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Isolation of *Proteus* sp. Closely Related to *P. Vulgaris* (*Vulgaris*/*hauseri* Complex) from Ornamental Koi (*Cyprinus rubrofuscus*) and its Pathogenicity

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Abstract

Koi (*Cyprinus rubrofuscus*) is a world-famous ornamental fish known for its unique characteristics and economic value. This species is susceptible to various infections, leading to high mortality and morbidity rates. In this study, bacterial pathogens isolated from naturally diseased koi were identified as belonging mainly to the genera *Aeromonas* and *Proteus* through comprehensive characterisation and molecular identification. Experimental infections with *Proteus* sp. closely related to *Proteus vulgaris* (*vulgaris*/*hauseri* complex) showed some clinical signs very similar to those in naturally infected koi, suggesting that *Proteus* sp. in this study is a possible pathogen in addition to *Aeromonas*, indicating a potential co-infection. The median lethal dose (LD50) for *Proteus* sp. was determined to be 1.7×10^8 CFU/mL, with histopathological analysis showing changes such as fusions, lymphocyte aggregation, and necrosis in the kidney and liver. The bacterium was found to be sensitive to various antibiotics, suggesting that broad-spectrum antibiotics may be effective in treating the pathogen. This study represents the isolation of *Proteus* sp. closely related to *P. vulgaris* (*vulgaris*/*hauseri* complex) from koi and provides valuable insights for the prevention and management of related disease outbreaks. The study on bacterial infections in koi fish, particularly the isolation and characterization of *Aeromonas* species alongside *Proteus* sp., highlights significant implications for koi health management. The findings underscore the pathogenicity, potential for coinfection, and the susceptibility of *Proteus* sp. to broad-spectrum antibiotics, providing key understanding for disease outbreak management in ornamental koi populations.

Introduction

The ornamental fish aquaculture has been in favour within the global aquaculture market [1–5] for its attractive features as living jewels that decorate aquariums [6–9] and garden pools for entertainment purposes, such as photography [10–16]. Over 7,000 aquatic species are bred and sold as ornamental fish at present, covering both freshwater and marine ornamental fish [3, 13, 17]. Each year, over one billion freshwater fish from more than 5,300 species are traded internationally as pets, with this sector of aquaculture growing by 14% since the 1970 s [18–21]. *Cyprinus rubrofuscus*, commonly known as Koi carp, Amur carp, "Nishikigoi" in Japan, or "Leekoh" in Malaysia, is a remarkable ornamental fish species distinguished by its distinctive bright colours, pattern varieties, vigorous swimming, and exceptionally high ornamental and commercial values [22]. Koi carp are benthopelagic and adaptable to various water layers,

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including slightly brackish environments, native to East Asia [23].

Although highly valued by aquarists for their vivid coloration and tranquil presence in ponds and water gardens, koi fish remain susceptible to a range of diseases and infections that can markedly affect their health and longevity, often leading to significant morbidity and mortality. Most prevalent diseases from aquaculture farms are commonly caused by bacterial pathogens [4]. *Aeromonas* septicemia, Edwardsiellosis, *Columnaris*, Streptococcosis, and vibriosis are some bacterial fish diseases that have been documented in the aquaculture industry [6]– [7, 24–27]. Meanwhile, *Aeromonas* sp., *Bacillus* sp., *Citrobacter* sp., *Klebsiella* sp., *Proteus* sp., *Edwardsiella* sp., *Flavobacterium* sp., *Providencia* sp., *Serratia* sp., and *Vibrio* sp. have been identified as pathogens in koi carp and can also be isolated from fish that do not show any outward symptoms of disease as opportunistic pathogens [4, 14]. For instance, swim bladder disorder in koi caused by *Aeromonas hydrophila* or the *caviae* group and *Shewanella xiamenensis* exemplifies a bacterial infection that manifests as abnormal buoyancy, lethargy, and fluid accumulation in the swim bladder [28].

In December 2023, more than 100 *C. rubrofuscus* juveniles from a local aquaculture farm in Kuching, Sarawak, Malaysia, were detected with clinical symptoms, possibly caused by disease infections. Since the causative agent or factors for the occurring disease were unknown, this study focused on the isolation of bacterial pathogens from the naturally disease-ornamental koi fish and the pathogenicity of *Proteus* sp. towards the overall health of the fish. The histopathology of infected fish organ tissue caused by *Proteus* sp., which could help in understanding the pathogenicity of the pathogen was also presented.

Materials and Methods

Fish Sampling and Bacteria Isolation

The naturally diseased *C. rubrofuscus* juveniles were obtained live from an aquaculture farm in Kuching, Sarawak, Malaysia, and were transferred to the laboratory in sterile plastic bags provided with oxygen. From the total sample of eight, a subset of fish ($n=3$) was euthanized and dissected for analysis. The internal organs (Intestine and kidney) were aseptically removed and homogenised under aseptic conditions.

The homogenized samples were aseptically diluted and inoculated onto *Aeromonas* isolation agar containing ampicillin (0.5 mg/mL). The cultured mediums were then incubated overnight for 18 h at 25 °C. Single dominant bacterial colonies were randomly selected from the cultured mediums, purified, and preserved in a 40% glycerol solution at −80 °C.

Morphological and Biochemical Characterization

Gram staining protocols were carried out for the morphological characterization of the isolates, observed under 1,000 × magnification with oil immersion. The isolates were also biochemically and physiologically characterized by using the VITEK® 2 Gram-Negative identification card (GN) that includes 47 biochemical tests based on the VITEK® 2 Systems (bioMérieux, France).

Molecular Identification

The DNA extraction was done using the TransGen EasyPure Genomic Extraction Kit according to the manufacturer's protocol. The extracted DNA was quality checked via agarose gel electrophoresis and quantified using a Nanodrop UV spectrophotometer prior to polymerase chain reaction (PCR). The 16S rRNA gene of the bacterial isolates was amplified by PCR using specific primers and subsequently visualized. The following procedure was then performed for the PCR amplification: Initial denaturation for three minutes at 94 °C, followed by 35 cycles of denaturation for 30 seconds at 94 °C, annealing for 30 seconds at 55 °C, extension for one minute at 72 °C, and final extension for 5 minutes at 72°C. The forward primer, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') (Integrated DNA Technologies (IDT), USA), and the reverse primer, 1492R (5'-CTACGGCTAC CTTGTTACGA-3') (Integrated DNA Technologies (IDT), USA) were used [29]. PCR products obtained were subjected to agarose gel electrophoresis, using a 1.5% agarose gel stained with ethidium bromide for visualization. PCR product purification was performed with the PrimeWay Gel Extraction/PCR Purification Kit (1st BASE, Singapore), and the purified PCR products were sent to Apical Scientific Sdn. Bhd for 16S rRNA sequencing. The sequencing results were analyzed by using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast) through the National Center for Biotechnology Information (NCBI) website. The MEGA12: Molecular Evolutionary Genetics Analysis Version 12 software was also used to construct multiple 16S rRNA sequencing alignments and a phylogenetic tree with other closely related bacterial species alongside 1,000 bootstrap replications.

Scanning Electron Microscopy

Scanning electron microscopy analysis was carried out for the confirmed isolated bacteria, I1_3. The bacteria stored in glycerol were revived and grown in lysogeny broth (LB)

medium, incubated for 16 h. The bacterial suspension was centrifuged at 8,000 rpm for five minutes to obtain bacterial pellets. The bacterial pellets collected were rinsed with phosphate-buffered saline (PBS) twice, followed by fixation with 4% paraformaldehyde (PFA) and incubation for six hours at 4 °C. To remove the fixative, the bacterial pellets were washed three times with PBS. Lastly, dehydration was done on the bacterial pellets using graded ethanol series solutions at 30%, 50%, 80% and 100% was performed, followed by an air-drying process. The sample was then viewed under the scanning electron microscope JSM-IT500HR (Jeol, Peabody, MA, USA). Dried specimens were mounted on aluminum stubs and sputter-coated with a ~ 10 nm layer of gold or gold-palladium using a sputter coater to increase conductivity and prevent charging during imaging. SEM imaging was conducted at an accelerating voltage of 10–15 kV, which provided optimal resolution and minimized sample damage. These conditions allowed for detailed surface morphology analysis without compromising sample integrity.

Experimental Infection with I1_3 Isolate

A total of 60 healthy juvenile *C. rubrofuscus* at an average weight of 22 ± 1 g and a length of 13 ± 1 cm was obtained from the same local aquaculture farm in Kuching, Sarawak. The fish were acclimatised for seven days prior to the experimental injection at 25 ± 1 °C with oxygen supply, and fed once daily.

The I1_3 isolate was subjected to an experimental median lethal dose (LD_{50}) assay. Single colonies of the I1_3 isolates grown on LB agar were inoculated in LB broth and incubated for 16 h. The bacterial suspension was centrifuged at 8,000 rpm for 10 min, and the harvested bacterial pellets were washed and resuspended in PBS. The bacteria concentrations for the assay were then prepared, at 6.1×10^8, 2.6×10^8, 1.3×10^8, 9.8×10^7, and 5.5×10^6 CFU/mL, respectively, with a dosage volume of 200 µL per fish. The bacterial concentrations (CFU/mL) were determined by plate counting of the colony-forming units (CFU) through serial dilution prior to inoculation. The 60 fish were randomly divided into ten fish per tank, each group injected intraperitoneally with the five bacterial concentrations listed, along with one control group injected with PBS. Clinical symptoms and cumulative mortalities of the treatment and control groups were monitored over a seven-day period. Fish mortalities were recorded at a 24-hour interval, and the LD_{50} value was calculated based on the data recorded using the Probit analysis [30].

Koch's Postulates

Ten healthy juvenile *C. rubrofuscus* (average weight: 22 ± 1 g; length: 13 ± 1 cm) were maintained at 25 ± 1 °C and acclimatized for 7 days prior to conducting Koch's postulates. The preserved isolated I1_3 strain was revived in LB broth and incubated at room temperature for 16 h. The bacterial suspension of 4.8×10^{14} CFU/mL was prepared and inoculated via intraperitoneal injection at 200 µL per fish. The bacterial concentration was verified using the plate-counting method as described. Mortalities and clinical symptoms were monitored throughout the trial. Bacteria were subsequently re-isolated from the intestine and kidney of the experimentally infected fish, and molecular identification was carried out based on 16S rRNA gene sequencing.

Histopathology Assay

Both control and infected fish were collected and dissected. The kidney and liver tissue samples were collected and fixed in 4% PFA at 4 °C for 72 h. The tissue samples were then rinsed and dehydrated in a graded sucrose series of 15% and 30% sucrose, then embedded in agar-sucrose solution [31]. Cryostat sectioning of the tissue samples was conducted by using the LETICA/CM1850 UV microtome (Leica Biosystems, Deer Park, United States). The sectioned tissue samples were stained with hematoxylin and eosin (H&E) before viewing under a compound light microscope.

Antibiotic Susceptibility Analysis

Antibiotic susceptibility testing was performed for *P. vulgaris* with the VITEK® 2 Compact system (bioMérieux, France). Antibiotics tested on the bacteria were: Ampicillin, ampicillin/sulbactam, piperacillin/tazobactam, cefuroxime, cefuroxime axetil, cefotaxime, ceftazidime, ceftriaxone, ceftazidime/avibactam, ceftolozane/tazobactam, cefepime, doripenem, ertapenem, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, trimethoprim/sulfamethoxazole.

Data Analysis

The cumulative mortalities of the infected fish recorded during experimental infection were further analysed by running the One-Way Analysis of Variance (ANOVA) test to confirm that the mean data of the five treatment groups to be statistically different, followed by Tukey's Honestly Significant Difference (HSD) test to understand the significant difference between the mean values of the groups. Both tests were conducted using the software Python, Jupyter notebook.

Results

Bacteria Isolation and Identification

Clinical symptoms exhibited by the naturally infected *C. rubrofuscus* samples were imbalanced swimming with "sleepy" behaviour, lethargy, haemorrhages, loss of scales, and bruised-looking skin discolouration (Fig. 1a). Bacteria colonies isolated from the intestine appeared to be opaque and translucent green with a dark centre, convex and round shaped; opaque yellow or white, irregular and round shape; and opaque yellow colour, opaque orange colour, convex, and round shape. For the kidney samples, the bacterial colonies were opaque dark green, convex, and round-shaped. A swarming growth phenomenon was noted in one of the purified bacterial isolates. To confirm the swarming growth phenomenon, the bacterial isolate was further subcultured on LB agar, resulting in swarming colonies portraying an obvious concentric 'bull's-eye' swarming pattern (Fig. 1b).

Gram staining of the bacterial isolates all revealed them to be red and pink-stained, rod-shaped, and arranged in single (Fig. 1c), thus confirming the isolates to be Gram-negative bacteria. The results for the biochemical properties of the isolates tested are listed in Table 1. The bacterial isolates were revealed to be under the *Aeromonas* and *Proteus* genus, having the probability percentage ranging under the confident level of excellent category, which were 97%–99% (Table 2). The size of the 16S rRNA gene sequence for all samples was approximately 1,500 bp, complementing the expected amplicon size of the universal 16S primers used. The bacterial isolate species detected consisted of four types, including a few dominant species from the *Aeromonas* genus and one species belonging to the *Proteus* genus (Table 3). The first five hits of the bacteria isolate I1_3 from BLAST NCBI were: *Proteus* sp. NT_S71F (PQ119108.1),

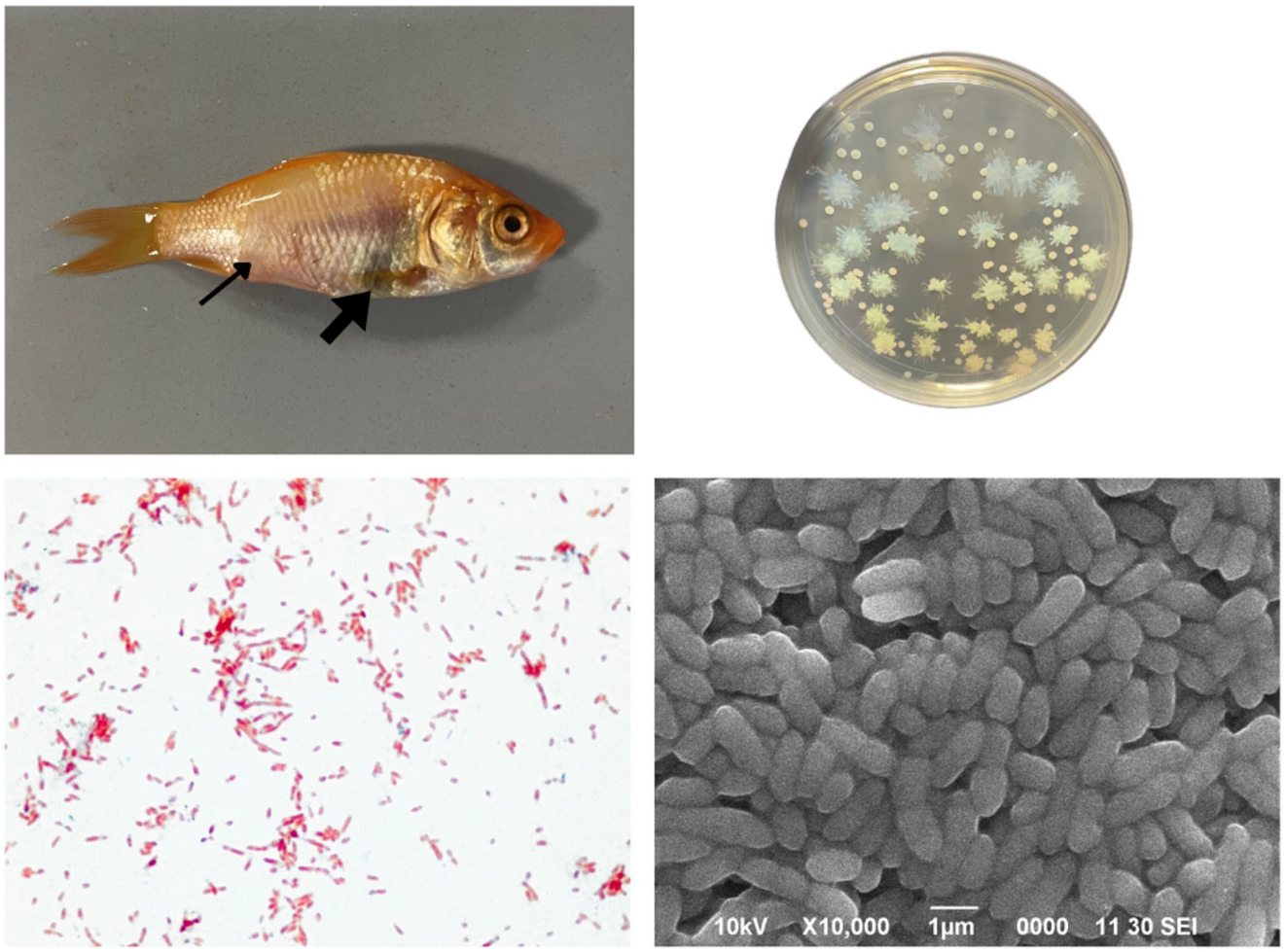

Fig. 1 Bacterial isolation from diseased *C. rubrofuscus*. **a** Clinical signs of the infection: Loss of scales (thin arrow) and bruised-looking skin discolouration (thick arrow). **b** Subcultured bacterial colonies showing swarming growth phenomenon after culturing on LB agar for 48 h. **c** Gram staining properties of the purified bacterial isolate under light microscope with 1,000 × magnification. **d** Scanning electron microscope observation of the bacterial cells at 10,000 × magnification

Table 1 Biochemical test results provided by VITEK® 2 Gram-Negative identification card (GN) (bioMérieux, France)

Well	Mnemonic	Bacteria isolates							
		I1_1	I1_3	I2_1	K1_1	K1_3	I3_1	I3_3	K3_4
2	APPA	-	-	-	+	-	+	-	+
3	ADO	-	-	-	-	-	-	-	-
4	PyrA	-	-	-	-	-	-	-	-
5	IARL	-	-	-	-	-	-	-	-
7	dCEL	-	-	-	-	-	+	-	-
9	BGAL	+	-	+	+	+	+	+	+
10	H2S	+	+	+	-	+	+	+	+
11	BNAG	+	-	+	+	+	+	+	+
12	AGLTp	-	-	-	-	-	-	-	-
13	dGLU	+	+	+	+	+	+	+	+
14	GGT	-	+	-	+	-	-	-	-
15	OFF	+	+	+	+	+	+	+	+
17	BGLU	+	-	+	-	+	+	-	-
18	dMAL	+	+	+	+	+	+	+	+
19	dMAN	+	-	-	+	+	+	+	+
20	dMNE	+	-	+	+	+	+	+	+
21	BXYL	-	-	-	-	-	-	-	-
22	BAIap	-	-	-	-	-	-	-	-
23	ProA	-	-	+	+	-	+	-	-
26	LIP	+	-	+	-	+	+	+	+
27	PLE	-	+	-	+	-	-	+	+
29	TyrA	-	+	-	+	-	+	-	-
31	URE	-	+	-	-	-	-	-	-
32	dSOR	-	-	-	-	-	-	-	-
33	SAC	-	+	+	+	+	+	+	+
34	dTAG	-	-	-	-	-	-	-	-
35	dTRE	+	-	+	+	+	+	+	+
36	CIT	-	-	-	+	-	-	-	-
37	MNT	-	-	-	-	-	-	-	-
39	5KG	-	-	-	-	-	-	-	-
40	ILATk	+	+	+	-	+	+	+	+
41	AGLU	-	+	-	-	-	-	-	-
42	SUCT	+	+	+	+	+	+	+	+
43	NAGA	-	-	-	-	-	-	-	-
44	AGAL	-	-	-	-	-	-	-	-
45	PHOS	-	+	-	-	-	-	-	-
46	GlyA	-	-	-	+	-	+	-	-
47	ODC	-	-	-	-	-	-	-	-
48	LDC	-	-	-	-	-	-	-	-
53	IHISa	-	-	-	-	-	-	-	-
56	CMT	-	+	+	+	-	+	+	+
57	BGUR	-	-	-	-	-	-	-	-
58	O129R	+	+	+	-	+	+	+	+
59	GGAA	+	+	+	+	+	+	+	+
61	IMLTa	-	-	-	-	-	+	-	-
62	ELLM	+	+	+	+	+	+	+	+
64	ILATa	-	-	-	-	-	-	-	-

Proteus sp. PYCC 8249 (MN510816.1), *P. vulgaris* T3-41 (KC210828.1), *P. vulgaris* LZH-W8 (MW493218.1), and *P. vulgaris* BLPS10 (ON460269.1). In the 16S rRNA gene Neighbor-Joining tree (Maximum Composite Likelihood, transitions + transversions; 1,000 bootstrap replicates), isolate I1_3 clustered with *Proteus* sp. ATCC 51,470 with strong bootstrap support (100%). However, the relatively long terminal branch for I1_3 indicates 16S divergence from the closest reference, suggesting it may represent a distinct lineage or strain-level variant (Fig. 2). Besides, the

Table 2 Organism detection results provided by VITEK® 2 Gram-Negative identification card (GN) (bioMérieux, France)

Bacteria isolates	Organism detected	Probability	Bionumber	Confidence level
I1_1	*Aeromonas hydrophila/punctata* (*caviae*)	98%	0435712040500031	Excellent identification
I1_3	*Proteus hauseri*	99%	0017204310740231	Excellent identification
I2_1	*Aeromonas hydrophila/punctata* (*caviae*)	98%	0435313050500231	Excellent identification
K1_1	*Aeromonas veronii*	99%	1,427,615,151,401,221	Excellent identification
K1_3	*Aeromonas hydrophila/punctata* (*caviae*)	98%	0435712050500031	Excellent identification
I3_1	*Aeromonas hydrophila/punctata* (*caviae*)	97%	1,635,713,150,501,271	Excellent identification
I3_3	*Aeromonas hydrophila/punctata* (*caviae*)	98%	0435616050500231	Excellent identification
K3_4	*Aeromonas hydrophila/punctata* (*caviae*)	98%	1,435,616,050,500,231	Excellent identification

Table 3 BLASTn search results from the 16 S rRNA gene sequences of the bacterial isolates

Isolate	Microorganism detected	Score	E Value	Query cover	Percentage Identity
I1_1	*Aeromonas hydrophila* 86	2156	0.0	97%	96.31%
I1_3	*Proteus* sp. NT_S71F	2054	0.0	100%	96.46%
I2_1	*Aeromonas hydrophila* CH-GX-LZ-HJX-3–2021	2176	0.0	95%	97.75%
K1_1	*Aeromonas caviae* S5-5	2165	0.0	96%	96.53%
K1_3	*Aeromonas hydrophila* CH-GX-YL-BL-1–2021	2191	0.0	99%	96.43%
I3_1	*Aeromonas* sp. NT_S124F	2198	0.0	99%	96.73%
I3_3	*Aeromonas* sp. NT_S65F	2202	0.0	100%	96.40%
K3_4	*Aeromonas* sp. SDT13	2141	0.0	98%	95.90%

I1_3 isolate viewed under the scanning electron microscope (SEM) confirmed that the bacterial cells were rod-shaped, exhibiting a relatively smooth and intact surface morphology (Fig. 1d). The smooth outer surface and absence of visible ruptures or surface irregularities suggest that the cells were in a healthy physiological state, with no apparent damage to the cell wall or membrane structure .

Experimental Infection with the I1_3 Isolate, Koch's Postulates, and Histopathology

The cumulative mortality rate of the infected fish across seven days is outlined in Table 4 ($p < 0.01$), and all the mean values between the five treatment groups compared with the control group revealed that there was a significant difference between the groups, except for treatment group 5. The median lethal dose (LD_{50}) of the bacteria towards the fish using the Probit analysis method was 1.7×10^8 CFU/mL (Fig. 3) [30]. In comparison with the control juvenile *C. rubrofuscus*, the fish artificially injected with the bacteria exhibited various clinical symptoms and varied between different bacterial concentration loads. For low bacterial concentration load, the infected fish showed eye lesions, loss of scales, and the body covered with a mucous layer (Fig. 4a and b). The fish injected with high bacterial concentration load experienced more severe symptoms, including the red vent syndrome, dropsy, haemorrhages, pale gills, and intestinal bloat (Fig. 4c and d).

Following inoculation of the isolated *P. vulgaris* strain into healthy juvenile *C. rubrofuscus*, the fish developed clinical signs consistent with the described disease within 24 h post-infection. A cumulative mortality of 100% was recorded over a 7-day observation period. Bacterial isolates recovered from the intestines of experimentally infected fish showed the top two BLAST hits as *Proteus* sp. NT_S71F (PQ119108.1) and *P. vulgaris* T3-41 (KC210828.1) based on 16 S rRNA gene sequence analysis, which satisfied Koch's postulate. The isolate had high sequence similarity and is therefore revealed to be closely associated with *P. vulgaris*. The tissues within the organs of diseased *C. rubrofuscus* had resulted in severe damage due to bacterial infection. The kidney tissues were affected, showing basophilic clusters and dilated renal tubules (Fig. 5a and b). Infected liver cells were observed to have lymphocytic aggregation occurring with the necrotic hepatic cells. (Fig. 5c and d).

Antibiotic Susceptibility Analysis

Based on the results from this study, the isolate I1_3 is resistant to three antibiotic groups (namely ampicillin, ampicillin/sulbactam, cefuroxime, cefuroxime axetil, ceftriaxone, and imipenem) out of the seven examined, while being susceptible to the remaining antibiotics. The results were summarized in Table 5.

Discussion

By performing characterisation based on the morphology, physiology, biochemical characteristics, and molecular identity, the bacterial isolates in this study were identified as belonging to the *Aeromonas* and *Proteus* genera, namely *Aeromonas* sp., *A. hydrophila*, *Aeromonas caviae*, and *Proteus* sp. Although *Aeromonas* spp. is frequently reported as

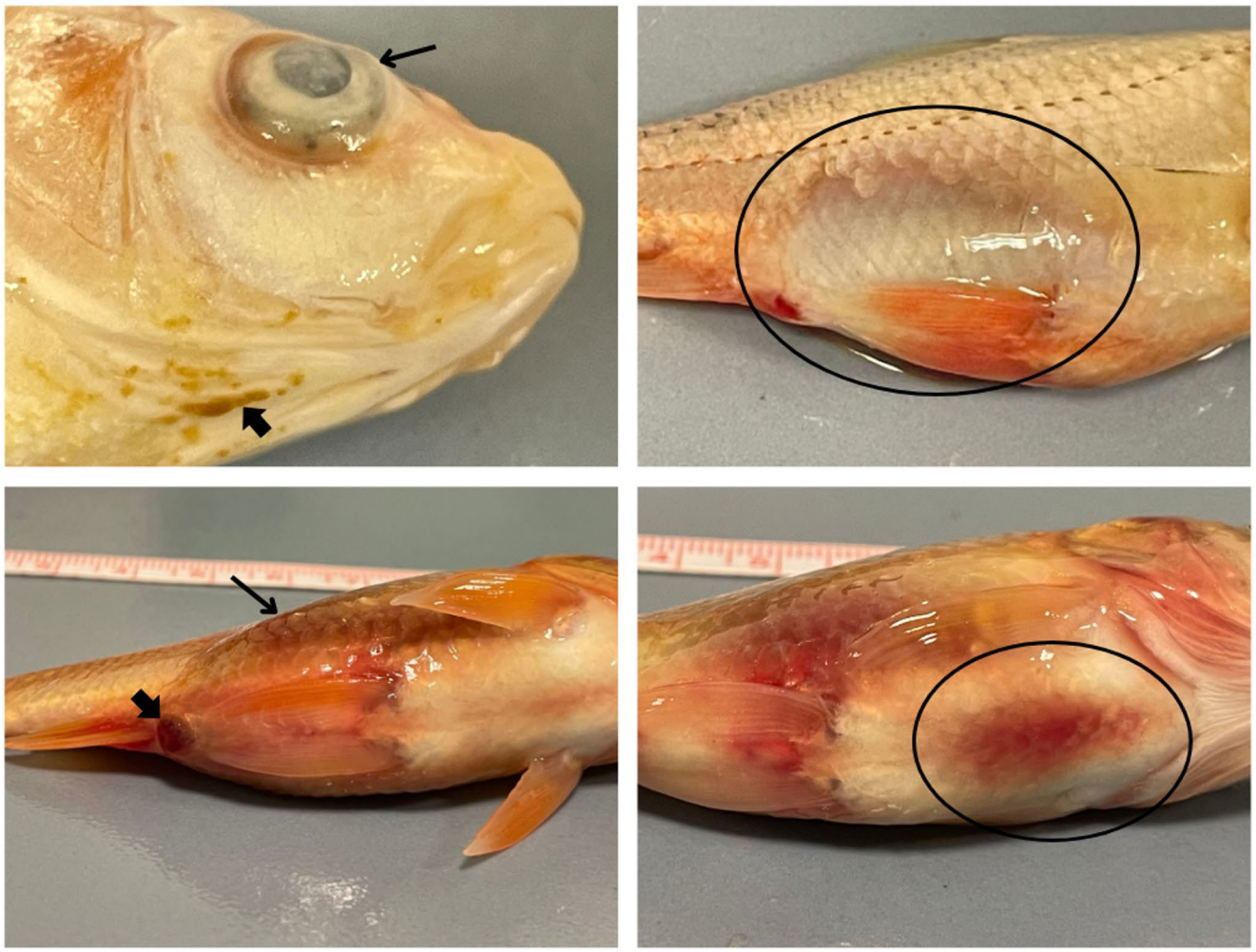

Fig. 2 Neighbor-Joining phylogenetic tree based on 16 S rRNA gene sequences of *Proteus* type strains, constructed using the Maximum Composite Likelihood (MCL) model with both transition and transversion substitutions. Bootstrap analysis was performed with 1,000 replicates, and the percentage of replicate trees in which the associated taxa clustered together is shown next to the branches. *Escherichia coli* NBRC 102,203^{T}, *Morganella morganii* ATCC35200^{T}, and *Morganella morganii* NBRC 3848^{T}, were used as outgroups. The scale bar represents 0.01 nucleotide substitutions per site

Table 4 Cumulative mortalities of infected fish with the I1_3 isolate

Group	Bacterial concentration (cfu/mL)	Dose (µL)	Fish number	Number of deaths at each time interval			Mortality (%) (*p*-value)
				24 h	48 h	3–7 days	
1	6.1×10^8	200	10	10	10	10	100 (<0.01)
2	2.6×10^8	200	10	4	4	5	50 (<0.01)
3	1.3×10^8	200	10	1	2	2	20 (<0.01)
4	9.8×10^7	200	10	1	1	1	10 (<0.05)
5	5.5×10^6	200	10	0	0	0	0 (no significant difference)
Control	1 × PBS	200	10	0	0	0	0

a predominant bacterium in carp culture systems, and its infections have been observed across a wide range of ornamental species including *Carassius auratus* and *Cyprinus carpio*, other bacterial species may also contribute significant roles in disease development [32, 33]. *Proteus* spp. are recognised as opportunistic pathogens in humans and animals, commonly isolated from healthy hosts without clinical symptoms [34]. However, recent studies have reported an increasing incidence of *Proteus*-associated pathology in both humans and animals, including active infections in fish

Fig. 3 Graph of log dose versus probits for LD_{50} calculations of the bacteria in juvenile *C. rubrofuscus* with 95% CI upper and lower bound

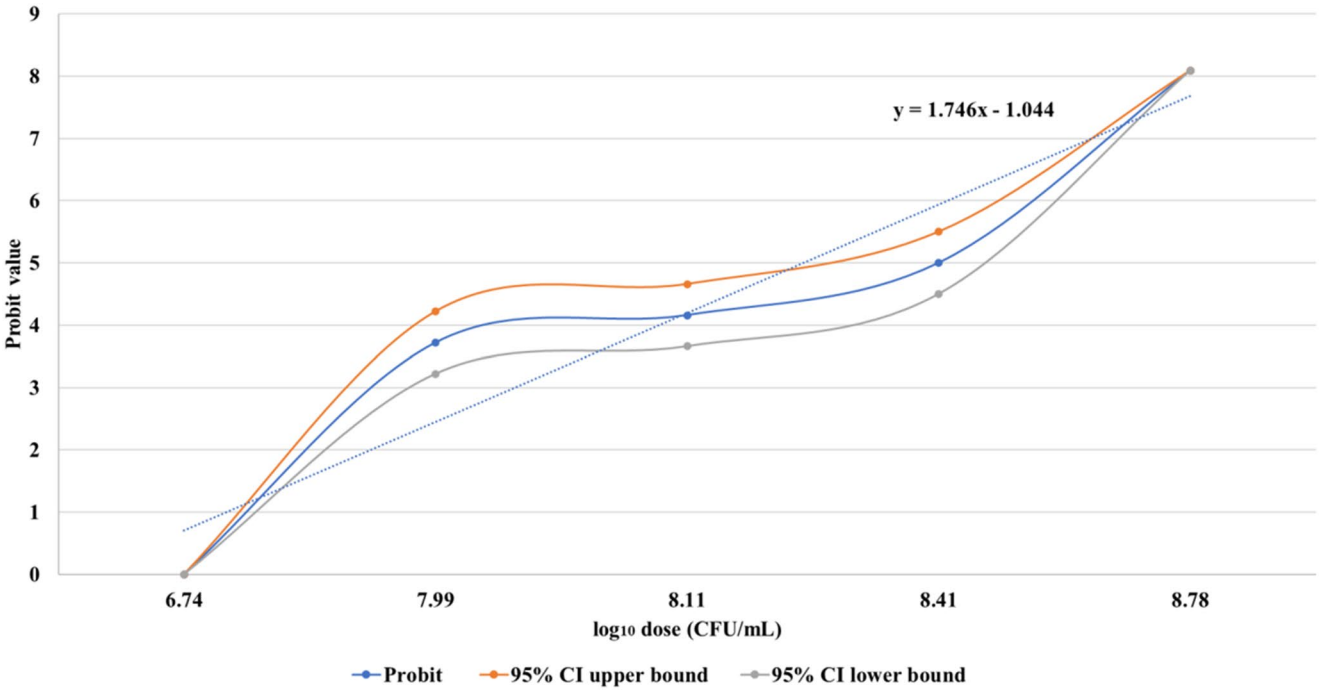

Fig. 4 Clinical symptoms exhibited on *P. vulgaris* infected fish: **a** Eye lesion (thin arrow) and appearance of mucous layer (thick arrow), **b** Loss of scales (black circle), **c** Red vent syndrome (thick arrow) and dropsy (thin arrow), **d** Haemorrhages (black circle)

species such as *C. carpio*, *Labeo rohita*, and *Pangasianodon hypophthalmus* [14, 34–38]. The occurrence of *Proteus* with *Aeromonas* is also noteworthy, as both species are reliable indicators of water quality, suggesting that the observed infections may be linked to environmental stressors such as poor water quality and faecal contamination [39, 40].

The 16S rRNA sequencing results showed that the bacterial isolate I1_3 had close matches to both *Proteus* sp. and *P. vulgaris* sequences. The top hits included two *Proteus* sp. strains, followed by several *P. vulgaris* strains. This pattern indicates that the isolate is genetically very similar to *P. vulgaris*, but some reference sequences in the database are still classified under the broader genus *Proteus* without a specific species designation. Therefore, the isolate I1_3 can be considered closely related to *P. vulgaris*, and the appearance of *Proteus* sp. in the top hits likely reflects incomplete or

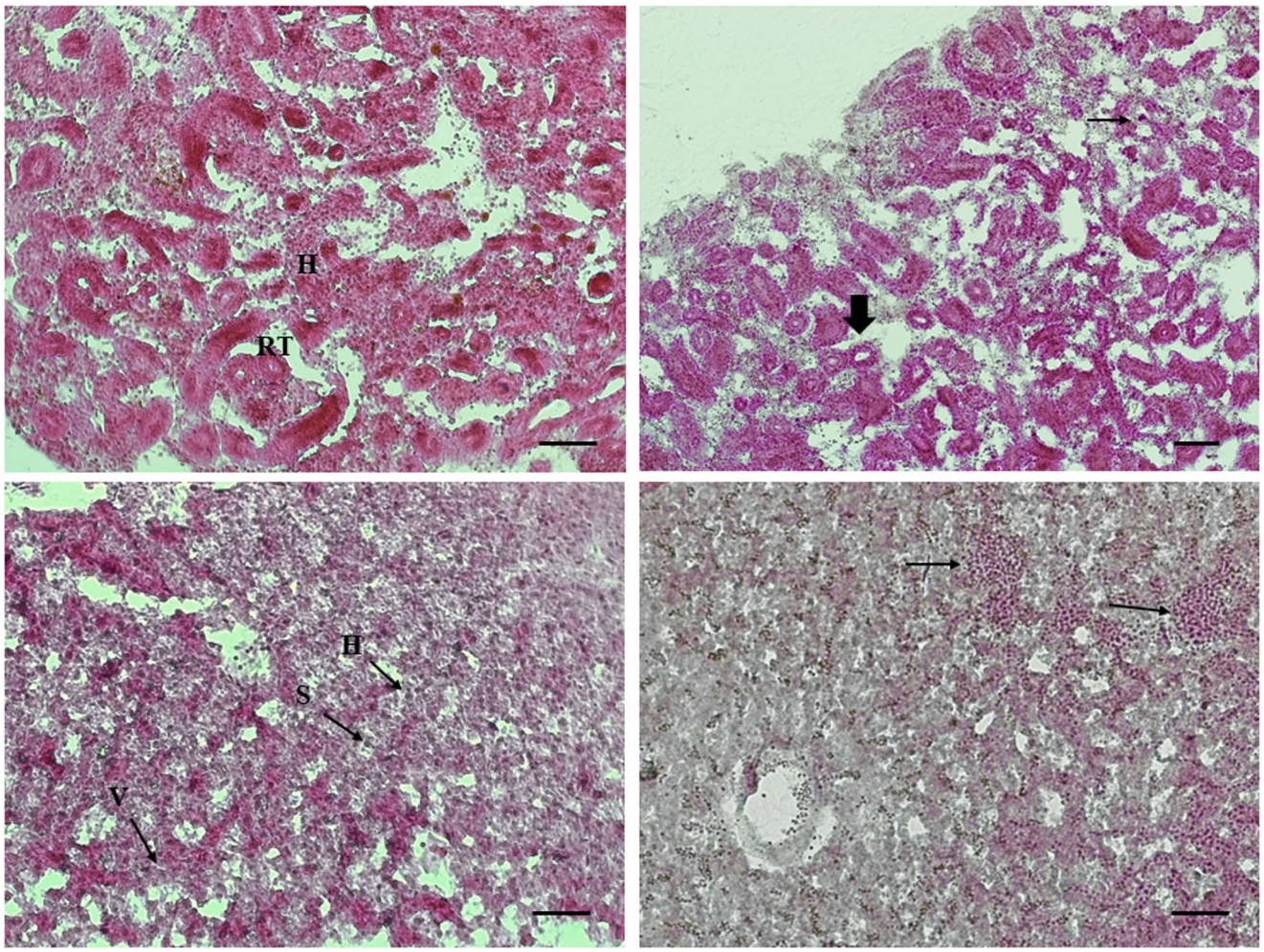

Fig. 5 Photomicrograph of control and infected fish organs, 400 × , H&E. Scale bar: 50 µm. **a** Control kidney cells consisting of renal tubules (RT) and an abundance of hematopoietic tissues (H) at surrounding, **b** Infected kidney tissues showing basophilic cluster (thin arrow) and dilated tubule (thick arrow), **c** Control liver tissues with a normal distribution of vein (V), hepatic cells (H) and sinusoid (S), and **d** Infected liver tissues showing lymphocytic aggregation with necrotic hepatic cells (arrow)

provisional species identification in the reference database rather than a distinct species difference. Within the phylogenetic tree, isolate I1_3 is grouped as a well-supported sister taxon to *Proteus* sp. ATCC 51,470 (MG269477.1) with a bootstrap value of 100. Despite this strong support, the relatively long branch lengths indicate that I1_3 is genetically divergent from *Proteus* sp. ATCC 51,470 (MG269477.1) at the 16 S rRNA locus. To further clarify the taxonomic position and evolutionary relationship of isolate I1_3, additional analyses such as multilocus sequence analysis (MLSA) using selected housekeeping genes are recommended for future work, as these genes provide higher resolution for distinguishing closely related *Proteus* species [41]. The biochemical tests conducted using the Gram-negative identification card detected the isolate as 99% *Proteus hauseri*. Comparing these results with O'Hara et al.'s findings reveals consistent biochemical profiles between *P. hauseri* and *P. vulgaris* [42]. Specifically, both species showed similarities in lysine decarboxylation, urease production, fermentation of glucose, mannose, maltose, and sucrose, tyrosine clearing, ornithine, lipase production, and citrate utilisation, indicating that *P. hauseri* and *P. vulgaris* share similar biochemical characteristics. Collectively, the molecular and biochemical evidence support the conclusion that *Proteus* sp. isolate I1_3 is closely related to *P. vulgaris*, likely belonging to the *P. vulgaris/hauseri* complex.

In the challenge experiment, a high number of mortalities occurred in the first 24-hour interval after the injection assay, and symptoms such as haemorrhages and imbalanced swimming were exhibited during the first 12 h. The trend gradually decreased for the next few days until no fish mortality was observed. Meanwhile, the control group had no mortalities or clinical signs. Compared to Sun et al., where early mortalities were lower, the faster disease progression

Table 5 Antimicrobial susceptibility analysis report consisting of 19 antibiotics tested

Antimicrobial agents	Minimum Inhibitory Concentration (MIC)	Interpretation
Penicilins		
Ampicillin	≥ 32	Resistant
Ampicillin/Sulbactam	N/A	Resistant
Cephalosporin		
Ceftazidime	≤ 0.1	Susceptible
Cefotaxime	32	Resistant
Cefepime	≤ 0.1	Susceptible
Ceftriaxone	N/A	Resistant
Cefuroxime	≥ 64	Resistant
Cefuroxime Axetil	≥ 64	Resistant
β-lactam combination agents		
Piperacillin/Tazobactam	≤ 4	Susceptible
Ceftazidime/Avibactam	N/A	Susceptible
Ceftolozane/Tazobactam	N/A	Susceptible
Carbapenems		
Doripenem	N/A	Susceptible
Ertapenem	≤ 0.1	Susceptible
Imipenem	8	Resistant
Meropenem	≤ 0.3	Susceptible
Fluoroquinolones		
Ciprofloxacin	0.3	Susceptible
Aminoglycosides		
Amikacin	≤ 1	Susceptible
Gentamicin	≤ 1	Susceptible
Others		
Trimethoprim/Sulfamethoxazole	≤ 20	Susceptible

in this study may reflect strain-specific virulence or heightened host susceptibility [35]. Several symptoms, including excessive mucus production, red vent syndrome, and haemorrhagic lesions, were consistent with those observed in *P. vulgaris*-infected common carp, indicating that isolate I1_3 is capable of causing active infections [35]. Furthermore, the clinical symptoms exhibited differently in the artificially diseased fish between different bacteria concentration loads, including eye lesions, loss of scales, and a body covered with mucous layer in low bacteria concentration load; red vent syndrome, dropsy, haemorrhages, pale gills, and intestinal bloat for higher bacteria concentration load. Despite the differences, the clinical signs of scale loss and haemorrhages aligned with the symptoms shown in the naturally infected koi. *C. rubrofuscus* injected with the I1_3 isolate also manifested severe histopathological changes. The artificially infected koi fish that showed necrosis occurring in the kidney haematopoietic cells reflected similarly in the kidney tissues of common carp infected with *P. vulgaris* reported by Sun et al.; as well as the liver tissues that resulted in lymphocytic aggregation similar to the symptoms shown in the study by Abdelhamed et al. about experimentally infected channel catfish with virulent *A. hydrophila* [1, 35]. Thus, the histopathological symptoms caused by the I1_3 isolate indicate its potential to induce tissue damage in koi.

The LD_{50} for *A. hydrophila* in koi was previously reportedly to be 2×10^7 CFU/mL, indicating a slightly higher virulence compared to the I1_3 isolate [43]. The LD_{50} findings of the *Proteus* isolate in this study align with previous observations of *A. hydrophila* consistently causing more severe disease outcomes than *Proteus* [44]. This established pattern supports the observation that the I1_3 isolate exhibits lower pathogenicity, reinforcing the interpretation that *Proteus* functions primarily as an opportunistic pathogen. However, although intraperitoneal injection is practical for LD_{50} assays, it does not fully replicate natural exposure routes such as immersion challenges [45]. This limitation should be considered when interpreting virulence and disease progression in experimental trials. By incorporating immersion-based challenge models that closely simulate environmental exposure in aquaculture settings, future studies can possibly achieve greater ecological realism and strengthen the translational impact of the findings.

The successful re-isolation and molecular identification of *Proteus* sp. from the intestine of artificially infected *C. rubrofuscus* confirm that the isolate persists in the host and is capable of inducing pathological effects, fulfilling Koch's postulates [46]. While these findings establish *Proteus* as a pathogen in koi, its lower virulence compared to *Aeromonas* indicates a potential opportunistic role in disease progression, as mentioned. The coexistence of these two bacterial genera raises the possibility of coinfection, which may influence host outcomes, including immune response, survivability, clinical signs, and the effectiveness of disease management. Supporting this, a recent study investigating single and co-infections of *Proteus mirabilis* and *A. hydrophila* in *Clarias gariepinus* reported an antagonistic interaction between the two species during co-infection, showing that interspecies bacterial interactions can modulate disease severity and outcome [44]. Future studies should systematically compare single infections and co-infections with *Aeromonas* to clarify the opportunistic role of *Proteus*, evaluate potential synergistic or antagonistic interactions, and understand the impact on disease progression. These studies will not only showcase the dynamics of bacterial coinfections but also lead to the development of efficient disease management and intervention techniques in ornamental fish cultivation.

Antibiotic susceptibility tests are crucial for identifying effective treatments for certain infections. The *Proteus* sp. isolates exhibit high resistance to ampicillin, piperacillin, cefoxitin, and trimethoprim/sulfamethoxazole, while conversely the highest susceptibility is levofloxacin and norfloxacin [47]. Ampicillin, ampicillin/sulbactam, cefuroxime,

cefuroxime axetil, ceftriaxone, and imipenem were found to be resistant towards the I1_3 isolate, and this aligns and adds up to the data by Sun et al., where the *P. vulgaris* strain CQC01 was reportedly resistant to cefazolin, cefuroxime, and ampicillin only [35]. However, the strain CQC01 was sensitive to ceftriaxone, indicating a slight difference in the susceptibilities between the two strains of *P. vulgaris.* Despite that, the bacteria can be recognised as a multidrug-resistant (MDR) bacterium [48]. In *Proteus* sp., an imipenem-resistant but meropenem- or ertapenem-susceptible pattern is uncommon but has been observed and may be attributed to natural reduced permeability to imipenem due to porin loss or intrinsic expression of low-level beta-lactamases [49]. Unlike other *Enterobacterales*, *Proteus* species may exhibit variable susceptibility to carbapenems due to species-specific membrane characteristics or enzyme activity that preferentially affects imipenem [49]. This pattern typically suggests the absence of broad-spectrum carbapenemases and highlights the need for further molecular or enzymatic assays to confirm resistance mechanisms and guide treatment. Therefore, the *P. vulgaris* isolate could be treated with broad-spectrum antibiotics for its susceptibility towards piperacillin/tazobactam, ceftazidime, ceftazidime/avibactam, ceftolozane/tazobactam, cefepime, doripenem, ertapenem, meropenem, amikacin, gentamicin, ciprofloxacin, and trimethoprim/sulfamethoxazole. In addition, a study from Iran suggested β-lactam/β-lactamase inhibitors, carbapenems, and fluoroquinolones as the most suitable drugs for treating *Proteus* sp. in human UTIs, which can be considered in fish infections as well [47].

Conclusion

We have successfully isolated, characterised, and identified the bacterial pathogens causing significant health challenges in koi, and also provided valuable insights into the pathogenicity of the I1_3 isolate as an opportunistic pathogen that might contribute to coinfection in the disease progression. The key findings of the I1_3 isolate were successfully documented through molecular and experimental infection assay, whereby the median lethal dosage was determined using the Probit analysis method, along with severe histopathological changes observed in infected specimens, meanwhile satisfying Koch's postulate.

As this research represents the first confirmed isolation of *Proteus* sp. closely related to *P. vulgaris/hauseri* complex from koi, the isolation and pathogenicity assessment of the pathogen in the fish highlights the critical need for integrated disease management strategies in aquaculture. Future research should focus on the development of effective preventive measures, including improved biosecurity, vaccination strategies, and the exploration of alternative treatments to combat antimicrobial resistance. Coinfection is another important aspect; the potential interactions between *Aeromonas* and *Proteus* in the development and virulence of the disease should also be further investigated. By addressing these attributes, the aquaculture industry can enhance the health and sustainability of ornamental fish farming, ensuring the welfare of these culturally and economically valuable species.

Author Contributions LCJJ: Formal analysis, Investigation, Writing-original draft, Writing-Review & Editing. CHH: Supervision, Funding acquisition, Writing-Review & Editing. LMML: Supervision, Writing-Review & Editing. KCJY: Supervision, Writing-Review & Editing. LLWK: Writing-Review & Editing. YKL: Writing-Review & Editing. HPX: Writing-Review & Editing.

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Data Availability The data that support the findings of this study are available upon reasonable request from the corresponding author, H.H. Chung. The nucleotide sequences generated in this study have been deposited in the NCBI GenBank database under the following accession numbers: I1_1 (PX460643), I1_3 (PX460644), I2_1 (PX460645), K1_1 (PX460646), K1_3 (PX460647), I3_1 (PX460648), I3_3 (PX460649), and K3_4 (PX460650).

Declarations

Conflict of interest The authors declared no conflict of interest.

Ethical Approval All experimental procedures in this study were in compliance to the guidelines and permission approved by the Animal Ethics Committee of Universiti Malaysia Sarawak (UNIMAS/TNC(PI)-04.01/06–09(17).

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