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Isolation and identification of marine fish tumour (odontoma) associated bacteria

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ABSTRACT

Objective: To identify fish tumour associated bacteria.
Methods: The marine fish *Sphyraena jello* with odontoma was collected from in Tamil Nadu (Southeast India), and tumour associated bacteria were isolated. Then the isolated bacteria were identified based on molecular characters.
Results: A total of 4 different bacterial species were isolated from tumour tissue. The bacterial species were *Bacillus* sp., *Pontibacter* sp., *Burkholderia* sp. and *Macrococcus* sp., and the sequences were submitted in DNA Data Bank of Japan with accession numbers of AB859240, AB859241, AB859242 and AB859243 respectively.
Conclusions: Four different bacterial species were isolated from *Sphyraena jello*, but the role of bacteria within tumour needs to be further investigated.

1. Introduction

The bacterial disease in fish was first reported in 1957, affecting cultured rainbow trout in Japan[1]. Relationship between fishes and bacteria varies because of the diverse range of fish species, the environment, the fish habitat and the variety of metabolic requirements of bacteria. Bacteria can colonize on surfaces of fish eggs, skin, gills, and gastrointestinal tract, while internal organs such as kidney, liver, and spleen of healthy fish are normally free of bacteria[2,3].

Naturally the fish has mucus layer on the above mentioned surfaces, which serves as a protective layer that can provide adhesion sites for bacteria[3]. The bacterium of *Macrococus* sp. is a member of the staphylococcal families. The genus *Macrococcus* has been described on the basis of comparative 16S rRNA analysis, DNA-DNA hybridization studies, ribotype patterns, cell wall composition, and phenotypic characteristics[4]. It was reported that probiotic activity of protease producing *Pontibacter* sp. influenced on fish growth and was isolated from the gastrointestinal tract of fish species[5,6]. *Bacillus* sp. belongs to the Bacillaceae family, which causes various infections in fish; it has been isolated from the skin of sea water fish, and more different species of bacteria have been isolated from the skin of the fresh water fish (catfish) [7-10]. *Burkholderia* is a genus of Proteobacteria that occurs mostly in animals. *Burkholderia cepacia* is an aerobic Gram-negative bacillus found in various aquatic environments[11-13]. The examined marine fish of *Sphyraena jello* (*S. jello*) in our study was frequently affected by mouth tumour (odontoma). We have reported the tumour prevalence in our previous research[14]. Hence in the present study, we focused on isolation and identification of marine fish tumour associated bacteria by using the molecular taxonomy.

2. Materials and methods

2.1. Sample collection

The fish with odontoma was collected from fish landing centers in south east coast of India for isolating the tumour associated bacteria. Nature of the tumour was recorded, then the fish samples were immediately brought to the microbiological laboratory in ice box with temperature at 4 °C within 6–8 h.

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2.2. Tumour bacterial isolation

The handling of specimens was carried out using an aseptic technique on tumor surfaces cleaned with Hycolin phenolic disinfectant (2%, v/v). The tumour tissue was cut with using the sterile surgical blade, and tissues were vortexed in saline water. The tissue specimen was subjected to immediate culture-dependent analysis, spread onto Zobell marine agar plates and incubated at 27 ℃ for 24 h. After incubation, all colony types on the plates were sub-cultured for purity; the cellular morphologies of Gram-stained smears were recorded, and the isolates were stored at -80 ℃ for identification by 16S rRNA gene sequence analysis.

2.3. Molecular identification of isolates

2.3.1. DNA extraction

The isolated pure colonies were used for DNA isolation using the combination of $NaCl_2$ and cetyl-trimethyl ammonium bromide digestion. The isolated colonies were inoculated into nutrient broth; the culture was kept incubated for 12 h at 37 ℃ and centrifuged at 5 000 r/min, and the supernatant were discarded. To the bacterial pellet, 576 μL of lysis buffer and 30 μL of 2.3 mg/mL proteinase K were added and the mixture was kept for incubation at 37 ℃ for 1 h. After the incubation, the mixture was centrifuged at 5 000 r/min for 10 min at 4 ℃. The supernatant were separated into fresh tube and DNA were extracted with chloroform: isoamyl alcohol (24:1) by centrifuging at 10 000 r/min for 5–10 min and supernatant were re-centrifuged with phenol: chloroform: isoamyl alcohol (25:24:1) at 10 000 r/min for 10 min and precipitated with 100 μL of 100% ice cold ethanol and washed with 75% ethanol. The bacterial pellets were dried overnight and re-suspended in 50 μL of T10E1 buffer and the DNA was analyzed through 1% agarose gel electrophoresis with 5 μL of ethidium bromide.

2.3.2. PCR amplification

PCR amplification of the 16S rRNA gene was carried out using universal bacterial primers 27F (5'-AGAGTTTGATCCTTGGCTCAG-3') and 1492R (5'-GCYTACCTTGTTACGACTT-3'). PCR reaction mixtures containing 0.5 μL of 1.2 IU of *Taq* (Merck Inc.), 5 μL of *Taq* buffer along with the 0.5 μL of 10 mmol/L deoxynucleotide solution, 1 μL of forward and reverse primer, 40 μL of Milli-Q water and 2 μL of DNA were incubated in a gradient PCR (Eppendorf Inc.) at 95 ℃ for 1 min initial denaturation and followed by 30 cycles at 94 ℃ for 30 s, 52 ℃ for 1 min, 72 ℃ for 2 min and then final extension period at 72 ℃ for 12 min. The amplicons were loaded onto 1% agarose and the concentration was checked using band intensity through image analysis.

2.3.3. Agarose gel electrophoresis

A total of 100 mL of electrophoresis buffer (1× Tris-acetate-ethylene diamine tetraacetic acid buffer), 0.8 g of electrophoresis grade molten agarose containing 5 μL of ethidium were added. The molten agarose was allowed to cool down to 50–60 ℃ and poured into a gel tray. The gel was allowed to solidify for 20–30 min at room temperature. After removing the comb, a gel casting tray was placed in the electrophoresis tank and sufficient 1× Tris-acetate-ethylene diamine tetraacetic acid buffer was added to immerse the gel completely. DNA samples were mixed with 6× loading dye in the ratio of 1:5, and 5 μL mixture was loaded into the gel wells with a micropipette at the negative electrode. About 3 μL of 100 bp DNA marker was loaded as a control. The leads were connected and voltage was applied, so that DNA could migrate toward the anode. The gel was electrophoresed at 1–10 V/cm of gel length until the dye front had migrated to about 3/4 of the gel. The DNA fragments were visualized using the gel documentation system Gel Logic 1500 (Genei, India).

2.3.4. DNA sequencing, editing and basic local alignment search tool analysis

Sequencing was carried out in a commercial company (Bioserve Biotechnologies Pvt. Ltd., India). The sequences were compared with other sequences in National Centre for Biotechnology Information Database through basic local alignment search tool analysis (http://blast.ncbi.nlm.nih.gov/Blast).

3. Results

3.1. Tumour description

The odontoma was mostly bony, with only a few being soft tissue, and located mostly in the upper jaw, lower jaw and tongue regions of the fish. In some cases, the tumour affected both jaws. Generally a single tumour occupied the entire mouth of the fish, but multiple tumours were also observed in many fishes (Figure 1).

Figure 1. Odontoma in fish.
A: *S. jello* with odontoma; B: Close up view of odontoma.

A total of 4 different bacterial species were isolated from *S. jello* tumour tissue. The isolated bacterium was identified as *Bacillus* sp., *Pontibacter* sp., *Burkholderia* sp., *Macrococcus* sp. based on the molecular characters.

3.2. DNA isolation

The genomic DNA was isolated using phenol-cholorform method and was visualized in 1% of agarose gel (Figure 2).

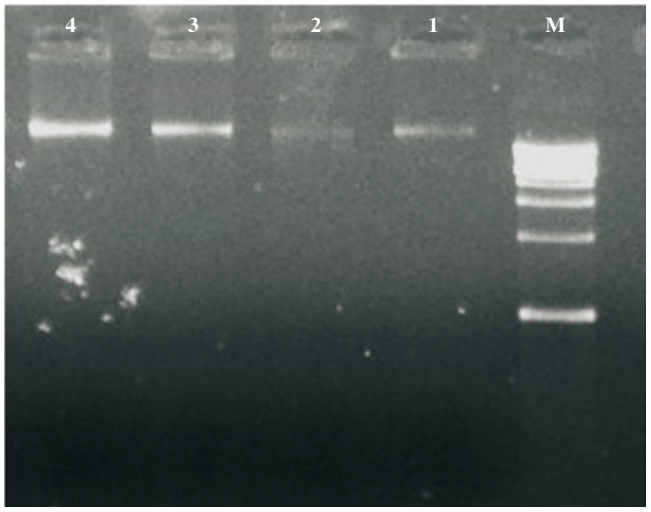

Figure 2. The DNA bands of isolated strains on 1% agarose gel.
Lane M: DNA marker (1 kb); Lane 1 to 4: Isolated DNA from the pure colony.

3.3. PCR amplification

A total of 4 bacterial isolates, from tumour tissue, are subjected to molecular characterization. The DNA isolated from the 4 cultured bacterial isolates were subjected to PCR with 27F and 1492R primer, and the amplification of 16S rRNA gene for 4 isolates is shown in Figure 3.

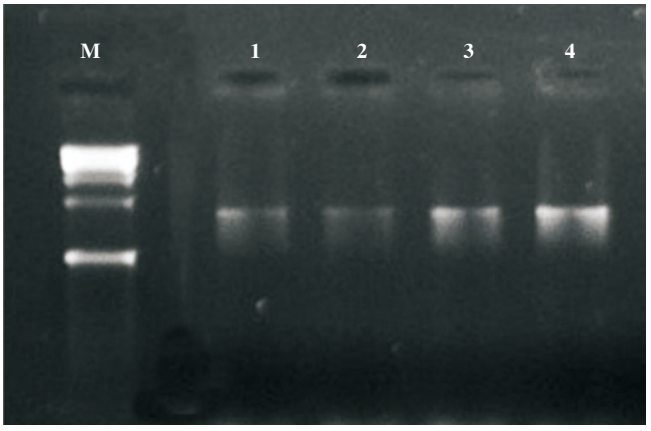

Figure 3. Gel picture showing amplification of 16S rRNA gene for 4 isolates.
Lane M: Ladder (1 kb); Lanes 1-4: Bacterial samples.

3.4. Bacterial stain accession number

The amplicons were sequenced in Applied Biosystems platform and identified as *Bacillus* sp., *Pontibacter* sp., *Burkholderia* sp. and *Macrococcus* sp., and the sequences were submitted in the DNA Data Bank of Japan with accession number of AB859240, AB859241, AB859242, and AB859243 respectively.

4. Discussion

The present study explored the tumour tissue associated bacteria isolated from tumour tissue of *S. jello* through 16S rRNA gene sequences and the bacterial genus were confirmed as *Bacillus* sp., *Macrococcus* sp., *Burkholderia* sp. and *Pontibacter* sp. The occurrence of bacterial disease in fish was common, sometimes due to the secondary infection of fish. Some bacterial species were isolated from some parts of fish organs, for example, the *Aeromonas hydrophila* was isolated from the stomach. *Bacillus pumilus* was isolated from the gonads of *Oreochromis niloticus*, and it has also been isolated from sea animals[15].

Sneath *et al*.[16] and Ghosh *et al*.[17] reported that *Bacillus* species were found in many fishes. Many reports revealed the isolation of *Bacillus* sp. from other fishes like carp, gold fish, catfish, mullet and tilapia[18]. These bacterial species were present in marine environments, but until now there is no report on these bacterial species from fish tumours. Fish tumours have been reported frequently in India as well as worldwide in both marine and fresh water fishes. For example, in India from the same study area, skin tumour was reported by Gopalakrishnan *et al*.[19] in *Sardinella longiceps*, by Jithendran *et al*. in *Muhill cephalus*[20] and mouth tumour by Vijayakumar *et al*.[14] in *S. jello;* they concluded that tumours were unknown aetiology.

This study only focused on identification of the tumour associated bacteria, role of bacteria within the tumour was unknown. According to some reports, the tumour commonly associated bacteria have some important roles within the tumour. The main mechanism was thought to be due to the hypoxic nature of many solid tumours, which results in low oxygen levels compared with normal tissues, providing a unique growth environment for anaerobic and facultative anaerobic bacteria[21].

The tumour-specific bacterial replication involves abnormal neovasculature and local immune suppression. This leaky tumour vasculature may allow bacteria to enter tumour tissue, and embed locally[22]. Furthermore, a variety of mechanisms are employed by tumour cells to avoid recognition by the immune system, resulting in insufficient immune activity within tumours, potentially providing a refuge for bacteria to evade immune clearance[23].

In the present study we concluded that only four different bacteria were associated with odontoma tissue of *S. jello*. The bacterial genus was confirmed by 16S rRNA gene sequencing, but the species level was not confirmed. The bacteria were commonly presented in the marine environments; however, still now there is no report on

the association of these bacteria with any fish tumours, but some reports revealed the same bacterial species which were isolated from diseased fishes. The tumours associated bacterium may have an important role within the tumours or cause any secondary infection to infected fish, but in the present study, the role of these bacteria was unknown. Hence, further investigations are needed to explore the role of bacteria within the fish tumors.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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