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ORIGINAL ARTICLE

Isolation and Identification of Bacteria from Freshwater Fish *Glossogobius giurus* (Hamilton, 1801) From YSR Kadapa District, Andhra Pradesh, India

[1]Rajeswari Dasari, [2*]Anu Prasanna Vankara, [3]Chandrasekhar Thummala and [4]Vijayalakshmi Degati

[1,2]Department of Zoology, Yogi Vemana University, YSR Kadapa, Andhra Pradesh-516005
[3]Department of Environmental Science, Yogi Vemana University, YSR Kadapa, Andhra Pradesh-516005
[4]Department of Microbiology, Yogi Vemana University, YSR Kadapa, Andhra Pradesh-516005
***Corresponding author:** annuprasanna@gmail.com and annuprasanna@yvu.edu.in

ABSTRACT

The present survey was conducted to evaluate the health and hygiene of freshwater Tank goby 'Glossogobius giurus' by examining the prevalence of bacteria, which is a vital indication of fish quality. From January to March 2023, fishes were bought biweekly from YSR Kadapa's local fish markets and their skin was thoroughly examined. The bacterium species Enterobacter cloacae was discovered using biochemical testing (IMViC testing) and molecular analysis. The findings of this study demonstrated that raw fish sold-out in local fish markets of YSR Kadapa region has high microbial contamination, highlighting the need to strengthen the standards of quality control systems in the fish markets.
***Keywords**: Glossogobius giurus, Bacteria, Enterobacter cloacae, Identification, YSR Kadapa.*

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INTRODUCTION

Approximately 17% of the global population considers fish as an essential source of protein because of their crucial role in food safety, nutrition, and income generation [1, 2]. They own an excellent source of protein rich in vital nutrients, such as vitamins (A, C, D, E, K and Vitamin-B complex), minerals (calcium, fluorine, iron, phosphorous, selenium), and essential amino acids and fatty acids (docosahexaenoic acid-DHA, Omega 3-fatty acids- eicosapentaenoic acid- EPA and PUFAs [3]. As one of the most accessible and reasonably priced forms of animal protein, raw fish is considered a staple food for the underprivileged worldwide. But, the consumption of cryo-preserved fish and shellfish may develop some diseases to the human beings due to bacterial infections or food poisoning. The bacteria found on the fish's slime, gills, stomach and among other exterior surfaces are the prime cause of illness in humans. The natural defense mechanism in the live fish prevents these bacteria from penetrating the sterile flesh, however on dead fish; the microbes or their toxins can easily spread into the animal tissue and interact with a composite mixture of naturally occurring molecules to steadily degrade the odoriferous and flavorous components of the fish flesh. There are numerous types of bacterial pathogens which are saprophytic in nature and are disease causing agents. Fish bacterial pathogens can be classified as Indigenous and non-indigenous types [4]. Indigenous types occur naturally in fish's habitat (*Aeromonas* sp., *Vibrio* sp.) while the non-indigenous types alter the fish and its habitat in one way or the other (*Salmonella typhi, Escherchia coli, Shigella dysenteriae, Staphylococcus aureus* etc.) [5,6]. Fish become susceptible to both indigenous and non-indigenous bacterial infection when they are nutritionally under-supplied, physiologically unstable and exposed to external stressors such poor water quality, stress due to overcrowding etc and these bacterial pathogens inturn become potentially pathogenic to humans [7-9]. *Glossogobius giurus*, commonly known as Tank goby is a native fish of Gobiidae inhabiting freshwaters and brackish waters, small pond and

ditches [10]. It is highly esteemed as food and is largely caught as sporting with rod and line using small prawns as bait. It has an ecological role as they feed on bottom dwelling fish, small crustaceans, rotten plants, insects, algae, worms etc. [11]. The bacteria on the fish are often related to those in their natural habitat and are influenced by the time of year and the circumstances during harvest and these bacteria can act as indicators of fish quality [12, 13]. Yagoub (2009) emphasized that the risks associated in fish handling during aquaculture, capture fisheries and the environmental toxins in fish-food might pose a severe risk to human health [14]. There are studies which emphasize on fresh fish quality in retail fish marketplaces [15-17]. It is very noticeable that there is a requisite to make quality standards because the quality of raw fish (fresh or chilled) at market places varies so much [18, 19]. There has been no research towards the microbial identification of these fishes. Hence, the goal of this study was to discover specific microbiological analyses for fish (*G. giurus*) sold-out at local markets in YSR Kadapa, Andhra Pradesh, India.

MATERIAL AND METHODS

Fish sampling

The *G. giurus* was obtained from local fish vendors in the fish market in YSR Kadapa. From January to March 2023, about 20 samples were collected biweekly and were immediately transported to the laboratory in a thermal bag and was processed within 3 hours of collection and stored in the refrigerator (4-8°C) (Figs. 1-4).

Sample preparation

A sterile blade was used to cut the 10 g fish sample off the skin's surface. The sliced samples were crushed into small pieces in a sterile mortar before being homogenized with approximately 10 mL of sterile water. Then, 1 ml of homogenized material was added to a dry sterile beaker containing 9 ml of distilled water, yielding in a 1:10 dilution. This was done for each of the twenty fish samples.

Isolation and Identification of bacteria from fish samples of *G. giurus*

The collected samples were processed under complete aseptic circumstances in the research laboratory, Department of Zoology, Yogi Vemana University, YSR Kadapa, Andhra Pradesh, India using conventional microbiological procedures. The swabs were inoculated on MacConkey agar and incubated at 37°C in an aerobic environment [20]. Various physiological, biochemical, and morphological parameters were used to identify the isolated bacterial colonies. These cultures were subjected to a battery of biochemical tests, including citrate, carbohydrate fermentation, gram staining, hydrogen sulfide production, indole, methyl red, motility, spore staining, voges proskauer, Triple sugar ion and oxidase tests to identify the phosphate solubilizing bacteria following the protocols from Bergey's manual of systematic bacteriology [21]. Bacteria like *Enterobacter* etc. were isolated from the fish sample.

Molecular Characterization of Bacterial Isolate

Purified bacterial isolates were inoculated on nutrient agar and cultured at 37°C for 24 hours prior to DNA extraction. Genomic DNA was extracted from the sample provided to Biokart India Pvt. Ltd following the standard protocol (YVUMB04). The sample was homogenized with 1ml of extraction buffer and the homogenate was transferred to a 2 ml microfuge tube, to which 1ml of Phenol: Chloroform: Isoamylalcohol (25:24:1) was added and centrifuged at 14000 rpm at room temperature for 15 minutes. The supernatant was taken into a new tube and an equal volume of chloroform: isoamylalcohol (24:1) was added and centrifuged again. The supernatant was transferred to another test tube to which 0.1 volume of 3M sodium acetate (pH 7.0) and 0.7 volume of isopropanol was added to precipitate the DNA. After 15 minutes of incubation at room temperature, the tubes were centrifuged at 4ºC for 15 minutes at 14,000 rpm. The DNA pellet was rinsed twice with 70% ethanol, then briefly with 100% ethanol before air drying. The DNA was dissolved in TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA). To eliminate RNA, add 5 µl of DNAse-free RNAse A (10 mg/ml) to the DNA. About, 169 ng/µl of extracted DNA (1.5kbp, 16s rDNA) was used for amplification along with 10pM of each primer and high-fidelity PCR polymerase. The 16S primers 16s forward primer (5′ GGATGAGCCCGCGGCCTA -3′) and 16s reverse primer (5′-CGGTGTGTACAAGGCCCGG -3′). The PCR amplification was done in a total reaction volume of 50µl TAQ master mix containing 1 µl DNA template, 10 µl of 10X Taq DNA polymerase assay buffer (MgCl2), 1 µl of Taq DNA polymerase enzyme (3U/ml), 4 µl of dNTPs (2.5mM each), 2 µl of each primer, and 30 µl PCR water. Amplification conditions included initial denaturation at 94°C for 3 minutes, followed by 1 minute at 94°C, 1 minute of annealing at 50°C, and 2 minutes of extension at 72°C. This was followed by a final extension at 72°C for 7 minutes in 30 cycles. The PCR products were sequenced bidirectionally using an automated DNA sequencer, ABI 3130 Genetic Analyzer, Big Dye Terminator version 3.1" Chemistry Cycle sequencing kit, POP_7 pol Capillary Array, BDTv3-KB-Denovo_v 5.2 analysis methodology, and Seq Scape_v 5.2 data analysis and Applied Biosystem Micro Amp Optical 96-well reaction plate software

reaction plate was used to analyze the forward and reverse sequences of the bacterial 16S rRNA gene-based sequence data. The sequence data was aligned and examined to identify the bacteria and its nearest neighbors using BLAST, which is available on the NCBI website. The matched organism sequences were collected from the same database and served as reference sequences. The MegaX software's CLUSTAL W option was used to do multiple sequence alignments. The phylogenetic analysis was performed using the Tamura-Nei neighbor-joining method with 1000 bootstrap replications, and the phylogenetic tree was created using the Phylogenetic Tree Builder [22, 23]. The generated consensus sequence was uploaded to the NCBI Gene Bank database and assigned an accession number (SUB13984602-OR864299).

RESULT AND DISCUSSION

Fish and shellfish are extremely perishable and vulnerable to considerable quality variations due to differences in species, ambient conditions, and feeding habits. They can also transmit a wide range of microbiological and other health hazards. As a result, quality control is crucial in the production and trade of fisheries products. Although just a few infectious agents in fish can infect humans, there are several exceptions that can be fatal. The greatest risk to human health, however, is the consumption of raw or improperly prepared fish and fish products. Bio-oxidation processes are extremely significant in bacteria. These processes aid bacteria in producing energy through oxidation of organic molecules or by fermentation. The bacteria were identified based on these responses, and the findings are shown in (Table-1; Figs. 5-19). An *Enterobacter cloaca (E. cloacae)* was detected from the fish in this investigation. *E. cloacae* is a gram-negative opportunistic bacterium belonging to the family *Enterobacteriaceae* with a worldwide distribution in aquatic and terrestrial environments and cause a range of infections in various body organs such as lower respiratory tract, urinary tract, skin, bile ducts, soft tissues and central nervous system of both animals and human beings [24, 25]. *E. cloacae* was recovered from 78% of the collected fish samples in this investigation, which is relevant since this bacteria plays an important role as a potentially hazardous bacterium for humans and as an indicator of food quality as a rotting organism [26-28].

Molecular identification and phylogenetic relationship among the gene sequencing of bacteria

The samples were subjected to bacteriological examination and evaluated the isolated culture by various biochemical tests which led to the confirmation of isolated bacterial species close to *Enterobacter* species. Further the evaluated isolates were tested with PCR assay using 16s rRNA. The confirmation process of the *Enterobacter cloacae* isolate recovered from fish sample and detected the presence of similar 16s rRNA genes. All the isolates possessed this gene equal to target product size 1387 bp (Fig.20). Microheterogenicity is essential to the microbiologist as it allows the ability to identify the important pathogenicity, phenotype and nitch difference between strains which is also helpful for exploitation of strain tracking and epidemiogical research. Based on the 16s rRNA gene sequence analogs, the phylogenetic tree has been constructed. As shown in the figure, the sequencing data is validated by building a maximum likelihood phylogenetic tree which is anticipated as a common branch. For instance, the maximum likelihood principle enlightens the probability of evolutionary changes describing data observed as origin. The present method (Neighbour-joining method) is used to construct the initial tree. To maximize the likelihood and for the designed evolutionary model, each branch length is tentatively considered as gene distances among the related species within their evolutionary relationship will be intimated in the form of tree topology. According to the sequence of 16s rRNA, the isolated strain of *Enterobacter* was more closely related to other *Enterobacter* strains (99% similarity). The test organism showed (99.27%) similarly of the 16s rRNA sequencing of *Enterobacter cloacae* strain ATCC13047 (Sequence ID: NR 118568.1) and (99.20%) similarity with the next closest homologue *Enterobacter sichuanensis* strain WCHECL1597 16s ribosomal RNA (Sequence ID: NR_179946.1) (Table-2). Hence the isolated organism has been proved to be *Enterobacter cloacae* strain based on phylogenetic tree analysis (Fig-21). The current findings of the study, together with a few other studies, imply that the microbiological quality of fish provide a possible concern to public health and may alter the immune-compromised communities of people [13, 17]. According to the findings of this investigation, raw fish marketed in local fish markets in YSR Kadapa has high contamination, which may be due to factors such as temperature, water quality of River Penna where the fishes are collected from and personal hygiene of fish handlers and maintenance of fish in contaminated waters might cause the microbial contamination in the fish. Hence, the present investigation suggests that there is an imperative need to reinforce the quality control mechanisms in the local fish markets of YSR Kadapa District, Andhra Pradesh.

Table 1: Bacteria isolated from the *G. giurus* purchased on first week of January 2023

Microorganism	Gram staining	Motility	Indole production	Methyl red	Voges-Proskauer	Catalase	Citrates	Mannitol	Urease	H_2S production
Enterobacter sp.	+	-	-	-	-	+	-	+	+	-

+: Positive; -: Negative; D: Different

Table 2: Blast data generated for the bacteria isolated from the *G. giurus*

Sl. No.	Organism Name	Accession No.	% Match
1	*Enterobacter cloacae* strain ATCC 13047 16S ribosomal RNA	NR_118568.1	99.27%
2	*Enterobacter sichuanensis* strain WCHECL1597 16S ribosomal RNA	NR_179946.1	99.20%
3	*Enterobacter hormaechei* strain 0992-77 16S ribosomal RNA	NR_042154.1	99.10%
4	*Enterobacter quasihormaechei* strain WCHEs120003 16S ribosomal RNA	NR_180451.1	99.06%
5	*Enterobacter hormaechei subsp. xiangfangensis* strain 10-17 16S ribosomal RNA	NR_126208.1	99.06%
6	*Enterobacter mori* strain YIM Hb-3 16S ribosomal RNA	NR_146667.2	99.06%
7	*Enterobacter quasiroggenkampii* strain WCHECL1060 16S ribosomal RNA	NR_179166.1	99.06%
8	*Enterobacter chuandaensis* strain 090028 16S ribosomal RNA	NR_180237.1	99.05%
9	*Silvania confinis* strain H4N4 16S ribosomal RNA	NR_184632.1	99.03%
10	*Enterobacter chengduensis* strain WCHECl-C4 16S ribosomal RNA	NR_179167.1	98.98%

1

2

3

4

Fig. 1: Map of Southern India showing the flow of River Penna into the Bay of Bengal; Fig. 2: Photograph of River Penna near Adinimmayepalli dam, Chennur, YSR Kadapa district; Fig.3: Live Fish, *Glossogobius giurus* maintained in fish tanks; Fig.4: *Glossogobius giurus* Hamilton, 1801

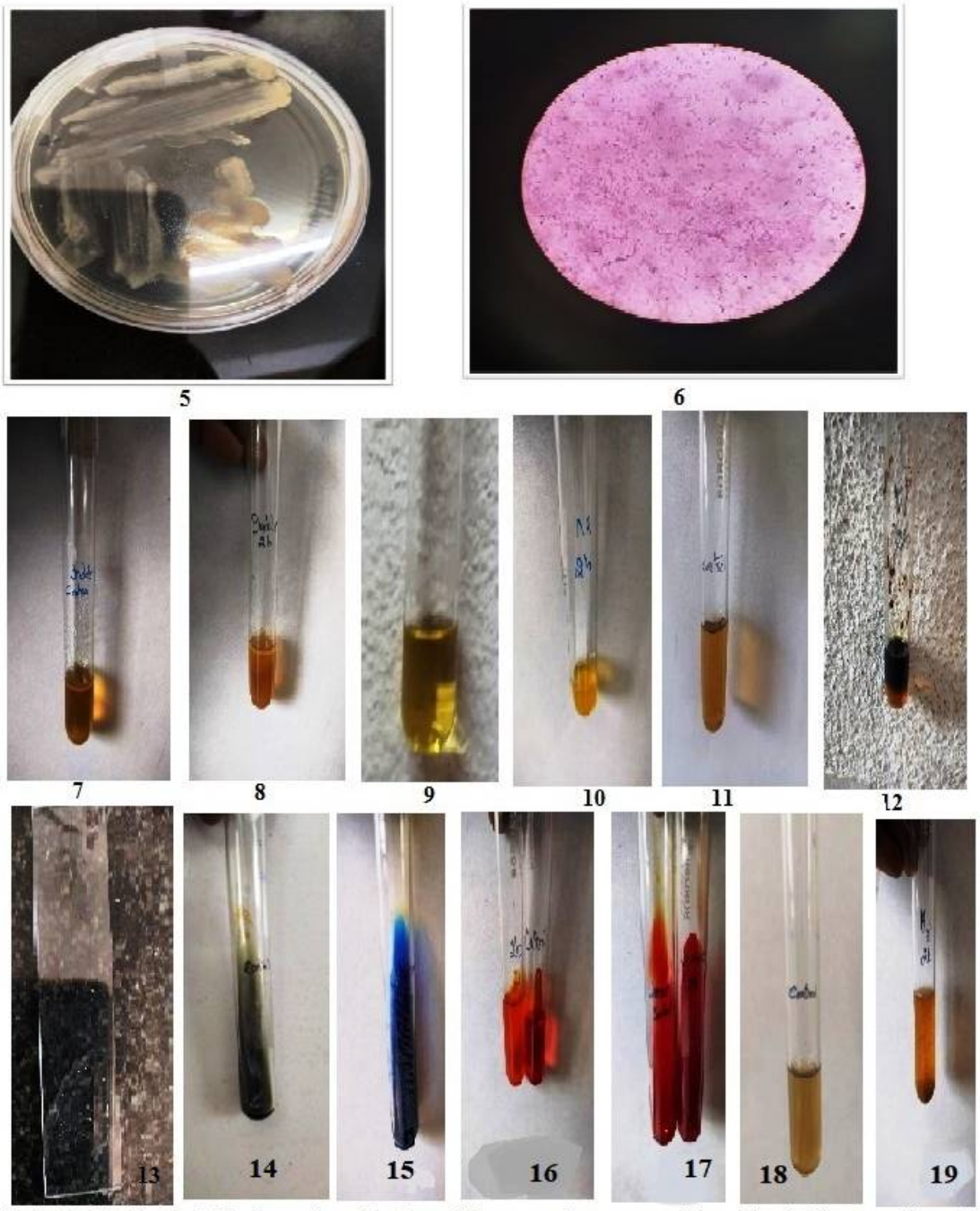

Fig. 5: Purified bacterial isolates from *G. giurus* fish on nutrient agar medium; Fig. 6: Gram staining of *Enterobacter* sp. rods; Fig.7: Indole reaction- control; Fig.8: Indole reaction with pathogen (-ve); Fig.9: Methyl red reaction- control; Fig.10: Methyl red reaction with pathogens (-ve); Fig.11: Voges-Proskauer reaction- control; Fig.12: Voges-Proskauer reaction with pathogens (-ve); Fig.13: Catalase test (+ve); Fig.14: Citrate test- control; Fig.15: Citrate test with pathogen (-ve); Fig.16: Mannitol test with control and pathogen (+ve); Fig.17: Urease test with control and pathogen (+ve); Fig.18: H_2S test – control; Fig.19: H_2S test with pathogen (-ve)

Fig.20: **Aligned sequence data of *Enterobacter cloacae* isolated from the *G. giurus***

Aligned Sequence Data of Sample – YVUMB04 **(1387 bp)**

>YVUMB04

AAGGTTAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGT
ATTCACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGG
ACTACGACGCACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTG
TAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTT
GAGTTCCCGGCCGAACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAA
CACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCT
GGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCC
GTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCAC
GCCTTCAAGGGCACAACCTCCAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCC
CACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGC
ATTTCACCGCTACACCTGGAAATTCTACCCCCCTCTACAAGACTCTAGCCTGCCAGTTTCGAATGCAGTTCCCAGG
TTGAGCCCGGGGATTTCACATCCGACTTGACAGACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCT
TGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATTGCTGAG
GTTATTAACCTCATCACCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGG
CTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTC
CAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCGTTACCCCACCTACTAGCTAAT
CCCATCTGGGCACATCCGATGGCAAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGTATTAGCT
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GCAGCAAGCTGCTTCCTGTTACCG

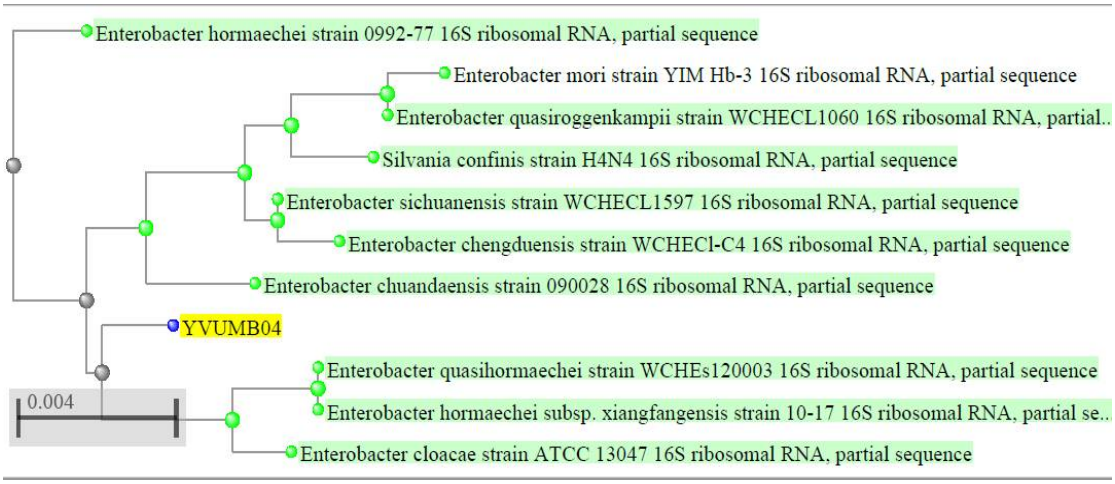

Fig.21: **Phylogenetic tree generated for the bacteria isolated from the *G. giurus***

CONCLUSION

Fish, being a crucial source of protein, minerals, and vitamins, has earned significant commercial importance around the globe. However, their sensitivity to different fish diseases caused by bacteria, viruses, fungus, and parasites has made them a source of concern in intensive aquaculture due to economic losses. The presence of bacterial pathogens in the fish affects the quality and freshness of the fish and might pose a potential risk to fish eaters.

Ethical approval

Ethics required is approved by the Ethical Committee of the Yogi Vemana University to carry out the fish handling procedures.

Consent to participate

The authors all participated in the work.

Consent to publish

The authors certify that the publisher is permitted to publish this work.

Conflict of interest
The authors declare no competing interests.

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