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Isolation, identification and characterisation of *Pseudomonas koreensis* CM-01 isolated from diseased Malaysian mahseer (*Tor tambroides*)

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A R T I C L E I N F O

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A B S T R A C T

Pseudomonas species are one of the most threatening fish pathogens which reside a wide range of environments. In this study, the dominant bacteria were isolated from diseased Malaysian mahseer (*Tor tambroides*) and tentatively named CM-01. It was identified as *Pseudomonas koreensis* based on its biochemical, morphological, genetic and physiological information. Its pathogenicity was found to be correlated with twelve virulence genes identified including iron uptake, protease, acylhomoserine lactone synthase gacS/gacA component regulation system, type IV secretion system, hydrogen cyanide production, exolysin, alginate biosynthesis, flagella and pili. The median lethal dose (LD50) for the CM-01 isolate on Malaysian mahseer was documented at 5.01×10^7 CFU/mL. The experimental infection revealed that CM-01 led to significant histological lesions in the fish, ultimately resulting in death. These lesions comprise necrosis, tissue thickening and aggregation. Drug sensitivity tests had shown its susceptibility to beta-lactam combination agents and further suggest its drug of choice. Its growing features had shown its growth at optimal temperature and pH. To the best of our knowledge, this is the first report of *P. koreensis* linked to diseased *T. tambroides*.

Statement of relevance: In this research, a novel strain of *Pseudomonas koreensis,* CM-01 was isolated from diseased *T. tambroides* for the first time. The antimicrobial susceptibility, pathogenicity, virulence genes and growth characteristics of CM-01 were studied. These findings established a scientific foundation for the recognition of *P. koreensis* and the management of fish infections caused by this pathogen.

1. Introduction

Aquaculture is important as animal protein source in global diets [1,2]. The fast-growing world population is urging the increment of aquaculture production by an additional 46.4 million metric tons by the year 2030 for sustainable food production and security [3]. In Malaysia, the demand for fish products was recorded as 59 kg per capita in the year 2016, which was one of the world's highest [4]. Thus, Malaysia greatly depends on its seafood production sector as one of its major protein source supplies whereas the aquaculture sector alone can represent 20% of the total seafood production [5]. Over the years, the industry had grown to an output of 1.69 million tons in 2017 with a steady production increment since 1980 [6].

The Malaysian mahseer (*Tor tambroides*) (Bleeker, 1854) is one of the most valuable riverine fishes in Southeast Asia with earlier research documented its genomic, metagenomic and mitogenomic data [7–10]. *T. tambroides* is one of the family members of *Cyprinidae*, sharing a similar biogeographical distribution with *T. douronensis* and *T. tambra* across freshwaters of Indonesia and Malaysia [11]. It had been characterized by its metagenomic, genomic, In addition to its food value, it is an important ornamental and sport fish. Its high nutritional value as well as unique flesh texture and taste are what made it highly sought after, earning it a top place among the most exorbitant fishes sold in the fish market (up to RM 400 per kg) and aquaculture industry [12]. However, disease outbreaks are one of the major bottlenecks faced in this growing industry in Malaysia which requires immediate attention. Several viral and bacterial infections were found to be persisting within the fish farms [13] which eventually would bring huge economic loss to the industry.

Pseudomonas sp. is one of the most threatening fish pathogens which can be found at a wide range of environments [14]. It induces ulcerative

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syndrome and hemorrhagic septicemia [15,16]. In a previous study, it had been reported up to half of the farm fish were lost due to bacterial infection before marketing [16,17]. The pathogenicity of *Pseudomonas* had been reported across wide variety of fish, for instance *Pseudomonas tructae* in *Oncorhynchus mykiss* [18], *Pseudomonas putida* in *Plecoglossus altivelis altivelis* [19], *Oncorhynchus mykiss* [20], *Pseudomonas baetica* in *Dicologlossa cuneata* [21]. *Pseudomonas* sp. are prevalent in normal fish microbiota but can act as opportunistic pathogens to cause infection to the host under stressful conditions [17].

Pseudomonas koreensis was previously isolated from Korean agricultural soil (first isolation) [22], rhizophores [23], agricultural soils [24], gold mining soil [25], yak milk [26], horse chestnut tree [27] and bronchiectasis patient [28]. It had been found in *Tor putitora* (golden mahseer) as a fish pathogen as well [29]. Further, in contrast to the extensively studied environmental *P. koreensis* found in soil, which is known for its role in promoting plant growth, the pathogenic *P. koreensis* from fish had been examined to a very limited degree. However, in present days, public health had drawn much of its attention towards *Pseudomonas* infection which causes serious food-borne illnesses via raw fish and its byproduct consumption [30]. There were previous reports on fish-isolated *Pseudomonas aeruginosa* causing hospital-acquired pneumonia in humans [31]. Besides, other cases were reporting on *P. koreensis* found in patient with bronchiectasis [28] as well as causing a mixed infectious keratitis together with *Aspergillus fumigatus* [32], depicting its ability to infect human beings.

In fish, reported clinical signs encompass opaque eyes, hemorrhagic septicemia, gills necrosis, abdominal distension, congested kidneys and friable liver [29,33]. Nevertheless, these symptoms may vary and are not consistently presented among infected fish: different bacterial isolates or strains exhibit varying degrees of pathogenicity [34]. Therefore, there is a necessity to investigate characteristics such as virulence, growth characteristics and histological lesions. The extensive usage of antimicrobial agents within the aquaculture sector and transmission of multidrug-resistant bacterial strains between terrestrial and aquatic ecosystems had been reported across the globe [35].

Virulence factors serve as a crucial parameter to judge the virulence and toxicity of bacterial pathogens [34]. The virulent genes tested can be mainly classified into adherence (*fliC*, *pilB*), antiphagocytosis (*algD*), quorum sensing (*hdtS*), protease (*aprA*), regulation (*gacA*, *gacS*), secretion system (*phnB*), iron uptake (*pvdA*, *pchE*) and toxin (*exlA*, *hcnA*). The survey of these virulence-related factors in fish-isolated *P. koreensis* is thus crucial for comprehending its pathogenesis and epidemiology [34] as there is still limited data and information available regarding this species. Moreover, the investigation into the growth characteristics of the isolate under diverse conditions is crucial. This can enhance the comprehension of the optimal growth condition for this pathogen. Therefore, in the current study, the dominant bacteria was isolated from the diseased *Tor tambroides* sourced from a local aquaculture farm and identified as *P. koreensis* CM-01 strain. The outcomes of the isolation, identification, pathogenicity, virulence genes and growth characteristics of *P. koreensis* were presented.

2. Materials and methods

2.1. Bacterial isolation & 16S rRNA gene analysis

P. koreensis was isolated from the internal organs (intestine and liver) of diseased adult *T. tambroides* sampled from a local aquaculture farm. Aeromonas isolation agar (Sigma Aldrich; US) was used for isolation of *P. koreensis* with the addition of Ampicillin as selective supplement (0.5 mg/mL). Cultured plates were incubated at 30 °C for 24 h. The colonies appeared on the agar plates was sub-cultured and purified until dominant colonies observed. Single colony was selected and inoculated in LB broth at 30 °C for 18hr. Then, the main culture was preserved in a final concentration of 20% sterile glycerol (v/v) at −20 °C (short-term storage) and −80 °C (long-term storage). Single colony was picked and added as template for colony PCR for its identity confirmation through 16S rRNA gene sequencing. A pair of universal 16S primer (27F & 1492R) was used.

2.2. 16S rRNA gene sequence analysis

The 16S rRNA was amplified and the conditions were set as follow: initial denaturation 95 °C for 3 min, followed by 35 cycles of denaturation 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min and subsequently final extension at 72 °C for 5 min. The amplified PCR products was visualized on 1.5% agarose gels by staining with ethidium bromide. The PCR products were purified with a GEL/PCR Purification Mini Kit (Favorgen Biotech Co.) and then sent for sequencing at Apical Scientific Sdn Bhd (Selangor, Malaysia). A BLAST search for sequences were performed through NCBI website. A phylogenetic tree was established using neighbour-joining method in the MEGA version 11.0.10 software [36].

2.4. Drug sensitivity tests

CM-01 strain was sub-cultured and tested for an expanded range of antimicrobial sensitivities using the VITEK2 system (bioMérieux; France). Specifically, the VITEK2 AST-N314 cards were used to test and the antibiotics included were cefotazime (1–64 μg/mL), ceftazidime (1–32 μg/mL), cefepime (1–64 μg/mL), ciprofloxacin (0.25–4 μg/mL), imipenem (0.25–16 μg/mL), meropenem (0.25–16 μg/mL) and piperacillin/tazobactam (4/4–128/4 μg/mL). The susceptibility category was designated according to the 2023 Clinical and Laboratory Standards Institute (CLSI) M100-Ed33 guidelines. *Pseudomonas aeruginosa* ATCC 27853 was used as the quality control strain for the antibiotics susceptibility testing.

2.3. Growth characteristics and microscopy observation

The isolated bacteria was grown on different agar, including lysogeny broth (LB) agar, Aeromonas isolation agar, Nutrient agar, eosin methylene blue (EMB) agar and MacConkey agar, to observe its growth and colony appearance. Next, the growing characteristics of the isolated bacteria was determined. The impact of pH (ranges from 3 to 11) and temperature (assessed at 27 °C, 30 °C and 42 °C) on the growth of isolated *P. koreensis* were identified. To achieve this, isolated *P. koreensis* was cultured in LB broth until its OD600 of the suspension reached 1.2. All the bacterial suspension was cultured at 150 rpm and the growth was observed at 4-h intervals at OD600 for 52 h.

For scanning electron microscopy examination, the CM-01 strain was cultured in LB medium at 37 °C for 24 h. Next, the culture was centrifuged at 8000 rpm for 5 min and the resulting pellets were washed twice with phosphate-buffered saline (PBS). The collected bacterial pellets were then fixed in 4% paraformaldehyde (PFA) and kept for 6 h under 4 °C. Subsequently, the pellets were washed again with PBS for three times to eliminate residue fixative and later dehydrated using a series of increasing concentration ethanol concentrations (30%, 50%, 80% and 100%). Finally, the dehydrated samples were observed under a JSM-IT500HR scanning electron microscope (Jeol, Peabody, MA, USA).

2.5. Screening of virulence genes

Next, isolated bacteria were screened for the presence of virulence genes. The selection of virulence genes was selected from the list of 150 virulence genes predicted from the whole genome data of *P. koreensis* using the Virulence Factors Database (http://www.mgc.ac.cn/VFs/) reported previously (Kho et al., 2023). Twelve virulence genes were screened, including *fliC, algD, pilB, phnB, pchE, hcnA, hdtS, exlA, aprA, gacS, pvdA* and *gacA* through conventional PCR. Each PCR reaction initiated with denaturation at 94 °C for 2 min, followed by 30 cycles of amplification, and finally extension at 72 °C for 10 min. Each cycle