30°C. Since the typical lesion gives a prognosis of *Aeromonas* infection therefore the bacterial colonies (n = 37) were transferred to a selective media, "Aeromonas-isolation medium (AIM) base" (HiMedia, Mumbai), supplemented with "Aeromonas-selective supplement" (HiMedia, Mumbai), followed by incubation at 30°C for 24 h till the appearance of a characteristic greenish-yellow colony with a blackish tinge. After the formation of the typical distinctive colonies, isolates were subjected to phenotypic tests following Khuntia (2011). Crude DNA was extracted through the colony PCR method for molecular characterization. Briefly, bacteria were grown on Luria Bertani agar (HiMedia, Mumbai) or in Luria Bertani broth (HiMedia, Mumbai). Where log phase young bacterial culture was taken from an agar plate in 0.05-0.1 mL of 1X TE (Tris-EDTA; HiMedia, Mumbai) or bacterial broth were mixed directly with TE buffer (9 broth: 1 1X TE) and vortexed, followed by a PCR denaturation cycle at 94°C for 10 min. Immediately after the completion of the denaturation cycle, samples were kept at 4°C for 3 minutes. Later, samples were centrifuged for 2 min at 10,000 rpm and the supernatants were used as a target template. Amplification of bacterial 16S rRNA gene was achieved using

the combination of universal primers 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' GGTTACCTTGTTACGACTT 3') (Weisburg *et al.,* 1991). Where, the PCR reaction mixture (26 µL) consisted of 1 µL template DNA (100 ng μL^{-1}), 1 µL of each primer (10 pmol μL^{-1}), 13 µL of Taq 2X Master Mix (HiMedia, Mumbai), and 10 µL of nuclease-free water. The PCR condition was: initial denaturation at 94°C for 10 min; 35 cycles of denaturation for 1 min at 94°C; annealing for 45 seconds at 55°C, and extension at 72°C for 1 min; final extension at 72°C for 7 min. After confirming the amplification of the PCR products (1000 bp) by the gel documentation unit (Bio-Rad, USA), the products were purified using a PCR product purification kit (Thermo Fisher Scientific Inc., USA) and sequenced at Bioserve Biotechnologies India Pvt. Ltd., Hyderabad. Bacterial identity was deduced by the BLAST (Basic Local Alignment Search Tool) algorithm against the GenBank database. The phylogenetic relationship of isolates was also determined with other strains based on homology exhibited during BLAST analysis with the help of Molecular Evolutionary Genetic Analysis (MEGA-X) software. The sequences were aligned with retrieved sequences of other *A. hydrophila* and *A. veronii* using the Clustal Omega program (http://www.ebi.ac. uk/Tools/ msa/clustalo/). Identified sequences were submitted to the NCBI (National Centre for Biotechnology Information) database, and accession numbers were obtained.

The antibiotic sensitivity of isolates was tested against eleven antibiotics of six different classes. Tested antibiotics include amoxicillin (30 mcg), chloramphenicol (30 mcg), erythromycin (15 mcg), gentamycin (10 mcg), kanamycin (30 mcg), azithromycin (15 mcg), Ciprofloxacin (5 mcg), nalidixic acid (30 mcg), oxytetracycline (1 mcg), penicillin (10 unit) and sulfonamide (10 mcg). Disc-diffusion method was followed for antibiogram analysis, and the sensitivity was interpreted based on the zone of inhibition (mm) according to the Clinical and Laboratory Standard Procedure (CLSI) guidelines.

In this study, strains *A. hydrophila* (COFKWD_AH22) and *A. veronii* (COFKWD_AV22) were isolated, and probably for the first time in India, synergistic infection of both these species was identified from cage-farmed pangas. Both these bacteria were frequently reported as fish pathogens and often associated with severe economic loss worldwide; however, their co-infection is a novel threat to aquaculture dynamics. Daily mortality with typical clinical signs, including haemorrhage, dropsy and fin erosion, was observed up to 3 weeks from the onset of infection, with total mortality up to 40%. Water quality during the outbreak fluctuated significantly ($P<0.05$) compared to previous months, including temperature of 17 to 25.6°C, dissolved oxygen of 3 to 4 ppm, and elevated levels of unionized ammonia of 0.35 to 0.40 ppm. Poor water quality parameters represented in the present study are likely to trigger stressful situations and make fish prone to diseases, as also reported by Nofal and Abdel-Latif (2017). Moreover, in corroboration to the present study, Abdel-Latif and Khafaga (2020) and El-Son *et al.* (2021) also suggested that the *A. hydrophila* infection may lead to severe economic loss when combined with other pathogens or poor water quality.

The morphological characteristics of the isolates are given in Table 1. The isolates showed similar morphological features to field isolates available in the laboratory. BLAST analyses of 16S rRNA sequences of *A. hydrophila* (COFKWD_AH22) and *A. veronii* (COFKWD_AV22) shared up to 99% homology with other published sequences retrieved from NCBI GenBank. Moreover, phylogenetic tree analysis also exhibited *A. hydrophila* (ON248038) and *A. veronii* (ON248036) were grouped together with other respective isolates originating from different parts of the world (Fig. 1). Table 2 shows the susceptibility of isolates against eleven different antibiotics. Where *A. hydrophila* (ON248038) and *A. veronii* (ON248036) showed resistance to six and seven antibiotics out of 11 tested antibiotics, respectively.

Table 1. Phenotypic characteristics of *Aeromonas hydrophila* (ON248038) and *A. veronii* (ON248036) compared

Characteristics	*A. hydrophila*		*A. veronii*	
	In present study (ON248038)	MK907589	In present study (ON248036)	MK907586
Gram stain	G-ve, rod-shaped	G-ve, rod-shaped	G-ve, rod-shaped	G-ve, rod-shaped
Growth on AIM	GY	GY	GY	GY
Swarming	-	-	-	-
Motility	+	+	+	+

Note: +: positive, -: negative, GY: greenish-yellow colony with blackish tinge. MK907589 and MK907586 are reference *A. hydrophila* and *A. veronii* isolates from our laboratory.

Table 2. Antibiotic susceptibility of *Aeromonas hydrophila* (ON248038) and *A. veronii* (ON248036)

Class of antibiotic	Antibiotics (Concentration/disc)	*A. hydrophila* (ON248038)	*A. veronii* (ON248036)
A. Aminoglycosides	1. Gentamicin (10 mcg)	S	R
	2. Kanamycin (30 mcg)	R	R
B. β-lactams	3. Amoxicillin (30 mcg)	R	S
	4. Penicillin (10 unit)	R	R
C. Macrolides	5. Azithromycin (15 mcg)	R	R
	6. Erythromycin (15 mcg)	R	R
D. Quinolones	7. Ciprofloxacin (5 mcg)	S	S
	8. Nalidixic acid (30 mcg)	R	R
E. Sulfa drugs	9. Chloramphenicol (30 mcg)	S	S
	10. Sulfonamide (10 mcg)	S	R
F. Tetracycline	11. Oxytetracycline (1 mcg)	S	S

Note: S= susceptible, R= resistant

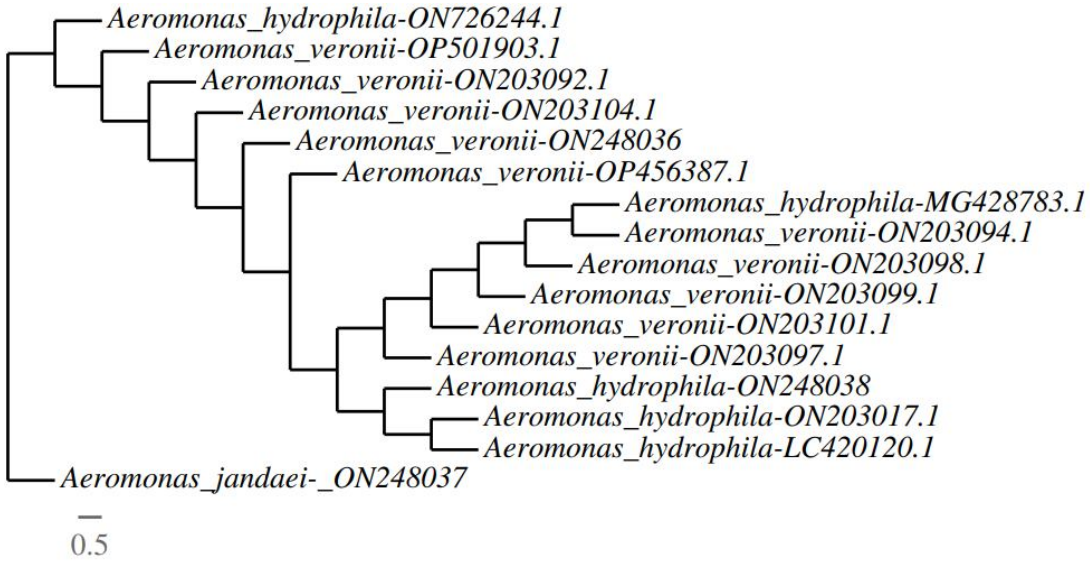

Fig. 1A. Phylogenetic tree of both the isolates, *Aeromonas hydrophila* ON248038 and *A. veronii* ON248036 based on 16S rRNA gene sequencing and neighbour-joining comparison exhibiting close taxonomic relations with other strains availed from in NCBI GenBank

In conclusion, it can be said that Aeromonad infection is a significant threat to the global aquaculture industry. The present study, reported simultaneous infection of two pathogenic strains *A. hydrophila* and *A. veronii*, from cage-farmed pangas, the first of its kind in India. Moreover, the deterioration of optimum water quality has also triggered stress in fish, which may be a predisposing factor for infection and subsequent mortality. Similar co-infection is an emerging trend and might aggravate the problem to a greater extent if not addressed properly. Therefore, the present report calls for further investigation including epidemiology and pathogenesis, to develop proper health management practices.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: MIRK: Conceptualization and designing of the experiments, execution of experiments, analyses of derived data, and drafting and finalizing the manuscript; KP: Review of the manuscript.

Ethical statement: All experiments were performed in accordance with the standard guidelines and policies suggested by the ethical committee of the institute.

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REFERENCES

Abdel-Latif HMR and Khafaga AF, 2020. Natural co-infection of cultured Nile tilapia *Oreochromis niloticus* with *Aeromonas hydrophila* and *Gyrodactylus cichlidarum* experiencing high mortality during summer. Aquac Res, 51(6): 1880-1892, doi: 10.1111/are.14538

Austin B and Austin DA, 1993. Bacterial fish pathogens disease in farmed and wild fish. In: Inglis V., Roberts, R. J. and Bromage N. R. (eds.) Bacterial diseases of fish, 2nd edn. Halsted Press, Sydney

Camus AC, Durborow RM, Hemstreet WG, Thune RL and Hawke JP, 1998. Aeromonas Bacterial Infections-Motile Aeromonad Septicemia (Vol. 478). Southern Regional Aquaculture Center

Chandrarathna HPSU, Nikapitiya C, Dananjaya SHS, Wijerathne CUB, Wimalasena SHMP *et al*., 2018. Outcome of co-infection with opportunistic and multidrug resistant *Aeromonas hydrophila* and *A. veronii* in zebrafish: identification, characterization, pathogenicity and immune responses. Fish Shellfish Immunol, 80: 573-581, doi: 10.1016/j.fsi.2018.06.049

Ekpenyong J, Iheanacho S, Asuquo F and Okey I, 2022. Ichthyotoxicity of ethanolic extract of *Justicia extensa* on behavioral and histopathological changes in *Clarias gariepinus* (Burchell, 1822). Environ Chem Ecotoxicol, 4: 211-215, doi: 10.1016/j.enceco.2022.11.001

El-Son MA, Nofal MI and Abdel-Latif HM, 2021. Co-infection of *Aeromonas hydrophila* and *Vibrio parahaemolyticus* isolated from diseased farmed striped mullet (*Mugil cephalus*) in Manzala, Egypt–A case report. Aquaculture, 530: 735738, doi: 10.1016/j.aquaculture.2020.735738

Khuntia BK, 2011. Basic Microbiology. Daya publishing house, Delhi

Kotob MH, Menanteau-Ledouble S, Kumar G, Abdelzaher M and El-Matbouli M, 2016. The impact of co-infections on fish: A review. Vet Res, 47(1): 98, doi: 10.1186/s13567-016-0383-4

Nofal MI and Abdel-Latif HMR, 2017. Ectoparasites and bacterial co-infections causing summer mortalities among cultured fishes at Al-Manzala with special reference to water quality parameters. Life Sci J, 14(6): 72-83

Pridgeon JW and Klesius PH, 2012. Major bacterial diseases in aquaculture and their vaccine development. CABI Reviews, pp 1-16, doi: 10.1079/PAVSNNR20127048

Saavedra MJ, Guedes-Novais S, Alves A, Rema P, Tacão M *et al*., 2004. Resistance to beta-lactam antibiotics in *Aeromonas hydrophila* isolated from rainbow trout (*Oncorhynchus mykiss*). Int Microbiol, 7(3): 207-211

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