Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(4): 3213 – 3222 (2025)
www.ejabf.journals.ekb.eg

Identification of Pathogenic Bacteria from Grouper (*Epinephelus* sp.) Based on 16s rRNA Genes

Dwi Tika Afriani[1*], Rahmi Nazliah[2], Siti Zahara Saragih[3], Rusdi Machrizal[2]

[1]Department of Aquaculture, Faculty of Fisheries, Dharmawangsa University, Medan, North Sumatera 21415, Indonesia

[2]Department of Biology Education, Universitas Labuhan Batu, Jl. SM. Raja No 126 A, Aek Tapa, Rantauprapat, North Sumatra, Indonesia

[3]Universitas Labuhan Batu, Jl. SM. Raja No 126 A, Aek Tapa, Rantauprapat, North Sumatra, Indonesia

[*]**Corresponding Author: dwitika_afriani@dharmawangsa.ac.id**

ARTICLE INFO

Article History:
Received: Jan. 19, 2025
Accepted: July 26, 2025
Online: Aug. 27, 2025

Keywords:
Grouper,
Aeromonas hydrophila,
Staphylococcus aureus,
Staphylococcus sciuri,
16s rRNA

ABSTRACT

Pathogenic bacteria are microorganisms capable of causing disease in animals and humans, including fish. This study aimed to identify pathogenic bacteria isolated from grouper fish using the 16S rRNA gene. Samples were collected from fish markets in the Central Tapanuli area, with bacterial isolates obtained from the liver, muscle, and stomach tissues. Isolation was carried out using TSA (Tryptone Soya Agar) and BHIA (Brain Heart Infusion Agar) media. Molecular identification of bacterial isolates was performed using the Polymerase Chain Reaction (PCR) method. DNA from selected isolates was extracted using the boiling method, followed by amplification of the 16S rRNA gene. PCR products from six samples yielded fragments ranging from 648 to 839 bp. BLAST analysis of the sequences revealed three types of pathogenic bacteria: *Aeromonas hydrophila*, *Staphylococcus aureus*, and *Staphylococcus sciuri*, with sequence similarity levels of 98.34–100% compared to reference data in GenBank. Phylogenetic analysis using the Kimura 2-Parameter (K2P) model showed that the genetic distance between the study samples and reference sequences in GenBank was less than 2%. This confirms that the isolates obtained were *A. hydrophila*, *S. aureus*, and *S. sciuri*. In conclusion, the 16S rRNA gene proved to be a highly effective molecular marker for the identification of pathogenic bacteria in grouper fish.

INTRODUCTION

Pathogenic bacteria in fish are microorganisms that can cause disease, particularly when fish are exposed to environmental stressors or have compromised immune function **(Kumar *et al.*, 2023)**. Bacterial diseases in fish are often associated with secondary infections caused by parasites or fungi and can result in substantial production losses and economic impacts on the aquaculture industry **(Agbabiaka *et al.*, 2022)**. Major fish

pathogens include *Aeromonas hydrophila*, *Flavobacterium psychrophilum*, and *Flavobacterium columnare* **(Chong *et al.*, 2023)**. Other important Gram-negative pathogens found in fish include *Vibrio* spp., *Pseudomonas* spp., *Aeromonas* spp., and *Salmonella* spp. **(Parlapani *et al.*, 2023)**. In addition to threatening fish health, pathogenic bacteria may also compromise food security and public health by contaminating fish products and causing illness in humans **(Yacoub *et al.*, 2023)**.

Both Gram-negative and Gram-positive bacteria are implicated in fish diseases. Common Gram-negative pathogens include the Aeromonadaceae, Vibrionaceae, Pseudomonadaceae, Enterobacteriaceae, and Hafniaceae families **(Kumar *et al.*, 2023)**, while Gram-positive pathogens include the Mycobacteriaceae, Streptococcaceae, and Erysipelothricaceae families **(Ziarati *et al.*, 2022)**. For example, *Streptococcus agalactiae* causes streptococcosis in hillstream fisheries **(Verma *et al.*, 2022)**. Other bacterial pathogens such as *Edwardsiella*, *Flavobacterium*, and *Mycobacterium* are also responsible for major fish diseases **(Chong *et al.*, 2023)**. These pathogens not only threaten fish populations but can also be transmitted to humans through the consumption of contaminated fish, causing zoonotic diseases such as tuberculosis, leprosy, and cholera **(Hajam *et al.*, 2022; Ziarati *et al.*, 2022)**. Proper fish processing and cooking are therefore essential to reduce the risks of such diseases.

Given these concerns, it is necessary to conduct research aimed at identifying pathogenic bacteria in fish. One widely used method is molecular identification using the 16S rRNA gene. This technique employs genus-specific primers to rapidly evaluate the most abundant bacterial genera in fish and water samples **(Duman *et al.*, 2022)**. Primers have been designed for 11 genera, including *Alkalimarinus*, *Colwellia*, *Enterovibrio*, *Marinomonas*, *Massilia*, *Oleispira*, *Phaeobacter*, *Photobacterium*, *Polaribacterium*, *Pseudomonas*, and *Psychrobium* **(Testerman *et al.*, 2021)**. Their specificity has been confirmed through phylogenetic analysis of sequenced PCR amplicons **(Sardjito *et al.*, 2022)**. Quantitative PCR (qPCR) using these genus-specific primers correlates well with results from 16S rRNA gene sequencing with universal primers **(Najafpour *et al.*, 2022)**. This approach provides a rapid and cost-effective method for identifying the most abundant bacterial genera in fish-related samples **(Piamsomboon *et al.*, 2020)**.

Accordingly, the present study focused on identifying pathogenic bacteria in fish collected from fish markets using the 16S rRNA gene.

MATERIALS AND METHODS

Sample collection

Thirty grouper fish (*Epinephelus* sp.) were collected from fish markets in Central Tapanuli Regency for bacterial identification. The organs used for bacterial isolation included the liver, muscle, and intestine. Each organ was aseptically dissected and streaked onto TSA (Tryptone Soya Agar) and BHIA (Brain Heart Infusion Agar) media. Plates were incubated at 28 °C for 24–48 hours under sterile conditions. Morphological

characteristics of purified colonies were observed macroscopically, followed by biochemical tests including Gram staining, motility, oxidase, catalase, oxidative-fermentative (O/F) tests, selective media assays, and API 20E kit analysis.

Molecular identification

Molecular identification was performed using the Polymerase Chain Reaction (PCR) method. DNA from selected isolates was extracted using the boiling method **(Suwanto *et al.*, 2000)**. A single colony was suspended in 500µL of sterile distilled water, heated at 98°C for 10 minutes, and centrifuged at 10,000 rpm for 10 minutes. The resulting supernatant (300µL) was collected and used to measure DNA concentration and purity with a Nanodrop spectrophotometer.

PCR amplification was carried out using a reaction mixture containing 12.5µL of Mastermix, 8.5µL of distilled water, 2µL of forward and reverse primers, and 2µL of bacterial DNA template. Amplification was performed in a thermocycler with the following program: initial denaturation at 95°C for 2min; denaturation at 95°C for 1min; annealing at 55°C for 1min; extension at 72°C for 1min; and a final extension at 72°C for 5min. Universal bacterial primers were used: forward primer 63f (5'-CAGGCCTAACACATGCAAGTA-3') and reverse primer 1387r (5'-GGGCGGWTGGTACAAGGC-3') **(Marchesi *et al.*, 1998)**. PCR products were electrophoresed and visualized using a gel documentation system.

Data analysis

Forward and reverse DNA sequences were assembled into contigs using the DNASTAR program **(Burland, 2000)** to obtain complete sequences. Pathogenic bacterial DNA sequences were compared against the NCBI database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine sequence similarity. Multiple sequence alignment was conducted using Clustal X 2.0 **(Thompson *et al.*, 1997)** and edited with BIOEDIT **(Hall, 1999)**. Nucleotide sequences were translated into amino acids using the in silico translation tool (http://insilico.ehu.es/translate).

Genetic distance was calculated using the Kimura 2-Parameter (K2P) model with 1000 bootstrap replications in MEGA XI **(Tamura *et al.*, 2021)**. Phylogenetic trees were constructed using the Neighbor-Joining (NJ) method with 1000 bootstrap repetitions in MEGA XI.

RESULTS

Of the 30 samples isolated, only six were successfully identified based on sequence similarity through BLAST analysis. Sequencing of the 16S rRNA gene produced sequence lengths ranging from 648 to 839 bp. BLAST analysis revealed that the isolates belong to three species of pathogenic bacteria. The detailed BLAST results are presented in Table (1).

Table 1. Percentage of sequence similarity based on BLAST analysis

Sample code	*Species name*	*Similarity percentage (%)*
KRP 01	*Aeromonas hydrophila*	100.00
KRP 02	*Staphylococcus aureus*	98.34
KRP 03	*Staphylococcus sciuri*	100.00
KRP 04	*Staphylococcus sciuri*	100.00
KRP 05	*Aeromonas hydrophila*	100.00
KRP 06	*Staphylococcus aureus*	98.34

Sequence analysis showed that samples KRP 01 and KRP 05 had AT content of 43.9–44.8% and GC content of 55.2–56.1%, and were identified as *Aeromonas hydrophila*. Samples KRP 02 and KRP 06 had AT content of 48.2–49.3% and GC content of 50.6–51.8%, and were identified as *Staphylococcus aureus*. Meanwhile, samples KRP 03 and KRP 04 had AT content of 48.1–48.3% and GC content of 51.7–52.0%, and were identified as *Staphylococcus sciuri* (Table 2).

Table 2. Percentage (%) of nucleotide base composition

Kode Sample	A (%)	T (U) (%)	G (%)	C (%)	AT (%)	GC (%)
KRP 01	25.2	19.6	33.2	22.0	44.8	55.2
KRP 02	27.9	21.4	30.1	20.5	49.3	50.6
KRP 03	27.3	21.0	30.5	21.2	48.3	51.7
KRP 04	27.1	21.0	30.8	21.2	48.1	52.0
KRP 05	24.9	19.0	34.1	22.0	43.9	56.1
KRP 06	27.2	21.0	30.8	21.0	48.2	51.8

DISCUSSION

The genetic distance between samples KRP 01 and KRP 05 and the GenBank sequence AJ518825.1 (*A. hydrophila*) ranged from 0.000 to 0.023. Similarly, sample KRP 02 showed a genetic distance of 0.000 when compared with GenBank sequence LC798341.1 (*S. aureus*). Meanwhile, samples KRP 03 and KRP 04 had a genetic distance of 0.000–0.016 when compared with GenBank sequence AM884572.1 (*S. sciuri*) (Table 3).

Table 3. The genetic distance of pathogenic bacteria isolated from grouper fish and Genbank data based on the Kimura 2- parameter model

No	Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	LC798342.1_*S.aureus*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
2	LC798341.1_*S.aureus*	0.027	*	*	*	*	*	*	*	*	*	*	*	*	*	*
3	KRP_04_Sequence	0.068	0.042	*	*	*	*	*	*	*	*	*	*	*	*	*
4	KRP_03_Sequence	0.087	0.060	0.016	*	*	*	*	*	*	*	*	*	*	*	*
5	KF918715.1_*S.sciuri*	0.068	0.042	0.000	0.016	*	*	*	*	*	*	*	*	*	*	*
6	AM884572.1_*S.sciuri*	0.087	0.060	0.016	0.000	0.016	*	*	*	*	*	*	*	*	*	*
7	AM778178.1_*S.sciuri*	0.068	0.042	0.000	0.016	0.000	0.016	*	*	*	*	*	*	*	*	*
8	LC583798.1_*S.sciuri*	3.126	3.363	3.126	3.235	3.126	3.235	3.126	*	*	*	*	*	*	*	*
9	KRP_06_sequence	0.075	0.054	0.016	0.027	0.016	0.027	0.016	3.235	*	*	*	*	*	*	*
10	KRP_02_sequence	0.027	0.000	0.042	0.060	0.042	0.060	0.042	3.363	0.054	*	*	*	*	*	*
11	AB182002.1_*A.hydrophila*	0.439	0.412	0.393	0.421	0.393	0.421	0.393	4.697	0.425	0.412	*	*	*	*	*
12	AB182021.1_*A.hydrophila*	0.439	0.412	0.393	0.421	0.393	0.421	0.393	4.697	0.425	0.412	0.000	*	*	*	*
13	AB182043.1_*A.hydrophila*	0.439	0.412	0.393	0.421	0.393	0.421	0.393	4.697	0.425	0.412	0.000	0.000	*	*	*
14	AJ518825.1_*A.hydrophila*	0.421	0.394	0.382	0.409	0.382	0.409	0.382	4.021	0.412	0.394	0.023	0.023	0.023	*	*
15	KRP_01_sequence	0.439	0.412	0.393	0.421	0.393	0.421	0.393	4.697	0.425	0.412	0.000	0.000	0.000	0.023	*
16	KRP_05_sequence	0.421	0.394	0.382	0.409	0.382	0.409	0.382	4.021	0.412	0.394	0.023	0.023	0.023	0.000	0.023

Genetic distance is a measure of variation between populations or species based on their genetic composition. It is commonly used to assess the degree of genetic divergence and provides insights into evolutionary relationships and population structure **(Dogan & Dogan, 2016; Pirany & Manafi, 2016)**. Genetic distance plays an important role in population genetics and evolutionary studies, as it helps reveal the genetic relationships among species **(Benjamin *et al*., 2015)**.

Several approaches have been developed to calculate genetic distance, each with distinct properties and applications **(David *et al*., 2023)**. These methods account for allele frequencies, codon or gene substitutions, and sequence variations to quantify differences between populations or species **(Rodriguez-Fontenla *et al*., 2014)**. The choice of method depends on the research objectives and the characteristics of the populations under study.

In this research, genetic distance values were used to construct a phylogenetic tree in order to determine the clustering patterns of the bacterial isolates (Fig. 1).

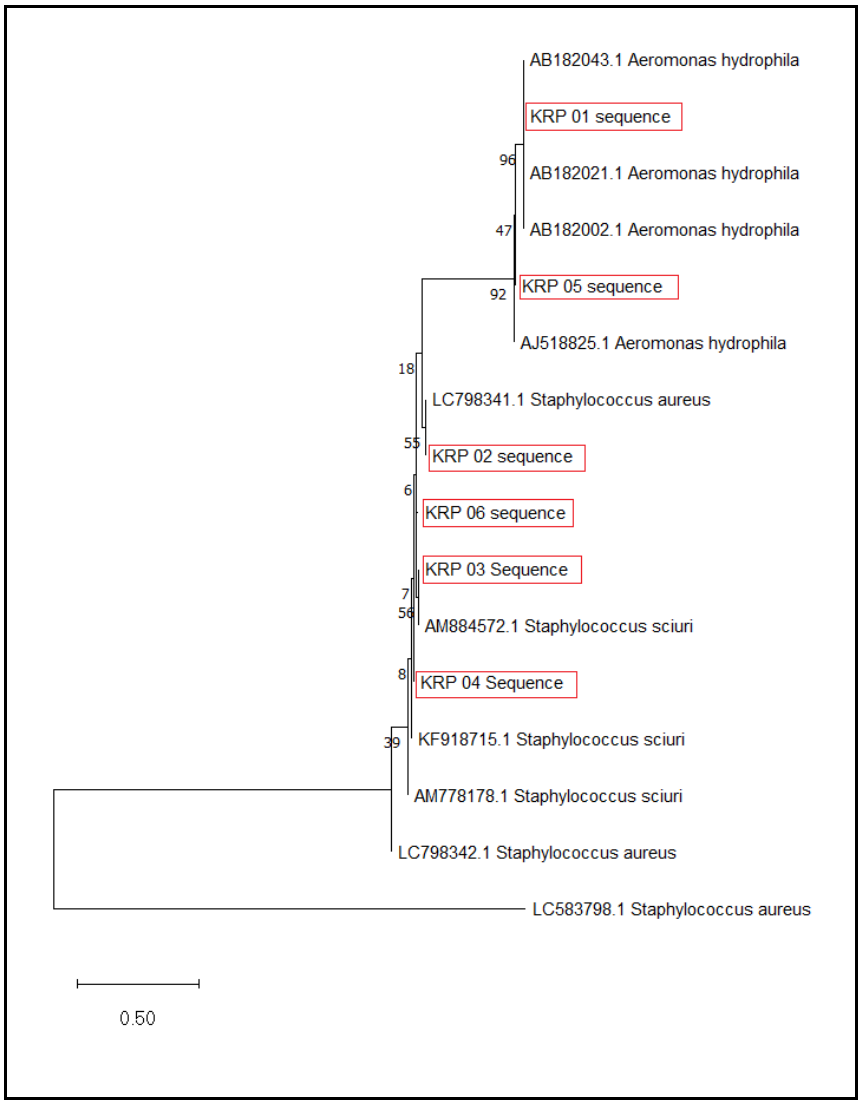

Fig. 1. Phylogenetic tree reconstruction using Neighbor-Joining method with comparison of 10 DNA sequences from Genbank data

Based on the Phylogenetic Tree (Fig. 1), the six bacterial isolates were identified as three pathogenic species: *A. hydrophila*, *S. aureus*, and *S. sciuri*. All isolates had genetic distances below 2% compared with reference sequences in GenBank. According to **Cai *et al.* (2016)**, a genetic distance of less than 2% indicates that the isolates belong to the same species.

Several factors influence genetic distance calculations based on 16S rRNA genes, including alignment quality, calculation methods, sequence masking, and the choice of variable regions. Alignment quality has a major effect on distance estimates, as different alignment approaches can yield varying predictions of genetic diversity **(Schloss, 2010;**

Angermeyer *et al*., 2016). The calculation method used for pairwise genetic distances may also influence results, although its effect is often subtle when assessing richness or phylogenetic diversity **(Sagova-Mareckova *et al*., 2015)**. Applying sequence masks to exclude variable positions can alter genetic distance values by reducing observed richness and phylogenetic diversity **(Ramírez-Moreno *et al*., 2004)**. Furthermore, the choice of variable region may not accurately reflect genetic distances when compared to full-length 16S rRNA sequences **(Keswani & Whitman, 2001)**. These methodological factors must be considered when interpreting phylogenetic and community structure analyses.

CONCLUSION

This study has successfully proven that the 16s rRNA gene effectively identifies pathogenic bacteria isolated from grouper fish samples.

REFERENCES

Angermeyer, A.; Crosby, S. C. and Huber J. A. (2016). Decoupled distance–decay patterns between dsrA and 16S rRNA genes among salt marsh sulfate-reducing bacteria. Environmental Microbiology, 18(1):75-86.

Agbabiaka, T. O.; Adebayo, I. A.; Ajijolakewu, K. A. and Agbabiaka, T. O. (2022). Profilling of common bacterial pathogens in fish, in Book: Bacterial Fish Diseases. Academic Press. London 229-255.

Burland, T. G. (2000). DNASTAR's Lasergene sequence analysis software. Methods in Molecular Biology 132:71-91.

Benjamin, D.; Kaehler; Yap, V. B.; Zhang, R. and Huttley, G. A. (2015). Genetic Distance for a General Non-Stationary Markov Substitution Process. Systematic Biology, 64(2):281-293.

Cai, Y.; Zhang, L.; Wang, Y.; Liu, Q.; Shui, Q.; Yue, B.; Zhang, Z. and Li, J. (2016). Identification of deer species (Cervidae, Cetartiodactyla) in China using mitochondrial cytochrome c oxidase subunit I (mtDNA COI). Mitochondrial DNA Part A: DNA Mapping, Sequencing, and Analysis 27(6):4240-4243.

Chong, M. C.; Shakir, M. Z.; Lai, S. K.; Liew, H. J. and Loh, J. Y. (2023). Microbes and fish diseases in Book: Recent Advances in Aquaculture Microbial Technology, Academic Press, London, Pages 65-102.

David, S.; Campo, A.; Mosa, I. and Khudyakov, Y. (2023). A Novel Information-Theory-Based Genetic Distance That Approximates Phenotypic Differences. Journal of Computational Biology, 30(4): 420–431.

Doğan, I. and Doğan, N. (2016). Genetic Distance Measures: Review. Turkiye Klinikleri Journal of Biostatistics, 8(1):87-93.

Duman, M.; Altun, S. and Saticioglu, I. B. (2022). General Assessment of Approaches to the Identification of Aquatic Bacterial Pathogens: A Methodological Review. North American Journal of Aquaculture, 84(4):405-426

Hajam, Y. A.; Kumar, R.; Rani, R.; Sharma, P. and Diksha. (2022). Efficacy of different treatments available against bacterial pathogens in fish in Book: Bacterial Fish Diseases Academic Press. London 379-398.

Hall, T. A. (1999). BioEdit: A user friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acid Symposium Series 41:95-98.

Keswani, J. and Whitman, W. B. (2001). Relationship of 16S rRNA sequence similarity to DNA hybridization in prokaryotes. International Journal of Systematic and Evolutionary Microbiology, 51(2): 667–678.

Kumar, R.; Sonika, R.; Gusain, M. P. and Gusain, O. P. (2023). The emergence of pathogenic bacteria in coldwater ichthyofauna: A Review. Journal of mountain research, 18(1):333-344.

Marchesi, J. R.; Sato, T.; Weightman, A. J.; Martin, T. A.; Fry, J. C.; Hiom, S. J. and Wade, W. G. (1998). Design and evaluation of useful bacterium specific PCR primer that amplify genes coding for bacterial 16S rRNA. Applied and Environmental Microbiology, 64:795-799.

Najafpour, B.; Pinto, P. I. S.; Canário, A. V. M. and Power, D. M. (2022). Quantifying dominant bacterial genera detected in metagenomic data from fish eggs and larvae using genus-specific primers. MicrobiologyOpen 11(3):e1274.

Parlapani, F. F.; Boziaris, I. S. and DeWitt, C. A. (2023). Pathogens and their sources in freshwater fish, sea finfish, shellfish, and algae in Book: Present Knowledge in Food Safety, Academic Press, London, Pages 471-492.

Piamsomboon, P.; Jaresitthikunchai, J.; Hung, T. Q.; Roytrakul, S. and Wongtavatchai, J. (2020). Identification of bacterial pathogens in cultured fish with a custom peptide database constructed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). BMC Veterinary Research, 16(52):1-10.

Pirany, N. and Manafi, M. (2016). Applying Different Genetic Distances and UPGMA Method to Find the Best Topology in Chicken Populations Using Microsatellite Markers. Genetics in the Third Millennium, 13(4):4106-4111.

Ramírez-Moreno, S.; Méndez-Álvarez, S.; Martínez-Alonso, M.; Esteve, I. and Gaju, N. (2004). Factors Affecting Interpretation of Restriction Fragment Length Polymorphism (RFLP) Patterns from PCR-Amplified Bacterial 16S rRNA Genes: Operon Number and Primer Mismatching. Current Microbiology, 48(4):285-90.

Rodriguez-Fontenla, C.; Calaza, M. and Gonzalez, A. (2014). Genetic distance as an alternative to physical distance for definition of gene units in association studies. BMC Genomics, 15(1):408-408

Sagova-Mareckova, M.; Ulanova, D.; Sanderova, P.; Omelka, M.; Kamenik, Z.; Olšovská, J. and Kopecky, J. (2015). Phylogenetic relatedness determined between antibiotic resistance and 16S rRNA genes in actinobacteria. BMC Microbiology, 15(81):1-13.

Sarjito, S.; Ariyati, R. W.; Desrina.; Haditomo, A. C.; Budiarjo, A.; Sabdaningsih, A. and Prayitno, S. B. (2022). Molecular identification of causative bacterial disease in giant gourami (*Osphronemus goramy*) based on 16S rRNA gene from extensive fishpond in Banjarnegara Regency, Indonesia. Egyptian journal of aquatic biology and fisheries, 26(6):555-566.

Schloss, P.D. (2010). The Effects of Alignment Quality, Distance Calculation Method, Sequence Filtering, and Region on the Analysis of 16S rRNA Gene-Based Studies. PLOS Computational Biology, 6(7):e1000844.

Suwanto, A.; Suryanto, D. Y.; Tan, I. & Puspitasari, E. (2000). Selected protocols. Training Course on Advances in Molecular Biology Tech niques to Assess Microbial Diversity. Bogor, 28 pp.

Tamura, K.; Stecher, G. and Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution 38(7):3022-3027.

Testerman, T.; Beka, L.; McClure, E. A.; Reichley, S. R.; King, S.; Welch, T. J. and Graf J. (2021). Detecting Flavobacterial Fish Pathogens in the Environment Using High-Throughput Community Analysis. Applied and environmental microbiology 88(2):1-14.

Thompson, J. D.; Gibson, T. J.; Plewniak, F.; Jeanmougin, F. and Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25(24):4876-4882.

Verma, R. K.; Sankhla, S. M.; Jadhav, S.; Parihar, K.; Gulliya, S.; Kumar, R. and Sonone S.S. (2022). Global Status of bacterial fish diseases in relation to aquatic pollution, in book: Bacterial Fish Diseases Academic Press. London 155-182.

Yacoub, O.; Torky, H. and Tawfik, R. (2023). Fish Bacterial Pathogen in Gills, Skin, Kidney, Intestines, and its Water at Elmahmoudia and Edfina. Alexandria journal of veterinary sciences, 76(2):58-62.

Ziarati, M.; Zorriehzahra, M. J.; Hassantabar, F.; Mehrabi, Z.; Dhawan, M.; Sharun, K.; Emran, T. B.; Dhama, K.; Chaicumpa, W. and Shamsi S. (2022). Zoonotic diseases of fish and their prevention and control. Veterinary Quarterly, 42(1):95-118.