



RESEARCH ARTICLE

A case investigation of mortality factors in Japanese Koi (*Cyprinus carpio*) from a recreational pond

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ARTICLE HISTORY

Received: 2 April 2026

Revised: 30 July 2026

Accepted: 31 July 2026

Published: 30 September 2026

ABSTRACT

An investigation covering water quality, parasitology, bacteriology, and virology was conducted to address cumulative mortality rates exceeding 80% among Japanese koi (*Cyprinus carpio*) in a recreational pond at AkuaTAR, Fisheries Research Institute, Penang. Low dissolved oxygen levels (DO < 4 mg/L) were identified as a critical environmental stressor likely contributing to the high mortality rates. Gross examinations of the Japanese koi revealed generalised hemorrhages on the body, tail, and fin bases, alongside clinical signs of fin rot and scale loss. The parasite *Argulus japonicus* was identified based on 18S rRNA with a 100% prevalence and a mean intensity of 79 parasites per fish. Conventional plate culture of the fish and parasite's internal tissue resulted in the isolation of three distinct bacterial strains, identified as *Aeromonas sobria*, *Aeromonas hydrophila*, and *Enterobacter aerogenes* based on biochemical tests. However, further taxonomic validation using whole genome sequencing and *in-silico* genome-to-genome hybridization approach reclassified both *A. sobria* and *A. hydrophila* as *Aeromonas jandaei*. The presence of multiple bacterial species within the fish parasite indicates its potential role as a vector in disease transmission. The pathology observed in the affected Japanese koi was primarily driven by tissue injury from *A. japonicus* attachments and bites, and alongside generalised haemorrhage associated with secondary *Aeromonas* spp. infections. The high parasite burden and tissue damage at the infection site suggest that *A. japonicus* potentially impairs host defenses and facilitates secondary bacterial infections, ultimately contributing to the mortality event. The findings suggest that the high mortality rate was associated with a multifactorial event involving low dissolved oxygen levels, a severe *A. japonicus* infestation, and subsequent secondary bacterial infections.

Keywords: *Argulus*; infestation; secondary infection; *Aeromonas* spp.; Koi.

INTRODUCTION

Health problems in cultured or ornamental fish maintained in ponds are often associated with various issues, including water quality deterioration, excessive algal growth, diseases caused by infectious agents (such as parasites, bacteria, viruses, or fungi), or non-infectious agents (such as nutrition and metabolic disorders), and environmental factors (such as toxins and predation). Japanese koi (*Cyprinus carpio*) are often selectively bred for attractive traits, which may potentially lead to lowered immunity and increased susceptibility to diseases (Khanjani & Sharifinia, 2020; Deocampo Jr *et al.*, 2021). Japanese koi has been reported with various diseases which include viral threats such as Cyprinid herpesviruses 2 and 3, gram-negative bacteria (genera *Aeromonas*, *Citrobacter*, *Edwardsiella*, *Flavobacterium*, *Pseudomonas*, and *Vibrio*), or parasitic infestations caused by ciliate (*Ichthyophthirius multifiliis*), fish lice (*Argulus* spp.), and anchor worm (*Lernaea* spp.) (Tonguthai, 1997; Ahne *et al.*, 2002; Decostere *et al.*, 2002; Roberts *et al.*, 2009; Dickerson, 2011; Elisafitri *et al.*, 2021; Larcombe *et al.*, 2024). On

the other hand, Japanese koi are also prone to co-infections, such as those involving any combination of parasites, bacteria, or viruses. Pathogenic microorganisms, such as bacteria and parasites, often act as secondary infections exacerbated by primary stressors. Furthermore, parasites can act as vectors, creating entry points through tissue damage or carrying pathogens that introduce systemic bacterial or viral infections. Recent findings by El-waziry *et al.* (2025) highlight the role of crustacean parasites as vectors, demonstrating their capacity to harbor and transmit *Vibrio alginolyticus* within marine aquatic environments. This physiological stress suppresses the host's immune system, potentially resulting in multiple infections and increased vulnerability, which can be fatal if not properly managed (Larcombe *et al.*, 2024).

Numerous parasites are known to act as vectors of infectious agents, such as bacterial and viral pathogens that affect fish. *Argulus* parasites are significant vectors of bacterial diseases, primarily by causing mechanical damage to the fish skin that allows opportunistic bacteria to enter the host, and by suppressing the host's immune system (Steckler & Yanong, 2012).

Argulus species (Family: Argulidae) are members of a large group of brachyuran parasites. There are approximately 100 different species of *Argulus* distributed worldwide, which, depending on the species, can infest both freshwater and saltwater fishes. The three most-studied species, *Argulus foliaceus*, *Argulus japonicus*, and *Argulus coregoni*, are found in freshwater systems (Noaman et al., 2010). Infections with these species are most common in wild and pond-raised freshwater (Wafer et al., 2015).

While the direct effect of argulid parasites on fish may be minimal, their interaction with bacterial pathogens can significantly increase host mortality. Bacterial diseases, which are a major concern in the aquaculture industry, can be exacerbated by parasitic infestations, as seen with *Flavobacterium columnare* (Bandilla, 2007; Mohd-Aris et al., 2019). However, data on multi-infections in Japanese koi involving parasites and bacteria are scarce in the literature. This study aimed to investigate the factors contributing to mortality, the nature of co-infection involved, and potential suitable treatment approaches.

MATERIALS AND METHOD

Investigation factors contributing to mortality

A case study involving Koi in a 'touch pool' recreational pond was reported from 3-11 October 2021, with cumulative mortalities of more than 80%. The concrete pond was circular in shape, with a depth of about one meter at the centre and an estimated water volume of about 80 tonnes/pond. It contained a mixed culture of other fish species such as 'Kelah' Malaysian Mahseer (*Tor* sp.), 'Baung' catfish (*Mystus* sp.), and Arapaima (*Arapaima gigas*). A new batch of 95 koi carps of various sizes at the grow-out stages were added to the pond for display on 20 September 2021. Other fish species appeared to be healthy without any clinical symptoms. Bigger fish, such as Arapaima, catfish, and 'kelah', were transferred to a separate culture tank to avoid the spread of disease once mortalities were noticed.

This study was conducted as a case investigation of a mortality event affecting Japanese koi in a recreational pond at AkuaTAR, Fisheries Research Institute, Penang. During the outbreak, only two moribund koi carp were available and submitted for detailed laboratory examination, including parasitological, bacteriological, virological, and genomic analyses. Consequently, the findings should be interpreted within the context of this specific mortality event or generalization to other koi populations.

Water quality analysis

In-situ analysis of physical water parameters such as temperature (°C), salinity (ppt), dissolved oxygen (mg/L), pH, and total suspended solid (TSS, mg/L) were carried out at surface level and water depth at 1 meter using YSI 5908 probe (Yellow Springs, OH, USA) from three different locations within the pond area (near water source, centre of pond, and water exit). Water samples were collected in 500 mL plastic bottles for the analysis of basic chemical parameters such as total ammonia-Nitrogen (NH₃-N), nitrite (NO₂⁻), sulphide (S²⁻), and iron (Fe), using a HACH test kit (Loveland, CO, USA).

Gross examination and post-mortem of fish samples

Two live moribund koi with sizes of 306 g (TL 31 cm) and 561.2 g (TL 34 cm) were sampled for necropsy examination and laboratory analysis. The sample size was limited (n=2) because selection focused on koi displaying clinical signs of illness during the visit with very few live fish remaining in the pond.

Parasitology examination

A standard method for examinations of ectoparasites from skin and gills of fish was performed (Kua & Nik-Haiha, 2018). A body smear from a live fish sample was placed on a glass slide, and examined

under the microscope. A small piece of the second and third segments of the gill branch from both sides of the koi fish was cut off using scissors, placed on a glass slide, air-dried, fixed in methanol, followed by Giemsa staining and microscopic examination. After taking samples, live fish was subjected to a five-minute freshwater dip in individual containers, followed by gentle manual brushing to collect, identify and determine the density of external parasites found on the body.

18S rRNA-based molecular identification of parasites

A representative parasite specimen from each fish sample (e.g., isolate Koi1 and isolate Koi2) was selected for full-length 18S rRNA gene-based molecular identification. Genomic DNA was extracted from individual parasite specimens using a salt precipitation-based extraction method. Briefly, samples were homogenized in tissue lysis buffer containing 1% SDS, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA, followed by incubation at 60°C for 30 min. The lysate was centrifuged at 10,000 × g to remove insoluble tissue debris, and the resulting supernatant was mixed with 0.5 volumes of saturated NaCl solution and incubated at 4°C for 15 min to facilitate protein and detergent precipitation. Following centrifugation at 10,000 × g for 10 min, the supernatant was mixed with 0.7 volumes of isopropanol to precipitate genomic DNA. The DNA pellet was recovered by centrifugation at 10,000 × g for 5 min, washed with 70% ethanol, air-dried, and resuspended in 50 µL TE buffer. The extracted DNA was used as the template for amplification of the full-length 18S rRNA gene using universal eukaryotic 18S rRNA primers EukA_21F (5'-AACCTGGTTGATCCTGCCAGT-3') and EukB_1791R (5'-GATCCTTCTGCAGGTTACCTAC-3'). PCR amplification was performed with an initial denaturation step at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 10 s, annealing at 50°C for 20 s, and extension at 72°C for 2 min, with a final extension step at 72°C for 3 min. PCR amplicons were purified and used for Oxford Nanopore sequencing library preparation using the SQK-LSK110 ligation sequencing kit with EXP-PBC001 native barcoding expansion, following the manufacturer's protocol. Sequencing reads were basecalled using Oxford Nanopore Guppy in Super Accuracy (SUP) mode with the R9.4.1 DNA SUP basecalling model. The resulting reads were demultiplexed based on barcode assignments, and full-length 18S rRNA consensus sequences for each parasite isolate were generated using NGSspeciesID version v0.3.1, a long-read amplicon consensus generation pipeline that clusters reads based on sequence similarity and generates polished consensus sequences through read-supported correction (Sahlin et al., 2021). The generated 18S rRNA consensus sequences were subsequently compared against reference sequences available in the NCBI nucleotide database using BLASTn for molecular identification. Reference sequences with alignment lengths of ≥1,600 bp were used for species-level comparison to ensure sufficient representation of informative regions within the 18S rRNA gene.

Bacteriology Examination

Isolation of bacteria from moribund fish specimen

Bacteria inoculation from the internal organs, such as liver, spleen, kidney, and brain of two Koi fish were cultured onto tryptose soy agar (TSA) (Oxoid, United Kingdom) at 30°C overnight, followed by subculturing until pure isolates were obtained. Pure isolates were subjected to Gram staining, oxidase, catalase, motility tests, and API 20 NE (Biomerieux, France) for identification at the genus and species levels. An analytical profile index 20 NE was used for the biochemical identification of non-Enterobacteriaceae. Identification with percentage of ≥90% was accepted as good identification (GID), ≥99% as very good identification (VGID), and ID percentage ≥80-89% indicate excellent identification to the genus of bacteria.

Isolation of bacteria from *Argulus parasite*

Inoculation of bacteria from the internal tissues of the parasite *A. japonicus* was performed by making a small incision on its body and transferring the direct inoculation from the *A. japonicus* internal tissue onto tryptic soy agar (TSA) using a sterile loop. Repeated subculturing was performed to obtain a pure bacterial colony isolated from *A. japonicus*, followed by similar procedures as mentioned in section above for the identification of bacteria to the genus and species.

Virology examination

Detection of Tilapia Lake Virus (TiLV) by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Sample Processing

Approximately 50-100 mg of liver, spleen, kidney, and brain tissues from koi carp were transferred into sterile microcentrifuge tubes containing sterile sand to facilitate mechanical disruption. An appropriate volume ($\approx 500 \mu\text{L}$) of sterile phosphate-buffered saline (PBS)/lysis buffer was added. The tissues were homogenised thoroughly by manual grinding using a sterile pestle until a uniform suspension was obtained. The homogenates were centrifuged briefly to remove tissue debris, and the clarified supernatant was used for nucleic acid extraction.

Nucleic Acid Extraction

Total nucleic acids (DNA/RNA) were extracted from the processed samples using an automated nucleic acid extraction system (GeneReach Biotechnology Corp., Taiwan) according to the manufacturer's instructions. The as-extracted nucleic acids were eluted in nuclease-free buffer and stored at -20°C until further analysis.

RT-PCR TiLV

Detection of TiLV was performed using RT-PCR assay targeting a conserved region of the TiLV genome. The reaction mixture was prepared according to the assay protocol MyTaq™ OneStep RT-PCR kit (Meridian Bioscience, Memphis, Tennessee, USA). PCR amplifications were performed using the T1000 Touch™ thermal cycler (BioRad Laboratories Inc., Hercules, California, USA). The amplification protocol is shown in Table 1. A set of new primers targeting segment 3 of TiLV was designed using National Centre Biotechnology Institute (NCBI) (Abdullah et al., 2022). The primers were constructed using the TiLV local isolate sequences that were previously obtained from the first outbreak case of TiLV in Malaysia in 2018. The primer sequences were named as F3-MaTiLV (5'-TGGGCACAAGGCATCCTAC-3') and R3-MaTiLV (5'-CACGTGCGTACTCGTTCAGT-3'). Amplified PCR products from the reaction were analysed by gel electrophoresis for 30 min at 100 V on a 1.5% (w/v) agarose gel in Tris-borate-EDTA buffer (TBE) and stained with RedSafe™ Nucleic acid staining solution (20,000x). The expected band size of 245 bp indicated a positive result for TiLV. Amplification profile of the RT-PCR thermal cycler for the detection of TiLV is shown in Table 1.

Table 1. Amplification profile of the RT-PCR thermal cycler for the detection of TiLV

Step	Temperature ($^\circ\text{C}$)	Time	Cycle
RT	45°C	30 min	1
Initial denaturation	95°C	1 min	1
Denaturation	94°C	10 sec	30
Annealing	55°C	10 sec	
Extension	72°C	30 sec	
Final extension	72°C	5 min	1
Cooling	4°C	∞	

DNA extraction of bacterial isolates for Illumina whole genome sequencing

Six bacterial isolates representing three species, namely *Enterobacter aerogenes* (K2A3_Ea, KA1_Ea, and KA2_Ea), *Aeromonas hydrophila* (KA2_Ah and KA5_Ah), and *Aeromonas sobria* (KA3_As), were selected for whole-genome sequencing following preliminary species identification using the API 20 NE biochemical identification system. DNA extraction was performed as per the method of Solokov (2000) with some modifications. The pellet was resuspended in 500 μL of lysis buffer (50 mM NaCl, 50 mM Tris-HCl, pH 8, 50 mM EDTA, 2% SDS) and incubated at 60°C for 30 min. Then, 3 μL RNase A (10 mg/mL) was added to the lysate, followed by incubation at room temperature for 10 min. Salting out was performed via the addition of 50 μL (0.1x vol.) saturated KCl at 4°C for 5 min. The lysate was subsequently extracted once with an equal volume of chloroform to remove remaining proteins. The aqueous layer containing the DNA was mixed with an equal volume of isopropanol and 20 μL of SPRI bead to promote binding of DNA onto the solid carboxylated layer (Oberacker et al., 2019). After incubation at room temperature for 10 min, the mixture was placed on a magnetic rack for 2 min, followed by the removal of supernatant. The bound magnetic bead was washed twice with 75% ethanol. DNA elution from the bead was performed by the resuspension of the bead with 100 μL of TE buffer, followed by incubation at 50°C for 5 min.

Library preparation and whole genome sequencing

For Illumina whole-genome sequencing, approximately 100 ng of genomic DNA, quantified using a Qubit Fluorometer (Qubit 4 Fluorometer, Thermo Fisher Scientific, USA), was used as input for library preparation using the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, USA) according to the manufacturer's instructions. Briefly, genomic DNA was fragmented to an average size of approximately 300 bp using a Bioruptor (Diagenode, Belgium). The fragmented DNA was subjected to end repair and dA-tailing, followed by the ligation of Illumina-compatible sequencing adapters. Adapter-ligated libraries were subsequently amplified by PCR using unique dual-index primers to enable sample multiplexing. The indexed libraries were sequenced on an Illumina NovaSeq 6000 platform (Illumina Inc., USA) using a paired-end 150 bp sequencing configuration ($2 \times 150 \text{ bp}$). Approximately 1 Gb of sequencing data was generated per sample, corresponding to an estimated sequencing depth of approximately 200x based on an expected genome size of 5 Mb.

De novo assembly, assembly quality assessment and genome-based species delimitation

Raw Illumina paired-end reads were trimmed with fastp v0.21 (Chen et al., 2018) to remove low-quality bases and Illumina adapter sequences. The trimmed reads were subsequently used for de novo assembly in SPAdes v3.15.0 (isolate setting) (Bankevich et al., 2012). Contigs smaller than 500 bp representing mostly sequencing artefact were removed and the filtered assembly was used for subsequent analysis. Genome assembly statistics were generated using QUAST (Gurevich et al., 2013). Average nucleotide index (ANI) values of the assembled genome were calculated using JSpeciesWS. Pairwise comparisons were made against the genome sequences of type strains of the suspected species and closely related taxa, with sequence regions exhibiting more than 90% identity to entries in the reference database.

Screening of virulence factors (VFs) and antimicrobial resistance (AMR) genes

ABRicate (<https://github.com/tseemann/abricate>) was used for mass screening of assembled contigs for antimicrobial resistance (AMR) and virulence factor (VF) genes. BLAST-based nucleotide similarity searches were performed against curated databases, including the NCBI, the Virulence Factor Database (VFDB), the *E. coli* VF database,

and PlasmidFinder, using the assembled genomes of five isolates obtained from *Argulus*. AMRFinder Plus and the Reference Gene Catalog were used to examine the genomic links between AMR and virulent genes in the samples by comparison with the NCBI database (Chen et al., 2005; Feldgarden et al., 2021). Identification of putative VF and AMR genes in each isolate was based on a nucleotide identity threshold of 80% against the VFDB and NCBI antibiotic database, respectively.

RESULTS

Water quality analysis

Water quality analysis of surface water from three different sites within the pond area showed slightly elevated ammonia (0.4-0.45 mg/L) and low dissolved oxygen (3.47-4.27 mg/L) levels. Low dissolved oxygen (<5 mg/L) was known as one of the factors that contribute to high mortalities in koi carp fish at AKUATAR 'touch pool'. Total ammonia level in a culture pond should be kept at a very low concentration (<0.02 mg/L) to maintain a safe level for long-term fish health. Hence, 30-40% water exchange in the affected pond was recommended to improve water quality. Results of basic physical and chemical water parameters are shown in Table 2.

Parasitology

The crustacean parasite identified as *Argulus* sp. was found in heavy infestation attached on the moribund's fish body surface, fins, and mouth, accompanied by generalised redness/haemorrhages over

the entire body, bases of the fins, along with scale loss (Figure 1). The prevalence of *Argulus* sp. was 100% with a mean intensity of 79 parasites per fish.

Identification of *Argulus* Species based on 18S rRNA gene sequences

Near full-length 18S rRNA sequences (1,800 bp) were successfully reconstructed from Nanopore sequencing reads using consensus-based assembly. Both Koi_1 and Koi_2 sequences were 1,800 bp in length and have been deposited in the NCBI nucleotide database under accession numbers PZ675502 and PZ675503, respectively. These sequences represent primer-to-primer near full-length 18S rRNA amplicons, providing a complete representation of the targeted region compared with the partial 18S rRNA sequences that comprise the majority of publicly available *Argulus* reference records. Both near full-length 18S rRNA sequences were identical to each other and provided strong molecular support for the identification of the parasite as *Argulus japonicus*. The consensus sequences showed complete query coverage (1,800 bp) and 99.889% nucleotide identity to the *A. japonicus* isolate HB1 reference sequence (MW866569.1) (Table 3). This identification was further supported by comparison with a published *A. japonicus* near full-length 18S rRNA sequence (OQ660496.1) reported by Das et al. (2025), which showed 99.597% sequence identity. Unexpectedly, a high sequence similarity was also observed with one *Argulus nobilis* (MH458748.1; 99.885% identity). However, this record is an unpublished GenBank submission without an associated taxonomic publication. In contrast, the published

Table 2. *In-situ* physical and chemical water parameters at the AKUATAR 'touch pool' on 11 October 2021

Parameters (mg/L) except pH and temperature	Station 1	Station 2	Station 3	*Normal level in aquaculture
1. Temperature (°C)	29.4	29.5	29.5	26-30°C
2. Dissolved oxygen (DO)	3.47	3.87	4.27	>5
3. pH	7.15	7.21	7.23	6.5 – 8.5
4. Total Ammonia, (NH ₃)/ unionized Ammonia	0.40 (0.004)	0.45 (0.006)	0.45 (0.006)	<1.0 / <0.02
5. Sulfate (S ₂)	4	7	16	< 10.0
6. Nitrite (NO ₂)	0.05	0.05	0.05	<0.05
7. Ferum (Fe)	0.02	0.01	0.007	< 0.5

Reference*: Boyd, 1990; PHILMINAQ, n.d.

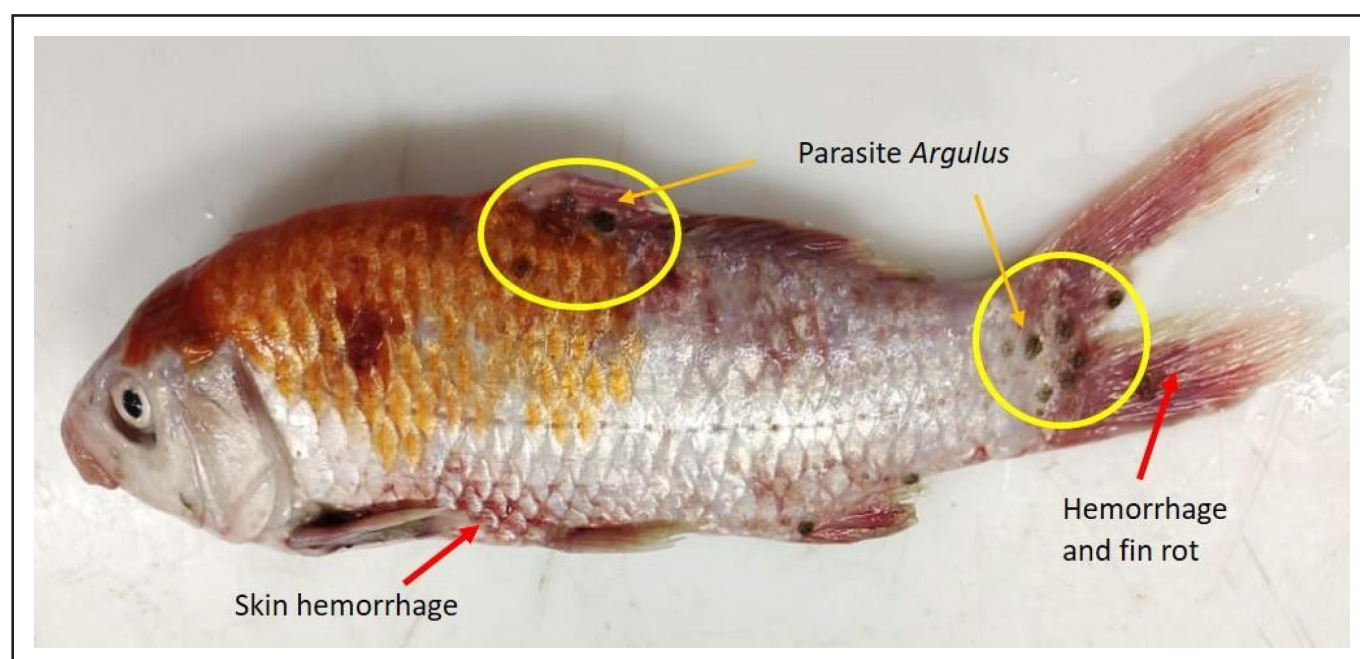


Figure 1. Adult *Argulus* sp. was found on the body of an infested koi with signs of generalised haemorrhage on the body, tail, fins, and mouth.

A. nobilis 18S rRNA sequence (M27187.1) showed much lower similarity (98.334%) with 25 mismatches to the present sequences. Considering the near full-length 18S rRNA agreement with validated *A. japonicus* references, together with morphological observations and the known occurrence of *A. japonicus* in freshwater and ornamental fish systems, the isolates were identified as *A. japonicus*.

Sequences generated isolates Koi_1 and Koi_2 were compared against 18S rRNA sequences available in the NCBI nucleotide database using BLASTn (as of 12 July 2026). Reference sequences with an alignment length of $\geq 1,600$ bp were prioritized for species-level comparison to ensure sufficient representation of informative regions within the 18S rRNA gene. Alignment length indicates the number of nucleotides compared between the query and reference sequence, and query coverage represents the proportion of the query sequence included in the alignment. Sequence identity indicates the percentage of identical nucleotides across the aligned region, while mismatches represent the number of nucleotide differences between the two sequences. Results are shown in Table 3.

Bacterial isolation and biochemical characterization of cultured isolates

Aeromonas sobria (1) and *Pasteurella pneumotropica* (1) were identified from the internal organs of koi carp (1), whereas *A. sobria* (1), *Aeromonas hydrophila* (2), and *Enterobacter aerogenes* (3) were identified from *Argulus* (5) internal tissues. Table 4 showed the

bacterial identification results obtained from culture and isolation using API 20 NE kit.

Biochemical analysis using API 20NE identified one isolate from Koi fish as *A. sobria* with very good identification to the species (99.4%). Another bacteria isolate was identified as *P. pneumotropica* / *Mannheimia haemolytica* (90.6%) belonging to the family Pasteurellaceae. *A. sobria* is a pathogenic bacterial disease in koi, often leading ulcers, fin rot and haemorrhagic septicaemia. These opportunistic bacteria naturally inhabit pond water, it primarily attacks koi fish with weakened immune systems, particularly during temperature swings or after parasitic stress (Liu et al., 2025). *P. pneumotropica* (now frequently classified as *Rodentibacter pneumotropicus*) is primarily a mammalian pathogen found in rodent, cats and dogs. It is not a common pathogen in koi fish of pond water. However, if detected in a pond, it is likely the results of external contamination from rodents or wildlife (Soto-Vega et al., 2025).

Whole genome-based identification and characterization of bacterial isolates from *Argulus japonicus*

Illumina whole genome sequencing generated approximately 1.2-1.5 Gb of paired-end reads per sample, corresponding to an estimated sequencing depth of 227x to 325x. De novo assembly produced draft genomes ranging from 4.4 Mb to 5.9 Mb, with GC contents between 57.7% to 58.8%. The assembled genomes consisted of 39 to 99 contigs with N50 values more than 145 kb and the largest

Table 3. BLASTn-based molecular identification of *Argulus japonicus* isolates using near full-length 18S rRNA gene sequences

Sample	Blast Hit	Accession Number	Alignment length (bp)	Query Coverage (%)	Percent Identity (%)	Number of Mismatches	Reference
Koi_1	<i>Argulus japonicus</i> isolate HB1	MW866569.1	1800	100	99.889	2	Unpublished
Koi_1	<i>Argulus japonicus</i> strain CIFRI.E2	OQ660496.1	1739	96.6	99.597	2	Das et al., 2025
Koi_1	<i>Argulus nobilis</i> strain AGGC_215	MH458748.1	1734	96.3	99.885	0	Unpublished
Koi_1	<i>Argulus nobilis</i> isolate proviral	M27187.1	1801	100	98.334	25	Abele et al., 1992
Koi_1	<i>Argulus siamensis</i> isolate WBUAFS-A3	KF583879.1	1688	93.8	98.637	3	Patra et al., 2016
Koi_1	<i>Argulus indicus</i> isolate A1 ERIB-1	MT538301.1	1729	96.1	97.108	38	Amriana et al., 2021
Koi_2	<i>Argulus japonicus</i> isolate HB1	MW866569.1	1800	100	99.889	2	Unpublished
Koi_2	<i>Argulus japonicus</i> strain CIFRI.E2	OQ660496.1	1739	96.6	99.597	2	Das et al., 2025
Koi_2	<i>Argulus nobilis</i> strain AGGC_215	MH458748.1	1734	96.3	99.885	0	Unpublished
Koi_2	<i>Argulus nobilis</i> proviral 18S ribosomal RNA	M27187.1	1801	100	98.334	25	Abele et al., 1992
Koi_2	<i>Argulus siamensis</i> isolate WBUAFS-A3 18S ribosomal RNA gene	KF583879.1	1688	93.8	98.637	3	Patra et al., 2016
Koi_2	<i>Argulus indicus</i> isolate A1 ERIB-1	MT538301.1	1729	96.1	97.108	38	Amriana et al., 2021

Table 4. Identification of bacteria from koi carp samples and *A. japonicus* using API 20NE

Number	ID sample	Source	Bacteria genus and species	ID percentage (%) using API 20NE
1.	Koi 1L/S	Liver/ Spleen	<i>A. sobria</i>	99.4
2.	Koi 1 L/S	Liver/spleen	<i>P. pneumotropa/Mannheimia haemolytica</i>	90.6
3.	Koi 2	Spleen/Liver/kidney	<i>Nil</i>	–
4.	K1A1	<i>Argulus</i> 1	<i>E. aerogenes</i>	91.9
5.	K1A2	<i>Argulus</i> 2	<i>E. aerogenes</i>	91.9
6.	K1A2	<i>Argulus</i> 2	<i>A. hydrophila/caviae</i>	83.9
7.	K1A3	<i>Argulus</i> 3	<i>A. sobria</i>	99.2
8.	K2A3	<i>Argulus</i> 3	<i>E. aerogenes</i>	91.9
9.	K2A4	<i>Argulus</i> 4	<i>Nil</i>	–
10.	K1A5	<i>Argulus</i> 5	<i>A. hydrophila/caviae</i>	83.9

Note: *ID sample: K- koi fish (1&2); A- *A. japonicus* collected from the body of koi fish sample.

Table 5. Whole-Genome Assembly Statistics and in-silico species identification

Assembly	K2A3_Ea	K1A1_Ea	K1A2_Ah	K1A2_Ea	K1A3_As	K1A5_Ah
Contigs	95	95	39	99	49	39
Largest Contigs	463914	310943	613642	310943	648338	1005985
Total Length	5390544	5391493	4853996	5390693	4492143	4871229
GC (%)	57.66	57.66	58.59	57.66	58.83	58.56
N50	155054	145447	218856	152825	205542	218856
Genome Coverage (x)	285	262	308	227	325	278
Taxonomic Assignment	<i>Klebsiella quasipneumoniae</i>	<i>Klebsiella quasipneumoniae</i>	<i>Aeromonas jandaei</i>	<i>Klebsiella quasipneumoniae</i>	<i>Aeromonas jandaei</i>	<i>Aeromonas jandaei</i>

contigs ranging from 311kb to 1.0 Mb (Table 5), indicating contiguous draft assemblies suitable for downstream comparative genomic and taxonomic analyses.

Based on an *in-silico* genome-genome hybridization approach, three bacterial isolates initially identified as *E. aerogenes* (K2A3_Ea, K1A1_Ea, and K1A2_Ea) using API 20 NE, were later confirmed as *Klebsiella quasipneumoniae* subsp. *similipneumoniae*, with an average nucleotide identity (ANI) value of 99%. On the other hand, isolate *A. sobria* (K1A3_As) and two isolates of *A. hydrophila* (K1A5_Ah and K1A2_Ah) were identified as *Aeromonas jandaei* with an ANI value of 96.7% and genome aligned fraction of at least 0.87.

Virology

The RT-PCR assay showed that the two koi carp samples were negative for TiLV. Although koi carp are considered to have a low risk of developing TiLV, they may still play a role in the potential transmission of the virus to other species, particularly tilapia in a mixed-species environment. On the contrary, Koi carp are highly susceptible to Koi Herpesvirus Disease (KHVD), which is highly contagious and fatal. However, the mortality pattern, clinical signs, and pathology changes observed were likely to be caused by parasitic infestation and secondary bacterial infections. KHVD was not tested due to the unavailability of the test kit at the time of the study.

Virulence factors (VFs) genes of bacteria isolated from *Argulus japonicus*

Two virulent genes, *pscR* and *pscS*, associated with the Type 3 secretion system (TTSS) have been identified from *A. jandaei* isolated from *Argulus* (KA2-Ah and KA5-Ah). These genes are involved in the secretion of effector proteins that contribute to host–bacterium interactions and are considered important virulence-associated determinants in *Aeromonas* species. On the other hand, eight virulent genes were identified from three analysed strains of *K. quasipneumoniae* subsp. *similipneumoniae* from *Argulus* (KA1_Ea, KA2_Ea, K2A3_Ea). They were *ompA*, *entB*, *yagV/ecpE*, *yagW/ecpD*, *yagX/ecpC*, *yagY/ecpB*, *yagZ/ecpA*, and *ykgK/ecpR* (Table 6). It is worth noting that although virulence-associated factors were identified in the *K. quasipneumoniae* subsp. *similipneumoniae* isolates, its contribution to fish mortality requires further confirmation. Members of the *K. pneumoniae* species complex are primarily recognized as opportunistic pathogens of humans and mammals, with limited understanding of their role in aquatic disease. Therefore, its detection in *A. japonicus* internal tissue should be interpreted cautiously, as it may represent an opportunistic colonizer, an environmental associate, or a secondary bacterium introduced following parasite-induced tissue damage rather than a primary fish pathogen.

Table 6. Identification of putative virulence factor (VFs) genes in *A. jandaei* (KA5_Ah and KA2_Ah) and *K. quasipneumoniae* subsp. *similipneumoniae* isolates (K2A3_Ea, KA1_Ea, and KA2_Ea) based on a nucleotide identity threshold of 80% against the VFDB

Sample	CONTIQ ID	START	END	GENE	%ID	ACCESSION
K2A3_Ea	K2A3_Ea_NODE_25_length_85618_cov_111.045218	13070	14140	ompA	83.19	AAF37887
		82530	83110	ykgK/ecpR	86.92	NP_286011
		83184	83767	yagZ/ecpA	90.24	NP_286010
		83829	84497	yagY/ecpB	88.04	NP_286009
		84523	87048	yagX/ecpC	86.9	NP_286008
		87038	88681	yagW/ecpD	88.14	NP_286007
		88607	89360	yagV/ecpE	84.22	NP_286006
	K2A3_Ea_NODE_31_length_59692_cov_117.141206	46812	47663	entB	82.52	NP_752613
KA1_Ea	KA1_Ea_NODE_27_length_85618_cov_102.863036	71479	72549	ompA	83.19	AAF37887
		82530	83110	ykgK/ecpR	86.92	NP_286011
		83184	83767	yagZ/ecpA	90.24	NP_286010
		83829	84497	yagY/ecpB	88.04	NP_286009
		84523	87048	yagX/ecpC	86.9	NP_286008
		87038	88681	yagW/ecpD	88.14	NP_286007
		88607	89360	yagV/ecpE	84.22	NP_286006
	KA1_Ea_NODE_33_length_59692_cov_108.436266	46812	47663	entB	82.52	NP_752613
KA2_Ah	KA2_Ah_NODE_5_length_298782_cov_135.421121	146975	147241	pscS	80.15	NP_250383
		147245	147896	pscR	82.52	NP_250384
KA2_Ea	KA2_Ea_NODE_25_length_85614_cov_87.097852	71479	72549	ompA	83.19	AAF37887
		210324	211077	yagV/ecpE	84.22	NP_286006
		211003	212646	yagW/ecpD	88.14	NP_286007
		212636	215161	yagX/ecpC	86.9	NP_286008
		215187	215855	yagY/ecpB	88.04	NP_286009
		215917	216500	yagZ/ecpA	90.24	NP_286010
		216574	217154	ykgK/ecpR	86.92	NP_286011
	KA2_Ea_NODE_34_length_59692_cov_97.185708	12030	12881	entB	82.52	NP_752613
KA5_Ah	KA5_Ah_NODE_4_length_298782_cov_119.626156	146975	147241	pscS	80.15	NP_250383
		147245	147896	pscR	82.52	NP_250384

On the contrary, the detection of *A. jandaei* isolates is noteworthy, as this species has been reported from aquatic environments and associated with opportunistic infections in fish, although it is less frequently encountered than *A. hydrophila*. The identification of virulence-associated factors suggests potential pathogenic capability and is consistent with previous reports of its association with fish disease. However, its contribution to the observed disease remains to be confirmed, and controlled infection challenge studies are required to establish whether *A. jandaei* acts as a primary causative agent or an opportunistic secondary bacterium facilitated by parasite-induced tissue damage.

In-silico identification of AMR genes

The *ompA* virulence factor gene associated with AMR from three isolates of *A. jandaei* (KA2-Ah; KA3-As; KA5-Ah) showed resistance to carbapenem, colistin, tetracycline, and extended-spectrum beta-lactam antibiotics. Another three isolates (KA1_Ea, KA2_Ea, and K2A3_Ea) identified as *K. quasipneumoniae* subsp. *similipneumoniae*

showed resistance to quinolone, phenicol, fosfomycin, and extended-spectrum beta-lactam antibiotics. The results are shown in Table 7.

DISCUSSION

Water quality problems

Water quality test revealed that koi fish were exposed to low dissolved oxygen (3.47-4.27 mg/L) and slightly high ammonia (0.4-0.45 mg/L), outside the optimal range for koi farming. Koi fish may suffer from an oxygen deficit (hypoxia), particularly at night. During the night, oxygen consumption increases due to respiration by bacteria, algae, or plants, as well as the decay of organic matter. This risk is especially high in stagnant water with elevated organic nutrients (FAO, 2025). Hypoxia reduces fish immune response, making them more susceptible to pathogens or secondary bacterial infections.

Table 7. Antimicrobial resistance (AMR) genes of isolates from *Argulus* sp.

Sample	Contig ID	Start	End	Gene	%Identity	Accession	Resistance
K2A3_Ea	K2A3_Ea_NODE_27_length_81 647_cov_120.525598	3942	5117	oqx A10	94.05	NG_050418.1	PHENICOL; QUINOLONE
		5141	8293	oqx B11	96.13	NG_050429.1	PHENICOL; QUINOLONE
	K2A3_Ea_NODE_6_length_210 898_cov_105.531788	110644	111504	blaOKP B-3	100	NG_049379.1	BETA-LACTAM
	K2A3_Ea_NODE_8_length_198 445_cov_125.922427	174890	175309	fosA_gen	95	NG_047881.1	FOSFOMYCIN
KA1_Ea	KA1_Ea_NODE_22_length_958 04_cov_121.548560	23137	23556	fosA gen	95	NG_047881.1	FOSFOMYCIN
	KA1_Ea_NODE_29_length_816 47_cov_113.732438	73355	76507	oqx B11	96.13	NG_050429.1	PHENICOL; QUINOLONE
		76531	77706	oqx A10	94.05	NG_050418.1	PHENICOL; QUINOLONE
	KA1_Ea_NODE_6_length_2108 98_cov_94.744157	99395	100255	blaOKP B-3	100	NG_049379.1	BETA-LACTAM
KA2_Ah	KA2_Ah_NODE_14_length_152 064_cov_148.459395	140200	140994	blaOXA 12	97.74	NG_049418.1	BETA-LACTAM
	KA2_Ah_NODE_16_length_138 246_cov_139.170545	126524	127275	cphA7	96.01	NG_050400.1	CARBAPENEM
	KA2_Ah_NODE_1_length_6136 42_cov_127.057502	322664	324222	mcr-7.1	84.82	NG_056413.1	COLISTIN
	KA2_Ah_NODE_26_length_375 11_cov_131.777983	31664	33260	mcr-7.1	82.55	NG_056413.1	COLISTIN
	KA2_Ah_NODE_2_length_4596 42_cov_135.384877	15975	17597	mcr-3.8	98.83	NG_055662.1	COLISTIN
		17681	19289	mcr-3.1	84.81	NG_055505.1	COLISTIN
		77190	78338	blaCMY -FOX	94.8	NG_047381.1	BETA-LACTAM
	KA2_Ah_NODE_31_length_103 74_cov_893.821210	6070	7287	tet(E)	99.26	NG_048188.1	TETRACYCLINE
KA2_Ea	KA2_Ea_NODE_27_length_816 47_cov_97.236165	73355	76507	oqx B11	96.13	NG_050429.1	PHENICOL;QUINOLONE
		76531	77706	oqx A10	94.05	NG_050418.1	PHENICOL; QUINOLONE
	KA2_Ea_NODE_5_length_2109 02_cov_82.238435	99395	100255	blaOKP B-3	100	NG_049379.1	BETA-LACTAM
	KA2_Ea_NODE_6_length_1985 62_cov_102.482717	23137	23556	fosA_gen	95	NG_047881.1	FOSFOMYCIN
KA3_As	KA3_As_NODE_16_length_128 725_cov_153.046359	88709	89908	tet(A)	100	NG_048154.1	TETRACYCLINE
		116779	117573	blaOXA 12	96.73	NG_049418.1	BETA-LACTAM
	KA3_As_NODE_19_length_881 21_cov_148.090353	76445	77196	cphA7	96.81	NG_050400.1	CARBAPENEM
	KA3_As_NODE_1_length_6483 38_cov_143.103483	31660	33256	mcr-7.1	82.95	NG_056413.1	COLISTIN
		421405	422551	blaCMY FOX	96.69	NG_047381.1	BETA-LACTAM
	KA3_As_NODE_2_length_3888 44_cov_136.700522	66504	68062	mcr-7.1	85.12	NG_056413.1	COLISTIN
KA5_Ah	KA5_Ah_NODE_13_length_152 064_cov_125.168929	140200	140994	blaOXA 12	97.74	NG_049418.1	BETA-LACTAM
	KA5_Ah_NODE_15_length_138 436_cov_121.035964	10972	11723	cphA7	96.01	NG_050400.1	CARBAPENEM
	KA5_Ah_NODE_1_length_1005 985_cov_111.684061	681758	683316	mcr-7.1	84.82	NG_056413.1	COLISTIN
	KA5_Ah_NODE_25_length_375 11_cov_115.840038	4252	5848	mcr-7.1	82.55	NG_056413.1	COLISTIN
	KA5_Ah_NODE_2_length_4596 42_cov_120.570202	381305	382453	blaCMY FOX	94.8	NG_047381.1	BETA-LACTAM
		440354	441962	mcr-3.1	84.81	NG_055505.1	COLISTIN
		442046	443668	mcr-3.8	98.83	NG_055662.1	COLISTIN
	KA5_Ah_NODE_30_length_103 74_cov_961.828008	5994	7211	tet(E)	99.26	NG_048188.1	TETRACYCLINE

Infestation of *Argulus* parasites and molecular identification

Argulus japonicus in koi is a highly prevalent ectoparasite affecting koi and freshwater fish across Malaysia. Thriving in the country's warm tropical climate, these disc-shaped crustaceans damage scales, causing haemorrhagic anemia, and introduce secondary bacterial infections such as *A. hydrophila* and *A. sobria* from freshwater aquatic environment. *A. japonicus* has been noted in Malaysian ornamental fish culture mainly fish that was imported from international trade compared to *A. foliaceus*, known as non-native parasite. Another species that has been found on red tilapia and reported in Malaysia was *A. coregoni*. This species was found through the importation of red tilapia (Everts & Avenant-Oldewage, 2009). It is important to note that molecular identification of *Argulus* species using 18S rRNA requires careful interpretation and possibly will only maximally benefit from full-length 18S rRNA given the high sequence conservation of the 18S rRNA. However, several *Argulus* 18S rRNA records, including those of *A. foliaceus* and *A. coregoni*, are partial (<1.5 kb) and may not encompass all informative regions required for species-level discrimination. In the present study, the availability of near full-length, highly similar *A. japonicus* reference sequences provided strong support for species assignment. That being said, comparable reference resources, such as near full-length 18S rRNA sequences, are not currently available for all *Argulus* species, limiting comprehensive evaluation across the genus. As additional full-length sequences linked to well-characterized specimens become available, molecular identification of closely related and currently underrepresented or undescribed *Argulus* species will become increasingly robust and better resolved.

Bacterial isolation from Koi fish and *A. japonicus*

Motile *Aeromonas* Septicaemia (MAS) caused by *Aeromonas* spp. (*A. jandaei*/*A. hydrophila*/*A. sobria*) are the cause of serious illness and high mortalities in Koi carp. *A. hydrophila* is by far the most pathogenic and common species isolated from fish, causing MAS and high mortalities (Byadgi et al., 2018). In this case, generalised haemorrhage, fin rot, kidney and liver congestion, and splenomegaly were observed in the infected koi (Koi_1 & Koi_2). The skin haemorrhage was initially caused by *Argulus* bites, and further aggravated by secondary systemic infection of *Aeromonas* spp. brought about by *A. japonicus*. These has been proven from the isolation of *A. sobria* from *Argulus* sample (K1A3) and *A. hydrophila* (K1A2, K1A5) from *Argulus* collected on the body of Koi fish (Koi_1 and Koi_2).

Aeromonas sobria is generally considered an opportunistic pathogen, as not every isolate carries the specific VFs identified in this study to cause an active infection (Majeed et al., 2023). Furthermore, the secretion and expression of VFs often closely related to the growth characteristics of strains. *Aeromonas sobria* causes illness during stressful conditions related to poor water quality, overcrowding, transportation movement stress, new environment, and rough handling (Noga, 2010; Beaz-Hidalgo & Figueras, 2013). Up to now, there is still a lack of relevant research on VFs, growth characteristics, and histopathological characteristics of *A. sobria* infection (De Silva et al., 2018).

Klebsiella quasipneumoniae subsp. *similipneumoniae* is a subspecies of Gram-negative bacteria in the *Klebsiella pneumoniae* complex that has been misclassified (KA1_Ea, KA2_Ea, and K2A3_Ea) in the current standard diagnostic method because it can only be accurately identified through genomic sequencing. *Pasteurella pneumotropica* (now frequently classified as (*Rodentibacter pneumotropicus*) and *Klebsiella quasipneumoniae* subsp. *similipneumoniae* are predominantly mammalian-associated bacteria, hence, isolation of these bacteria from the internal organ of fish (liver and spleen) and *A. japonicus* suggestive of opportunistic contaminants, spillover infections, or environmental indicators rather than primary fish pathogens.

The discrepancies observed between API 20 NE and WGS identifications highlight the limitations of phenotypic identification methods when applied to closely related bacterial taxa. API 20 NE identifies bacteria based on biochemical reactions and metabolic profiles, which may overlap among members of the Enterobacteriaceae and Aeromonadaceae. Consequently, isolates identified as *E. aerogenes* by API 20 NE were subsequently reclassified as *K. quasipneumoniae* subsp. *similipneumoniae* following WGS genome-based analysis. Similarly, *Aeromonas* isolates identified by conventional methods were shown to belong to *A. jandaei*. These findings demonstrate the superior discriminatory power of WGS and emphasize the importance of genomic tools for accurate bacterial identification in aquatic animal health investigations.

VFs determination of bacterial isolates

Aeromonas jandaei infection in fish also known as MAS associated with the *pscR* and *pscS* virulence genes TTSS (Chen et al., 2024). These flexible genes are shared between multiple groups/species that were belong to multiple genera within the phylum Proteobacteria, including *Pseudomonas*, *Klebsiella*, and *E. coli*. Infection with *A. jandaei* in fish is associated with clinical signs such as skin haemorrhage, loss of scales, and ulcers, as well as internal organ damage due to toxin production (Dong et al., 2017).

Outer membrane protein A (ompA) VF is a major component in Gram-negative bacterial cells, which mediate bacterial biofilm formation, eukaryotic cell infections, AMR, and immunomodulation, whereas Enterobactin (entB) VF relates to nutritional and metabolic factors (Smani et al., 2020; Zhou et al., 2023). *E. coli* common pilus (ecp), which is encoded by the *ecpRABCDE* operon, is an extracellular adhesive fibre in association with Enterobacteria of gastrointestinal and urinary tracts that plays a role in early-stage biofilm development and host cell recognition (Saldaña et al., 2014). These VFs identified from *K. quasipneumoniae* subsp. *similipneumoniae* has more concerning effect to human health than to aquatic animals. *P. pneumotropica* or *K. quasipneumoniae* ssp. *pneumonia* is not recognized as the primary pathogen of fish, and no cases have been reported of these bacteria causing infection in koi. This bacterium is predominantly an opportunistic pathogen in rodents and other mammals; thus, it is typically found as part of commensal bacterial flora in aquatic systems and pond water.

Genetic determinants for AMR factors

AMR genes primarily found in *A. jandaei* (KA2_Ah, KA5_Ah, and KA3_As) are *cphA7*, which is associated with carbapenem resistance, whereas *mcr-7.1*, *mcr-3.8*, and *mcr-3.1* confer resistance to colistin; *blaCMY*, *blaFOX*, and *blaOXA-12* are associated with beta-lactam resistance; and *tet(E)* and *tet(A)* are linked to tetracycline resistance (Piotrowska et al., 2017; Dang et al., 2018; McDonald et al., 2024). Results are shown in Table 8. *tet(E)* and *tet(A)* are the most frequently encountered tetracycline resistance genes in various Gram-negative species, including *Aeromonas* (Hedayatianfard et al., 2014). Some authors previously believed that efflux pump genes (*tet(A)*, *tet(D)*, and *tet(E)*) are carried by *Aeromonas* spp. in fish (Dang et al., 2007) and *Vibrio* spp. in the marine environment (Kim et al., 2007).

The *oqxAB* genes are part of an operon that encodes the OqxAB efflux pump, a system commonly found in *K. pneumoniae* and other Enterobacteriaceae that contributes to multidrug resistance. This system primarily confers resistance to fluoroquinolone and phenicols (ciprofloxacin, norfloxacin, and levofloxacin), chloramphenicol, trimethoprim, and nitrofurantoin (Li et al., 2019; Alade et al., 2023). *oqxA10* and *oqxB11* are specific variants (alleles) or genetic variations that have been discovered in both chromosomal and plasmid locations in bacteria belonging to the family Enterobacteriaceae, including *K. pneumoniae*.

The plasmid-mediated gene *mcr-3.1* (and its variants) primarily confers resistance to the 'last resort' antibiotic colistin (polymyxin E) in *Klebsiella* and *Aeromonas* sp. Resistance is achieved by an

enzyme that modifies the bacterial cell membrane, thereby reducing the antibiotic's ability to bind to and kill the bacteria (McDonald et al., 2024). The *mcr-3.1* gene encodes a phosphoethanolamine transferase (MCR-3.1 enzyme) that modifies the lipid, a component of the bacterial lipopolysaccharide (LPS) layer, which is the primary target for colistin. This addition increases the positive charge of the bacterial outer membrane, which reduces the binding affinity of the positively charged colistin antibiotic to the cell surface. The reduced binding prevents colistin from disrupting the membrane integrity and causing cell death, thus conferring resistance to the antibiotic (Sweeney et al., 2020; Mondal et al., 2024).

Prevention and treatment of parasitic and bacterial diseases in Koi carp

The findings of the present study highlight the importance of adopting an integrated disease management approach to reduce disease outbreaks in ornamental koi culture. The mortality event observed in this study was likely influenced by multiple interacting factors, including *A. japonicus* infestation, poor water quality, and opportunistic bacterial infections. Therefore, effective disease prevention should not rely solely on therapeutic interventions but should also emphasize good husbandry practices, routine health surveillance, and environmental management.

Maintaining optimal water quality is one of the most important preventive measures to minimize physiological stress and improve fish resilience against infectious diseases. Regular monitoring of dissolved oxygen, pH, ammonia, nitrite, and temperature, together with appropriate stocking density, adequate aeration, proper nutrition, and regular pond sanitation, can substantially reduce the risk of parasite proliferation and secondary bacterial infections. Newly introduced fish should also undergo quarantine and health screening before being introduced into existing stocks to prevent the introduction of pathogens and parasites.

Treating *A. japonicus* in freshwater aquaculture requires an integrated approach combining chemical treatments, mechanical removal, and pond management, as the parasite has a complex life cycle and can develop resistance to chemicals. Several chemical and non-chemical control strategies have been reported to effectively reduce *A. japonicus* infestations. Potassium permanganate is effective treatment in killing the adult and juvenile stage at a concentration of 10 ppm for 5 hours, or 0.01 g/L, organophosphates eg. Trichlorfon (0.25-0.4 mg/L) or Malathion (0.25-0.5 ppm, 3-4 times weekly). Emamectin Benzoate (SLICE®), as feed medication effective against freshwater fish lice and a 2.5-3% salt bath for 5-10 min can help dislodge parasites. Mechanical and physical control such as substrate removal, pond drying, mechanical removal of *Argulus* on fish body are among several methods that are being used in dealing with this parasite.

Based on the antimicrobial resistance profile observed in the present study, sulphonamides may represent a potential therapeutic option for MAS caused by tetracycline-resistant *Aeromonas* spp. However, antimicrobial therapy should only be initiated following bacterial identification and antimicrobial susceptibility testing, and treatment should be prescribed by a licensed veterinarian or qualified aquatic animal health professional. Farm management should adopt a prevention-first approach by strengthening farm biosecurity, improving water quality, maintaining good hygiene, disinfecting culture facilities, and minimizing environmental stressors. Reducing stocking density, improving nutrition, providing supplements, and increasing aeration can help prevent MAS.

Overall, effective management of koi health should integrate routine water quality monitoring, strict biosecurity practices, early parasite detection and control, and evidence-based antimicrobial therapy. Such an integrated strategy can reduce disease outbreaks while minimizing the emergence of antimicrobial resistance in ornamental aquaculture.

Limitations of study

This study was conducted as a case investigation of a mortality event affecting Japanese koi at AkuaTAR, Fisheries Research Institute, Penang. Only two moribund fish were available for detailed laboratory examination during the outbreak, which limited the sample size and precluded statistical analysis. Consequently, the findings should be interpreted within the context of this specific mortality event and not as representative of all koi populations.

In addition, although Koi Herpesvirus Disease (KHVD) is a significant viral pathogen of koi carp and an important differential diagnosis in mortality investigations, KHVD-specific testing was not available during the study period and therefore could not be performed. As a result, the potential contribution of KHVD to the observed mortality event could not be definitively excluded. Nevertheless, the integration of water quality assessment, parasitological examination, bacteriological analyses, and whole genome sequencing provided valuable insights into the potential factors associated with the observed mortality event.

CONCLUSION

Argulus japonicus is a major, highly destructive, and economically significant ectoparasite in freshwater aquaculture, posing a severe threat particularly to koi and ornamental fish. As blood-feeding parasites, *A. japonicus* attach to fish using suckers, causing irritation, inflammation, scale loss, and extensive skin damage. These lesions can compromise the host's protective barriers and increase susceptibility to opportunistic secondary bacterial infections.

Aeromonas jandaei is an emerging pathogenic bacterium in freshwater aquaculture that may be misidentified as *A. hydrophila* or *A. sobria* using the conventional biochemical methods. In the present case investigation, severe *A. japonicus* infestation, together with poor water quality conditions characterized by low dissolved oxygen and elevated ammonia levels, were identified as a highly probable primary contributing factors to the mortality event. *A. japonicus* may have contributed to disease progression through tissue damage and the potential carriage of bacterial pathogens, thereby facilitating secondary infections by *Aeromonas* spp. However, direct pathogen transmission by *A. japonicus* was not evaluated in this study.

The virulence factors *pscR* and *pscS* associated with the Type III Secretion System (TTSS) were identified in *A. jandaei*/*A. hydrophila*. In addition, antimicrobial resistance genes associated with resistance to carbapenems (*cphA7*), colistin (*mcr-3* variants), β -lactams (*blaCMY*, *blaFOX*, and *blaOXA-12*), and tetracycline [*tet(A)* and *tet(E)*] were detected. As many virulence and resistance determinants are shared among multiple bacterial taxa, accurate species identification can be challenging using conventional methods alone. Therefore, genomic approaches such as DNA sequencing remain valuable tools for the accurate identification and characterization of emerging bacterial pathogens in aquaculture.

ACKNOWLEDGEMENT

The authors would like to express a sincere gratitude and appreciation to Director the Department of Fisheries Malaysia, Dato' Haji Adnan bin Hussain, former Senior Director of the Fisheries Research Institute, Batu Maung (May, 2026), and Director of National Fish Health Research Centre (NaFiH), Dr. Azila Abdullah, as well as the manager and staff at AkuaTAR, FRIBM Penang, who assisted in this study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The raw sequencing data and assembled genome sequences of the bacterial isolates, together with the full-length 18S rRNA gene sequences generated for *Argulus japonicus* molecular identification, have been deposited in the NCBI BioProject database under the accession number PRJNA1493852.

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