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Phenotypic and genomic characterization of *Aeromonas veronii* from Thai rugose frog (*Hoplobatrachus rugulosus*)

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Abstract

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The farming of the Rugose frog (*Hoplobatrachus rugulosus*) is of considerable economic importance in Thailand; however, bacterial diseases, particularly those associated with the genus *Aeromonas*, continue to result in significant production losses. This study represents the report of *Aeromonas veronii* associated with disease in *H. rugulosus* in Phayao province, Thailand. The objective was to isolate and characterize the phenotypic and genomic bacterial pathogens from diseased frogs. The identification was conducted using morphological characterization, Gram staining, 16S rRNA gene sequencing, and whole-genome sequencing (WGS). Clinically, infected frogs exhibited external signs including erythema, swelling of the digits, and emaciation, while no obvious internal lesions were observed. The isolate demonstrated β-hemolytic activity and was confirmed as a Gram-negative bacterium. Therefore, the isolate was identified as *A. veronii* strain Fsp5. Whole-genome analysis revealed a genome size of approximately 4.7 Mb and identified numerous virulence-associated genes, as annotated using the Virulence Factor Database (VFDB), including genes related to adhesion, secretion systems, motility, and regulatory functions. These findings establish the pathogenic potential and genomic features of *A. veronii* Fsp5 in *H. rugulosus*, providing a reference isolate to enhance our understanding of *Aeromonas* infections in cultured frog species.

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1. Introduction

The rugose frog (*Hoplobatrachus rugulosus*), previously known as *Rana rugulosa*, is taxonomically classified within the anuran family Dicroglossidae. It is a large frog widely distributed across southern China, Myanmar, Thailand, and Peninsular Malaysia, with introduced populations in Borneo and the Philippines. In Thailand, this frog is of considerable importance in many regions and widely cultured due to high consumer demand. In 2024, total aquaculture production of frogs reached 3,106 tons, with an estimated value of approximately 218 million Thai baht [1]. Because of high market demand, the Department of Fisheries has implemented policies promoting frog farming to expand production for both domestic and international markets. However, infectious diseases remain a major challenge to the sustainable development of frog farming worldwide [2, 3]. In Thailand, bacterial diseases are common in cultured frogs, with *Aeromonas hydrophila* reported as the predominant pathogen [4]. Nevertheless, the information on bacterial pathogens of the rugose frog (*Hoplobatrachus rugulosus*) in Thailand is still limited.

A previous study reported that *Aeromonas* strains have been identified as the causative agent of red leg on *H. rugulosus* in Thailand [5]. Moreover, the genera *Aeromonas* and *Staphylococcus* were identified as bacterial pathogens responsible for frog diseases with hemorrhagic lesions on the body, ulcers on the mouth and limbs, corneal opacity, and exophthalmia in Maha Sarakham Province, Thailand [6]. Furthermore, some studies reported that *Mycobacterium* spp. caused prominent internal organ lesions in infected frogs, characterized by multiple cream-white hepatic nodules distributed throughout the liver parenchyma [7]. Additionally, a recent study compared the skin microbiomes of healthy and ulcerated *H. rugulosus* and reported a high

abundance of the genus Acinetobacter in ulcerated skin [8]. In addition to *H. rugulosus*, other amphibian species also face problems with bacterial infections, which cause economic damage to breeders. Co-infections with *Klebsiella pneumoniae* and *Elizabethkingia miricola* have been associated with clinical manifestations including anorexia, reduced mobility, intermittent circular swimming behaviour, and a sudden mortality outbreak of approximately 80% in the black-spotted frog (*Pelophylax ridibundus*) [9]. Despite increasing reports of bacterial diseases in frogs, comprehensive knowledge of their pathogenic mechanisms remains limited. Therefore, the comprehensive understanding of disease pathogenesis contributes to the development of effective preventive strategies and provides a scientific foundation for sustainable frog farming management in the future.

Aeromonas veronii is a Gram-negative, rod-shaped bacterium that belongs to the family Aeromonadaceae. It is prevalent in freshwater environments and aquaculture systems, and it is acknowledged as a significant pathogenic bacterium affecting several aquatic animals [10, 11]. It is responsible for significant morbidity and high fatality rates in various frog species, including the American bullfrog (*Aquarana catesbeiana*) and black-spotted frog (*P. ridibundus*) [9, 12]. This bacterium can cause disease through the production of a variety of virulence factors, including toxins, enzymes, and proteins involved in host invasion and tissue destruction [13]. Major virulence genes in *A. veronii* include aerolysin (*aerA*) and hemolysins (*hlyA*) genes, which are associated with the production of toxins that disrupt host cell membranes, leading to cell lysis and hemorrhagic conditions (β-hemolysis). In addition, the cytotoxic enterotoxins (*act*), cytotonic enterotoxins (*alt*), and heat-stable cytotonic enterotoxin (*ast*) genes belong to the enterotoxin gene group and are associated with pathological effects on internal organ systems [11]. The lipase (*lip*) and elastase (*ela*) genes are also involved in the production of lipase and elastase enzymes, which facilitate tissue degradation and promote bacterial dissemination within the host [14]. The flagellin (*fla*) gene plays a role in bacterial motility and adhesion to host tissue surfaces, while the ascending colonization protein V (*ascV*) gene is a critical component of the type III secretion system (*T3SS*), which is closely associated with the severity of infection [13, 15].

Whole-genome sequencing (WGS) has become an essential approach for improving the identification and characterization of bacterial pathogens, providing high-resolution insights into taxonomy, virulence profiles, molecular epidemiology, and phylogenetic relationships [16, 17]. The genomic investigation of *A. veronii* strain Aer12, which was obtained from the fish (*Channa argus*), demonstrates the importance of WGS by identifying 216 predicted antibiotic resistance genes and 676 probable virulence-associated genes [18]. Additionally, genomic analysis of *A. veronii* strain MRG1, isolated from the freshwater shrimp *Macrobrachium rosenbergii*, identified 121 putative virulence-associated genes [19]. To date, there are no confirmed scientific reports in Thailand documenting diseases caused by *A. veronii* in the frog *H. rugulosus*, a species of considerable economic importance that is widely farmed in the country. Therefore, this study aimed to isolate and identify bacterial pathogens from diseased frogs. Bacterial isolates were collected and characterized using both phenotypic methods and genomic analysis, with a particular focus on virulence-associated genes. The findings of this study provide baseline information to support effective disease surveillance, prevention, and management in frog aquaculture, contributing to more sustainable production systems in the future.

2. Materials and Methods

Sample collection and bacterial isolation

Eight diseased frogs were gathered from frog farms in Phayao Province from June to December 2024. The frogs were anesthetized by immersion in water with a diluted clove oil (40 drops per L). The frogs were submerged for roughly 5–10 minutes until total anaesthesia was attained. The external surface of each frog was thereafter cleaned with distilled water to minimize external contamination and gently dried. The liver, kidney, and spleen were removed under aseptic conditions. The organs were submerged in 70% ethanol for 1 minute to eradicate surface impurities, and then rinsed with sterile 0.85% NaCl solution. The organs were subsequently transferred into sterile test tubes for bacterial isolation. Each organ specimen was weighed and homogenized in a sterile diluent to achieve a final concentration of 1 mg/mL. Subsequently, 100 µL of the homogenized material was distributed onto Tryptone Soya Agar (TSA; HiMedia, India) plates and incubated at 30 °C for 16–24 hours. Following incubation, bacterial colonies were subcultured utilizing the quadrant streaking technique to confirm individual colonies and pure isolates. This purification was conducted twice. Pure bacterial isolates were subsequently inoculated into Tryptone Soya Broth (TSB; HiMedia, India) and incubated at 30 °C for 16–24 hours with agitation at 200 rpm. The cultures were combined with sterile glycerol, placed into cryovials, and preserved at −80 °C for future microbiological analysis and other experimental procedures.

Gram staining and haemolytic activity

Gram staining was conducted following the standard Gram method with a commercial staining kit (R.V.L. Supply, Thailand). Briefly, bacterial smears were produced on clean glass slides, air-dried, and subjected to heat fixation. The slides were subjected to crystal violet staining for one minute, washed with water, and then treated with iodine solution for one minute. The slides were then decolorized for 30 seconds, rinsed with water, and counterstained with safranin for a further 30 seconds. After a final rinse and air-drying, the slides were examined under a light microscope using a 100 × objective lens.

For the haemolytic assay, bacterial isolates were cultivated in 1 mL of TSB and incubated at 30 °C for 16–24 hours with shaking at 200 rpm for haemolysis assessment. Blood agar plates (M&P Impex, Thailand) were fabricated and partitioned into four segments. Subsequently, 3 µL of

the bacterial suspension was pipetted and applied to each designated region. The inoculation plates were incubated at 30 °C for 16–24 hours, following which haemolytic activity was assessed by examining clear zones surrounding bacterial colonies, signifying haemolysis. Haemolytic patterns were categorized as β-haemolysis (development of a clear zone), α-haemolysis (greenish discolouration), or γ-haemolysis (no observable change).

Challenge experiment

A total of 12 β-haemolytic isolates were separately inoculated into TSA plates at 30°C for 16–24 hours. A single colony was subsequently inoculated into 3 mL of TSB and incubated at 30 °C with agitation at 200 rpm for 16-24 hours. The bacterial culture was centrifuged at 4,000 rpm for 15 minutes at 4 °C, and the pellet was washed twice with 3 mL sterile 0.85% NaCl. The bacterial solution was calibrated to an optical density at 600 nm (OD600) of 1.0 and then diluted ten-fold using sterile 0.85% NaCl before injection. Prior to the challenge experiment, all frogs (9.05±3.20 g, 2 months) were acclimatised for a minimum of 7 days. Frogs were randomly assigned to two groups: a control group of five individuals, which received intraperitoneal injections of sterile 0.85% NaCl, and 12 experimental groups of five individuals, which received intraperitoneal injections of a bacterial suspension at a dosage of 1 μL/g of body weight. After bacterial infections, frogs were fed daily between 10:00 and 16:00 hours. Clinical symptoms, atypical behaviours, and mortality rates in each group were monitored and documented bi-daily for a duration of 14 days. The mortality was recorded to assess the virulence of each bacterial strain.

Bacterial identification

Bacterial dentification was conducted through analysis of the 16S rRNA gene and whole genome sequencing (WGS). Genomic DNA was extracted utilizing a GF-1 Bacterial DNA Extraction Kit (Vivantis Genomic DNA Extraction Kit, Malaysia) in accordance with the protocol. The isolated DNA was subsequently analysed by polymerase chain reaction (PCR) with universal bacterial primers, 8fm (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) [20]. PCR amplification was conducted under these parameters: initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 45 seconds, and elongation at 72 °C for 3 minutes, concluding with a final extension at 72 °C for 7 minutes. The PCR results were analyzed using 1% agarose gel electrophoresis. Samples exhibiting a DNA band of approximately 1,500 base pairs were purified utilizing the GF-1 AmbiClean Kit (Vivantis, Malaysia) in accordance with the manufacturer's guidelines. The purified PCR products were then analysed using nucleotide sequencing via the Sanger method. Bacterial species identification was executed using sequence similarity analysis utilizing the GenBank BLAST program, while phylogenetic analysis was carried out using MEGA12 version 12.1.2 software using Neighbor-Joining (NJ) with 1,000 Bootstrap replicates.

For the WGS, the bacterial isolate was inoculated into 3 mL of TSB and incubated at 30°C with shaking at 200 rpm for 16 to 18 hours. One milliliter of the culture was then centrifuged at 10,000 rpm for 5 minutes, and the resulting bacterial pellet was used for DNA extraction. The genomic DNA was extracted using the GF-1 Bacterial DNA Extraction Kit. Subsequently, the DNA was delivered to GENEWIZ™ from Azenta Life Science (China). Next-generation sequencing libraries were prepared according to the company's standard protocol. The qualified libraries were sequenced using paired-end (PE150) reads on the Illumina MiSeq platform. Then, the whole-genome taxonomic assignment was evaluated using Average Nucleotide Identity (ANI) comparisons. Pairwise genomic similarities were calculated using FastANI (v1.33) [21] executed within a Linux/Ubuntu (WSL2) environment. The query assembly was evaluated against representative reference genomes retrieved from the NCBI RefSeq database using the fully sequenced *Aeromonas veronii* reference strain VA60 (NCBI Accession: NZ_AP027937.1). The standard species-delineation threshold of ≥ 95.0 – 96.0% ANI was applied to confirm species identity [22].

Genome assembly, and annotation

Raw sequencing data were also processed via the BV-BRC web-based server (version 3.57.29) using its analysis pipelines [23, 24]. Briefly, the FASTQ files were subjected to genome assembly, and reads were de novo assembled into contigs using SPAdes genome assembler v4.0.0 with default parameters. Subsequently, the assembled contigs were annotated using the Genome Annotation Service, which identifies genomic features through the RAST toolkit (RASTtk).

Biochemical analysis

The biochemical profile of the bacterial isolate was determined using the API® 20 E system (BioMérieux, France), following the manufacturer's instructions with modification in incubation temperature at 30 °C. Briefly, the isolate was streaked on TSA and cultured at 30 °C for 16–24 hours. After incubation, the bacterial colony was suspended in 5 mL of sterile 0.85% NaCl solution and homogenized. The suspension was inoculated into the API® 20E plastic strip. Mineral oil was added to the ADH, LDC, ODC, H_2S, and URE reactions to establish anaerobic conditions. The strips were incubated at 30°C for 16–24 hours. Following incubation, reactions were interpreted based on the manufacturer's reference chart. TDA, IND, and VP reactions were developed using TDA, JAMES, and VP1/VP2 reagents, respectively, and scored as positive or negative according to the resulting color reactions.

3. Results and Discussion

Characteristics of diseased frogs and A. veronii Fsp5

Physical examination of diseased frogs revealed swelling and erythema at the toes, accompanied by an abnormally

emaciated body condition (Fig. 1 (a) and 1(b)). To identify the causative agent, Gram staining was performed on bacterial isolates obtained from the diseased frogs. The staining results showed numerous small rod-shaped bacteria stained red, indicating Gram-negative bacteria (Fig. 1 (c)), which are commonly associated with diseases in aquatic animals and amphibians. In addition, hemolytic activity was assessed using blood agar. The results demonstrated that the bacterial colonies were surrounded by distinct clear zones, indicating β-hemolysis (Fig. 1 (d)). This hemolytic pattern is a characteristic feature of highly virulence bacteria, as it reflects the ability to produce enzymes or toxins capable of destroying erythrocytes and host tissues.

Out of 42 bacterial isolates obtained from diseased frogs, 12 isolates demonstrating β-hemolytic activity were subsequently selected for a challenge test in *H. rugulosus*. Among the experimental groups, frogs infected with isolate Fsp5 showed the highest mortality rate, resulting in the death of all five frogs during the study. Mortality was first observed on day 10, during which four frogs died, followed by the death of the remaining frog on day 11. Consequently, cumulative mortality in this group reached 100% within 11 days, following the isolate Fsp2 caused the mortality of five frogs within 14 days (Table 1). No specific internal pathological lesions were observed prior to death; however, the external clinical signs and bacteriological findings strongly suggest that mortality was associated with infection by a highly virulence bacterial pathogen.

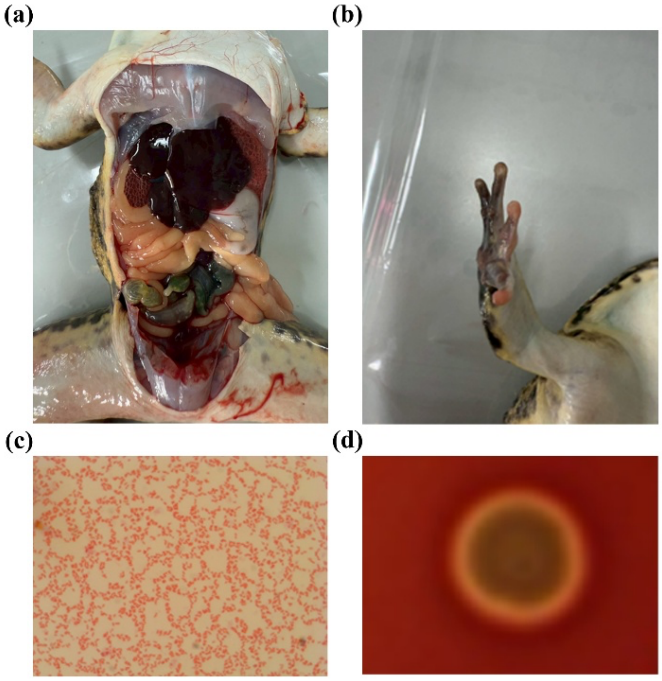


Fig. 1 Internal organs of diseased frogs (a), External lesions of diseased frogs (b), Gram staining of the bacterial isolate (c), β -hemolysis of erythrocytes on blood agar (d).

Table 1 The mortality of *H. rugulosus* challenged with β-haemolytic bacterial strains.

Strains	Concentration (CFU mL^{-1})	Death number	Mortality (%)
Fsp5	1.3 x 10^8	5	100
Fsp2	1.5 x 10^8	5	100
FLi2(2)	1.9 x 10^8	4	80
FLi4(2)	2.5 x 10^8	4	80
IFLV2-3	1.1 x 10^8	3	60
IFsp2-1	1.9 x 10^8	1	20
IFsp3-1	1.8 x 10^7	1	20
Fsp1	2.7 x 10^7	0	0
Fsp3(1)	2.8 x 10^7	0	0
Fsp3(2)	2.8 x 10^7	0	0
FLi2(1)	1.9 x 10^7	0	0
FLi4(2)	2.5 x 10^7	0	0
Control	0.85% NaCl	0	0

Molecular identification

Phylogenetic analysis based on 16S rRNA gene sequences showed that the isolate Fsp5 clustered closely with *A. veronii* strains JMC7375, ATCC 35624, and 115/II, with strong support indicated by a percent identity exceeding 99%. Isolate Fsp5 was clearly separated from other *Aeromonas* spp., reflecting genetic divergence at the species level. The result indicated that isolate Fsp5 can be reliably identified as *A. veronii* based on its phylogenetic position and the strong statistical support of the corresponding nodes (Fig. 2).

Additionally, the species identification of isolate Fsp5 was confirmed by WGS-based comparative genomic analysis. Average nucleotide identity (ANI) analysis with the *A. veronii* reference genome (NCBI accession no. NZ_AP027937.1), yielded an ANI value of 96.24%, based on 1,342 aligned orthologous fragments out of 1,539 query

fragments, corresponding to approximately 87.2% genome coverage. This ANI value exceeds the generally accepted threshold of 95–96% for species delineation, supporting the assignment of strain Fsp5 to be an *A. veronii*.

Genome assembly and annotation

A genome assembly of the Fsp5 isolate was obtained, revealing a total genome size of 4,727,634 bp, comprising 102 contigs and a G + C content of 58.56%. A circular genomic map is shown in Fig. 3. The L50 count was determined to be 15, while the N50 length was calculated as 113,381 bp. Genome quality assessment indicated high completeness (100%) and no detectable contamination (0%). Genome annotation identified 2,909 protein-coding sequences (CDS), along with 4 ribosomal RNA (rRNA) genes and 90 transfer RNA (tRNA) genes. Additional genomic features included 815 hypothetical proteins, 204 putative virulence factors, 48 antibiotic resistance genes, 34 transporter-related genes, and 4 predicted drug targets (Table 2). The raw data of whole genome sequencing of *A. veronii* Fsp5 were submitted to BioProject: PRJNA1455468.

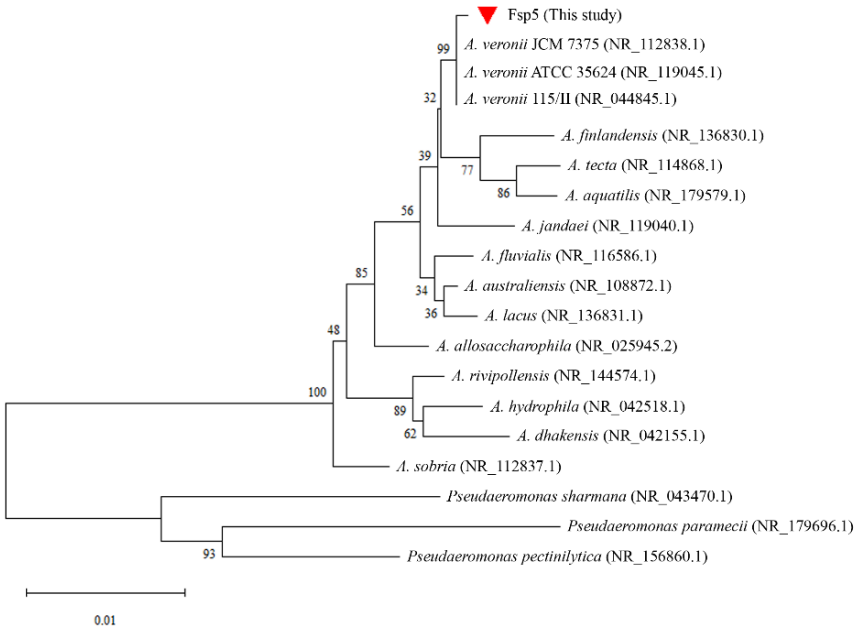


Fig. 2 Phylogenetic tree derived from 16S rRNA gene sequences. The tree was constructed using the Neighbor-Joining method. Percentage bootstrap values (1000 replicates) are shown at each branch point. The scale bar 0.01 represents substitutions per nucleotide position.

Table 2 Genomic description of *A. veronii* Fsp5.

Characteristics	Source	Total
Genome length	PATRIC	4,727,634
Number of contigs	PATRIC	102
G+C contents	PATRIC	58.56
Contig L50	PATRIC	15
Contig N50	PATRIC	113381
Number of protein-coding sequences	PATRIC	2909
Number of hypothetical proteins	PATRIC	815
Number of rRNA genes	PATRIC	4
Number of tRNA genes	PATRIC	90
Number of proteins with pathways	PATRIC	620
Virulence factor	Victors	35
Virulence factor	VFDB	169
Antibiotic resistance genes	PATRIC	36
Antibiotic resistance genes	CARD	7
Antibiotic resistance genes	NDARO	5
Transporter genes	TCBD	34
Drug target genes	TTD	4

Virulence factor genes

Functional annotation of virulence-associated genes using the Virulence Factor Database (VFDB) revealed that a total of 169 genes were identified across four functional categories, with motility-associated genes being the most abundant (75 genes), followed by effector delivery system genes (60 genes), adherence-associated genes (32 genes) and regulation (2 genes) (Table 3). This result suggests that this isolate possesses a broad genetic repertoire potentially contributing to host colonization and pathogenicity.

Subsystems in Fsp5 isolate

A subsystem is defined as a group of proteins that function collectively to perform a specific biological process or to form a structural complex. The distribution and coverage of these subsystems are summarized in a pie chart. In total, 91 subsystems comprising 698 genes were identified and assigned across various functional categories, with the highest representation observed in the Amino Acids and Derivatives class. In Fsp5 isolate, metabolism was characterized as a specific biological process governed by these 91 subsystems and their associated 698 genes (Fig. 3).

Table 3 The prediction of virulence factor genes based on the Virulence Factor Database (VFDB).

Classification	Number	Genes
Adherence	32	*fimA, fimC, fimD, fimF, flp1, flpA, flpB, flpC, flpD, flpE, flpF, flpG, flpH, flpL, mshB, mshE, mshG, mshI, mshL, mshM, mshO, tapB, tapC, tapD/pilD, tapF, tapM, tapN, tapO, tapQ, tapT, tapU, tapW*
Effector delivery system	60	*ascY, exsE, exeA, exeD, exeE, exeF, exeG, exeI, exeJ, exeL, exeM, exsD, sycX, acr2, clpB, hcp (2), atsQ, vipA, vipB, atsH, atsI, atsL, vasK/icmF, atsJ, atsG, dotU, AHA_RS09305, atsK, vasH, ascJ, ascB, ascN, acrG, ascF, ascI, ascK, ascL, ascH, ascV, ascD, ascQ, ascR, ascS, ascT, ascU, aopN, acr1, ascC, ascX, ascG, ascO, exsA, exsB, atsS, exsC, vgrG2 (3), vgrG3*
Motility	75	*AHML_RS07540, cheA-2, cheB-2, cheR-3, cheV, cheW, cheY, cheZ, flgB, flgC (2), flgE (2), flgF, flgG, flgH, flgI (2), flgJ (2), flgM, flgN, flhA, flhB, flhF, flhG, fliA, fliE, fliF, fliF, fliG, fliG, fliH, fliI, fliJ, fliL, fliM, fliN, fliO, fliP, fliP, fliQ, fliR, flmH, flrA, flrB, flrC, lafB, lafC, lafF, lafS, lafT, lafU, lfgB, lfgF, lfgG, lfgH, lfgK, lfgL, lfhA, lfhB, lfiE, lfiH, lfiI, lfiN, lfiQ, lfiR, maf-5, motA, motX, motY, nueA, pomA2, pomB, pomB2*
Regulation	2	*csrA, fur*

Subsystems in Fsp5 isolate

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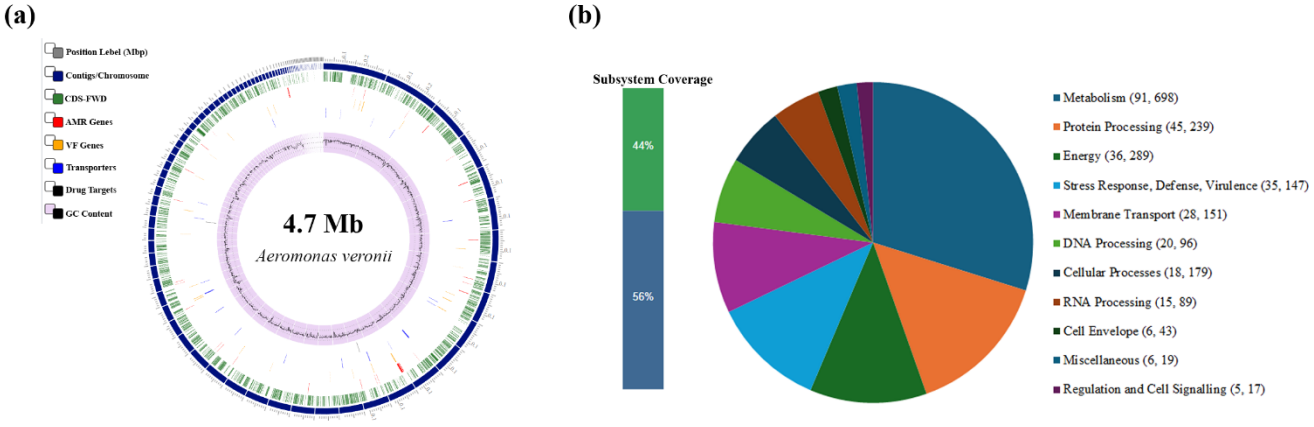


Fig. 3 Circular genomic map for *A. veronii* Fsp5. From the outside to the center are the assembled contigs, open reading frame (ORF), CDS in the forward strand, CDS with similarity to known antibiotic resistance genes, CDS with similarity to virulence factors, transporters, drug target, and GC content (a), subsystems information. Subsystem coverage showed that 44% of genes (2,055) were assigned to subsystems, while the remaining 56% (2,565 genes) were not classified within the subsystem categories (b).

Biochemical characterization

The biochemical analysis of the *A. veronii* strain Fsp5 was compared with previous research in Table 4. The result demonstrated the positive results for β-galactosidase, lysine decarboxylase, indole production, acetoin production, fermentation-oxidation, glucose, mannitol, and saccharose (Table 4).

Discussion

Aeromonas veronii has previously been isolated from diseased frogs (*A. catesbeiana* and *P. ridibundus*) and several aquatic animals [9, 12]. *A. veronii* infected the bullfrogs in China, with clinical manifestations including erythema of the legs and abdominal skin and lesions affecting internal organs [9]. In the present study, the unhealthy *H. rugulosus* frogs were collected from the frog farm in Phayao province, and the bacteria were isolated. A total of 42 bacterial isolates were examined for hemolytic activity following the challenge tests. The bacterial isolate exhibiting β-hemolytic activity and causing the highest mortality rate was identified as *A. veronii* through 16S rRNA gene sequencing. In addition, whole-genome sequencing (WGS) was used to characterize its virulence factor genes.

Table 4 Biochemical results of *A. veronii* Fsp5 isolated from diseased frog (*H. rugulosus*) using the API 20E system.

Test	Fsp5	[25]
β-galactosidase	+	+
Arginine dihydrolase	-	+
Lysine decarboxylase	+	-
Ornithine decarboxylase	-	-
Citrate utilization	-	-
H_2S production	-	-
Urease	-	-
Tryptophan deaminase	-	-
Indole production	+	+
Acetoin production	+	+
Fermentation-oxidation	+	-
- Glucose	+	+
- Mannitol	+	+
- Inositol	-	-
- Sorbitol	-	-
- rhamnose	-	-
- Saccharose	+	+
- Melibiose	-	-
- Amygdalin	-	-
- Arabinose	-	+

The isolation of bacteria from diseased frogs revealed the presence of *A. veronii* strain Fsp5. Clinically, the affected frogs exhibited swollen and reddened toes and emaciated bodies, which are consistent with the typical manifestations of infections caused by bacteria in the genus *Aeromonas* [9, 26]. The isolated strain also exhibited β-hemolytic activity, a characteristic feature of pathogenic Aeromonas species [27]. Additionally, *A. veronii* strain RDL-2, isolated from the skin of infected *P. ridibundus* larvae, exhibited β-hemolytic activity and was associated with ulcerative skin lesions and pale skin appearance [9]. The hemolytic activity observed in *Aeromonas* spp. suggests the production of hemolysin virulence factors that facilitate host tissue invasion [28]. Therefore, *A. veronii* Fsp5 might be associated with the erythematous lesions on the toes of the *H. rugulosus* frogs.

The presence of virulence genes plays a crucial role in determining bacterial pathogenicity [28]. A total of 169 virulence-associated genes were predicted in *A. veronii* strain Fsp5 and identified as four categories consisting of adherence, effector delivery systems, motility, and regulation. However, the two major virulence factor genes, namely *aerA* and *hlyA*, were not observed in the *A. veronii* strain *Fsp5* genome, despite the isolate exhibiting positive hemolytic activity *in vitro*. The *aerA* gene functions as a major virulence factor mediating toxin production that damages host cell membranes, causing cell lysis and hemorrhagic skin lesions [29]. Additionally, a study on *Aeromonas salmonicida* has demonstrated that both *aerA* and *hlyA* genes were associated with hemolytic activity [30]. Similar observations have been reported in previous studies, where *A. hydrophila* isolated from freshwater fish displayed β-hemolytic activity but lacked the *hlyA* gene [31]. These observations indicate that hemolytic activity in *Aeromonas* spp. is not solely mediated by hemolysin but rather results from the combined action of multiple virulence genes that collectively contribute to erythrocyte lysis and pathogenicity.

Furthermore, the genome was found to contain several ascending colonization protein (*asc*) genes. These genes are critical components of the Type III Secretion System (T3SS), a specialized molecular apparatus closely associated with increased infection severity and host cell manipulation [13, 15]. Additionally, the predicted virulence genes contained a cluster of the Type VI secretion system (T6SS) genes. This secretion system exports effectors that enable competition with other cells and can lead to the demise of both bacterial and eukaryotic host cells [32]. It is necessary for the secretion of hemolysin coregulated protein (*Hcp*) and the valine-glycine repeat protein (*VgrG*) [28]. Consistent with previous studies on virulence genes in *A. veronii* from fish, environmental, and human sources, both T3SS and T6SS have been identified in the genomes of pathogenic *A. veronii* strains [33]. Additionally, functional evidence of T6SS from *A. hydrophila* demonstrated that targeted deletion of the essential genes *hcp1* and *vgrG1* in the virulence ML09-119 strain resulted in a 2.24-fold reduction in virulence in catfish fingerlings compared with the parental wild-type strain [34]. Collectively, these findings suggest that the concurrent presence of T3SS- and T6SS-associated genes might contribute to the pathogenic potential of this isolate and may, at least in part, be associated with its pronounced β-hemolytic activity and high virulence in *H. rugulosus*.

Furthermore, the genome contained several genes associated with motility, especially the cluster of flagellar genes (*fli*). A previous study indicated that *fliD* is crucial for the pathogenicity of *A. veronii*, and its absence results in diminished colonization efficiency and a lower capacity for

the bacterium to initiate infection [35]. Additonally, disruption of the *fur* gene has been shown to significantly attenuate the pathogenicity of *A. veronii* in crucian carp and zebrafish, highlighting the importance of iron homeostasis in virulence [36]. The WGS explored the many genes associated with virulence factors. Therefore, *A. veronii* strain Fsp5, isolated from *H. rugulosus*, is considered a causative agent of disease and mortality in frogs. However, the underlying pathogenic mechanisms remain critical aspects for further investigation to better understand disease progression associated with *A. veronii*. The present study represents a preliminary investigation, primarily focusing on the genomic characterization of this pathogen.

4. Conclusion

Bacterial isolate obtained from diseased frogs (*H. rugulosus*) in Phayao Province was genetically analyzed and identified as *A. veronii* strain Fsp5. This bacterium was characterized as a Gram-negative, rod-shaped bacterium and exhibited β-hemolytic activity. The WGS was used to predict the potential of bacterial virulence. The study identified several genes associated with virulence factors, including those involved in adherence, effector delivery systems, motility, and regulation. Therefore, *A. veronii* strain Fsp5, isolated from *H. rugulosus*, is considered a causative agent of disease and mortality in frogs.

5. Suggestions

This study provides foundational insights into the genomic characteristics of *A. veronii* strain Fsp5 isolated from diseased frogs, including the identification of key virulence-associated genes. Nevertheless, certain limitations remain. Future studies should include larger sample sizes and a range of bacterial inoculum levels to better clarify pathogenic mechanisms. Validation of virulence-associated genes using real-time PCR is also recommended to strengthen the findings. In addition, evaluating a broader panel of antimicrobial agents would improve the clinical and epidemiological relevance of the results. Despite these limitations, the present study offers important fundamental data that can support the development and optimization of effective disease management strategies in aquaculture systems in the future.

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7. Declaration of Generative AI in Scientific Writing

Generative AI tools may be used only to improve the readability and language of a manuscript.

8. CRediT Author Statement

Wiranphat Pengput: Methodology, Formal analysis, Investigation, Visualization, Writing - original draft. **Kriengkrai Seetapan**: Conceptualization, Resources, Writing - review & editing. **Eakapol Wangkahart**: Resources, Writing - review & editing. **Wipasiri Soonthornchai**: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resource, Data curation, Writing - Original draft, Writing - review & editing, Visualization, Project administration, Funding acquisition.

9. Research Involving Human and Animal Rights

This study involved the use of animals and was conducted with approval from the Institutional Animal Care and Use Committee of the University of Phayao.

10. Ethics Approval and Consent to Participate

All experimental procedures were conducted in strict accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Phayao (IACUC-UP) and were approved under protocol number UP-AE 1-029-68. No endangered or protected species were involved, and all efforts were made to minimize animal suffering. Additionally, these research methods, involving the pathogenic bacteria from the infected frogs, were reviewed and approved by the Institutional Biosafety Committee, University of Phayao, Thailand (UP-IBC; Approval No. IBC 2/001/2569).

11. 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.

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