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MOLECULAR CHARACTERIZATION OF *MYROIDES GITAMENSIS* FROM FISH SAMPLES AND USE OF BIO PRESERVATIVES

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ABSTRACT: The rapid emergence of AR pathogens associated with fish causes a serious public health concern. The present study focused on the isolation and molecular identification of AR bacteria from three different fish samples of *Cephaloscyllium silasi* (shark), *Sardinella longiceps* (sardine), and *Rastrelliger kanagurta* (mackerel). Among the three fish tried the common pathogen of *Myroides gitamensis* was confirmed by using 16S RNA study, and the sequence was submitted to NCBI, the accession number was MG554743.1. MDR pattern was also carried out against 10 antibiotics, its shown resistant power against the entire antibiotic; highest zone (7mm) was observed only for gatifloxacin and cefotaxime. The bio preservatives from *Lactobacillus acidophophillus* were produced and it's showing better shelf life at room temperature.

KEYWORDS: DNA, PCR, Phylogenetic tree, Antibiotic susceptibility test, Biopreservatives

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1.INTRODUCTION

A huge and extensive source of natural compounds can be retrieved from the marine environment [1], Marine microorganisms exhibit unique metabolic and physiological capabilities conferring them the ability to survive in extreme conditions and consequently produce novel metabolites that cannot be found elsewhere [2]. Hence, the marine environment holds a great promise towards the discovery of novel bioactive and relevant compounds including antibiotics, enzymes, vitamins, drugs and bio surfactants, among others [3]. India is the second largest fish producing nation in the world, exporting to 127 countries. It has the potential to grow further in view of the growing demand in

export to European Union (EU), United States (US), China and Middle East. Hence seafood quality presently is most pivotal aspect of seafood trade. The main impediments in the proper utilization of the seafood are its high perishability and health risk due to contaminated pathogens [4]. Microbial contamination is a major problem as evidenced from the reports on a worldwide basis. Microbial hazards include many bacterial pathogens associated with food born disease [5]. These are broadly classified into two major groups: those that are naturally present in the environment which is indigenous to the food at the time of harvesting and those that get entry in to fish during various stages of handling or those introduced in to the environment from external sources [6]. Pathogenic and potentially pathogenic bacteria associated with fish and shellfish include *Mycobacteria, Streptococcus iniae*, *Vibrio vulnificus*, *Vibrio* spp., *Aeromonads, Salmonella* spp. and others [7]. Human infections and intoxications from fish with the following bacteria have been recorded: *Mycobacterium* spp., *Streptococcus iniae*, *Photobacterium damselae*, *Vibrio alginolyticus, V. vulnificus, V. parahaemolyticus, V. cholerae, Erysipelothrix rhusiopathiae, Escherichia coli, Aeromonas* spp., *Salmonella* spp., *Staphylococcus aureus, Listeria monocytogenes, Clostridium botulinum, C. perfringens*, *Campylobacter jejuni, Delftia acidovorans, Edwardsiella tarda, Legionella pneumophila, and Plesiomonas shigelloides* [8]. Most human bacterial infections can be successfully treated using current antibiotic therapies. However, in recent years, a significant increase in the emergence of pathogenic microorganisms resistant to the available antimicrobials has been observed, including multi-drug resistant (MDR) pathogens, which has been associated with the misuse or abuse of antibiotics. As a result, persistent and difficult to treat infectious diseases appeared, which constitutes a serious public health problem [9]. PCR has become a very rapid and reliable tool for the molecular biology-based diagnosis of a variety of infectious disease. PCR has been applied for the detection of microorganisms from microbial cultures and tissues and directly from clinical sample. PCR have overcome problems associated with culture-based techniques, enabling the detection of microorganisms directly in clinical samples without the need for previous culturing. The genus *Myroides*, proposed as belonging to the family *Flavobacteriaceae*, was established with the reclassification of the species *Flavobacterium odoratum* [10]. Members of this genus are non-fermenting, Gram-negative, aerobic, and non-motile bacteria [11] that previously have been discovered only from clinical sources, aquatic environment, grey mullet's gut, and flesh flies [12]. Food producers of today are met with inherently contradictory demands as seen from a microbiological point of view: producing foods that are less stable (due to nutritional and taste requirements) by processes that confer less control of the detrimental micro flora due to trends of convenience, minimal processing, and reducing or removing additives including preservatives. Bio preservation systems in foods are of increasing interest for industry and consumers. Bacteriocinogenic lactic acid bacteria and/or their isolated bacteriocins are considered safe additives (GRAS), useful to control the frequent development of pathogens and spoiling microorganisms in

foods and feed. This study was conducted with the aim of characterizing molecular and evaluating the antibiotic profile of multi-drug pathogens isolated from marine fish of sardine (*Sardinella longiceps*) mackerel (*Rastrelliger kanagurta*), and shark (*Cephaloscyllium silasi*) and production of bio preservatives– bacteriocin from *Lactobacillus acidophilus*, to increase the shelf life of the fish.

2. MATERIALS AND METHODS

2.1. Sample collection and isolation

Marine fish samples were collected from the local fish market in Coimbatore, Tamilnadu, India. Three different species, marine fishes were collected, including *Cephaloscyllium silasi* (F1), *Sardinella longiceps* (F2) and *Rastrelliger kanagurta* (F3). For the isolation of pathogens, the methods of culture and plating techniques described by [13], were followed. The tissues of *S. longiceps, R. kanagurta,* and *C .silasi* were dissected and processed. 10 g portions were dissolved in 100ml of sterile saline and serially diluted, followed by total viable count was identified and they were subjected to the specific culture medium.

2.2. Isolation and confirmation of the organisms

Culture technique procedures recommended by [14] were followed. The media employed for the isolation and primary confirmation of organisms was nutrient agar, nutrient broth, Mac Conkey agar, Citrimide agar, Skim milk agar, Mannnitol salt agar and thiosulfate-citratebile salts-sucrose (TCBS) agar (Hi-Media, India) and these were time tested ones in bacterial cultures. The isolated pathogens were subjected to biochemical tests for further confirmation apart from the culturing methods. The following are the tests performed during this study Simmon citrate, and triple sugar iron (TSI), were carried out following the standard process in the biochemical analysis.

2.3. Molecular identification of the organisms

2.3.1. DNA isolation [15]

DNA from the bacterial genome was extracted as per standard phenol- chloroform method. Bacterial cultures were prepared and suspended in Luria-Bertani broth (Hi-Media, India) and incubated at 37°C for 24 hrs. The 24-hr old bacterial cells were pelleted by centrifugation and this was suspended in lysozyme and saline EDTA, after mixing incubated at 37^0C for 30 minutes. To this 150µl of 10% SDS was added and incubated at 65^0C for 15 minutes. Phenol, Chloroform and Iso-amyl alcohol in the ratio of 25:24:1 were added and undergone centrifugation. The aqueous phase was precipitated by adding double volume of isopropanol and washed with absolute ethanol. The DNA was suspended in 30µl of TE buffer and visualized by 0.8% agarose gel electrophoresis.

2.3.2. Polymerase chain reaction (PCR) amplification of 16S rRNA gene [16]

PCR reaction was performed in a gradient thermal cycler (AB Applied Biosystems-Veriti 96 well Thermal cycler). The universal primers (Forward primer -F 5'-GGT TAT GCC TTA TAT TCG-3' and reverse primer R 5'-TTA GCG TTG CCA GTG CTC-3') were used for the amplification of the 16S rRNA gene fragment. PCR (30ul) was carried out by adding 4µl of Template DNA, 16µl of

PCR master mix, 2µl of primer, 6µl of distilled water. Amplification was done by initial denaturation at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, the annealing temperature of the primers was 55^0C for 30 second and extension at 72^0C for 1 minute. The final extension was conducted at 72^0C for 8 minutes. Agarose gel electrophoresis of PCR product was carried out as follows 8µl of the reaction mixture was then analyzed by gel electrophoresis using 1.5% agarose with ethidium bromide at 50V and the reaction product was visualized under gel documentation system. The PCR product was further sequenced.

2.3.3. Phylogenetic analysis

Nucleotide sequences were subjected to homology search using the nucleotide BLAST NCBI. The phylogenetic tree was constructed using MEGA 5 software, the partial 16s RNA sequences were submitted to the NCBI Gene Bank database.

2.4. Antibiotic Susceptibility Test

Antibiotic sensitivity tests were carried on Mueller-Hinton agar (MHA - Hi-Media, India) by Kirby-Bauer disk diffusion method [17],using antibiotic discs of ampicillin (AMP2mcg), chloramphenicol (C30mcg), ciprofloxacin (CIP5mcg), ceftazidime (CAC30mcg), cefotaxime (CTX30mcg), ofloxacin (OF2mcg), ceftriaxone (CI10mcg), levofloxacin (LE5mcg), gatifloxacin (GF5mcg), norfloxacin (NX10mcg). Approximately 50µl of the inoculums was spread on Mueller Hinton agar plate using a cotton swab and antibiotic discs were placed with sterile forceps. Then the plate was incubated at 37°C for 24 hours and zones of inhibition was measured in millimeter (mm). Characterization of the strain as sensitive, intermediately sensitive and resistant was based on the size of inhibition zones around each disc as per the manufacturer's instructions, which matched the interpretive criteria as per CLSI guidelines [18].

2.5. Bio preservatives used as a remedial measure

The MRS broth was prepared and inoculated with *Lactobacillus acidophilus* after adding the flask was incubated for 37°Cfor 24hs. Then the cultures were centrifuged to identify the biomass of the culture. After the identification of optimum day, the supernatant was used for the efficacy study. Well diffusion method was used to test against *E.coli* cultured on the Mueller Hinton agar plate at 37^0C for 24 hrs. After incubation zone of inhibition was measured to identify the efficacy of the sample. The same above described method was used to test against the fish pathogens also. The supernatant was partially purified using acetone followed by dialysis against phosphate buffer (pH 7.0) and subjected to study for the activity against the marine fish isolate *Myroides gitamensis*. The sample was used as a bio preservative to check the shelf life of the fish. The samples were poured on the fish and kept in a beaker and stored at room temperature for 3 days. Structural changes and odor of the sample was observed.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of organism

In this study three different species of marine fishes selected such as *Sardinella longiceps*, *Rastrelliger kanagurta,* and *Cephaloscyllium silasi* and the pathogens was isolated after serial dilution, followed by total viable count and they were subjected to the specific medium, for the confirmation of organisms. According to Bergy's manual, Mac Conkey agar is used as a selective medium for *E.coli* [16] reported that MacConkey agar and Simmon's citrate agar used to differentiate the *E. coli* isolated from fresh water market fish. TCBS used as a selective medium for *Vibrio* spp, [19] investigated, MDR *Vibrio parahaemolyticus* isolated from shrimp using TCBS and TSI. Cetrimide agar used to differentiate the *Pseudomonas* species, [20] reported isolation of the *pseudomonas* strain from the egg shell. Mannitol salt agar used as a selective medium for *Staphylococcus Spp*. [21] Reported, mannitol salt agar used to isolate the Methicillin resistant *Staphylococcus aureus* (MRSA) from ocular patients. Simmons' citrate agar is used for differentiating gram-negative bacteria on the basis of citrate utilization. The increase in pH causes color change in the bromothymol blue indicator, turning it blue. Under neutral conditions the medium remains green in color. It mainly used to differentiate the *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella* and *Shigella*. Skim milk agar used for *Enterobacteriaceae, Bacillaceae,* and several other families. The Triple Sugar Iron (TSI) test is a microbiological test roughly named for its ability to test a microorganism's ability to ferment sugars and to produce hydrogen sulfide. It is often used in the selective identification of enteric bacteria including *Salmonella* and *Shigella*. Blackening of the bottom due to H_2S production may mask the acid reaction (yellow) in the bottom of the tube.

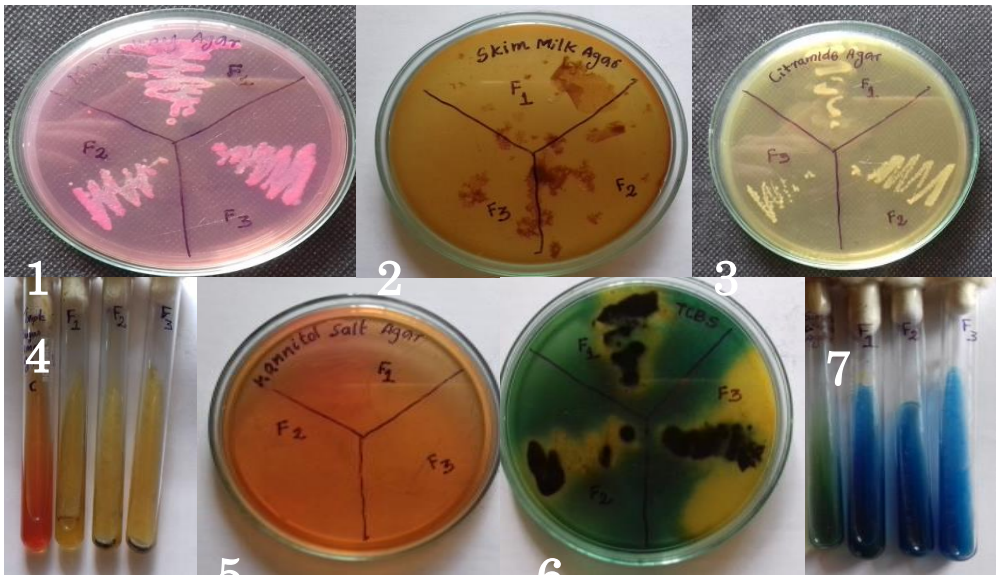

Figure 1. Confirmation of organisms using specific media (1) MacConkey agar, (2) Skim milk agar, (3) Cetrimide, (4) TSI, (5) Mannitol salt agar, (6) TCBS, (7) Simmon's Citrate

In this study except mannitol salt agar and skim milk agar plate, all the other media such as MacConkey, Cetrimide, TSI, Simmon's Citrate, TCBS agar showing the presence of growth. Based upon the above discussion this three fish samples contain the presence of E. coli, Staphylococcus aureus, Pseudomonas, Salmonella, Shigella, vibrio spp. None of the articles described about the presence of some other organism in TCBS and which is showing, black in colour, silver silky glazing nature and foul smell, especially the F3 shows more characteristics so we selected this organism for further study and which is confirmed by 16S rRNA sequencing.

Table 1: Results of Confirmation of organisms in specific media

Name of the test	*Cephaloscyllium silasi* (F1)	*Sardinellalongiceps* (F2)	*Rastrelligerkanagurta* (F3)
MacConkey agar	+ve	+ve	+ve
TCBS	+ve	+ve	+ve
Citrimide agar	+ve	+ve	+ve
Mannitol salt gar	-ve	-ve	-ve
TSI	+ve	+ve	+ve
Simmon Citrate agar	+ve	+ve	+ve
Skim milk agar	-ve	-ve	-ve

3.2. Isolation of DNA and Polymerase ChainReaction (PCR) amplification of 16S rRNA gene

Genomic DNA was isolated and characterized by phenol-chloroform extraction method, and its quality was checked using agarose gel methods, PCR, performed using universal primers SHV-F 5'andSHV-R 5'and the products were observed, which showing 795Kbs.Further the product was confirmed by sequencing analysis. *Myroides xuanwuensis* spp. novel, a mineral-weathering bacterium isolated from forest soil and 16S ribosomal RNA gene sequencing (16S rRNA sequencing), a standardized bacterial strain identification method used to identify the organism [22]. [16] Reported, Molecular characterization and antimicrobial resistance of *E. coli* isolated from fresh water market fish, characterized biochemically as well as by PCR from 25 (18.66%) samples. Virulence genes detection by PCR study revealed the presence of stx1 and stx2 genes in 12% and 8% of isolates, respectively and which showing 202Kbs.

3.2.1. Phylogenetic tree

A phylogenetic tree is a visual representation of the relationship between different organisms, showing the path through evolutionary time from a common ancestor to different descendants. The tips of the tree represent groups of descendent taxa (often species) and the nodes on the tree represent the common ancestors of those descendants. The gene bank accession number is MG554743.1 (*Myroides gitamensis*).

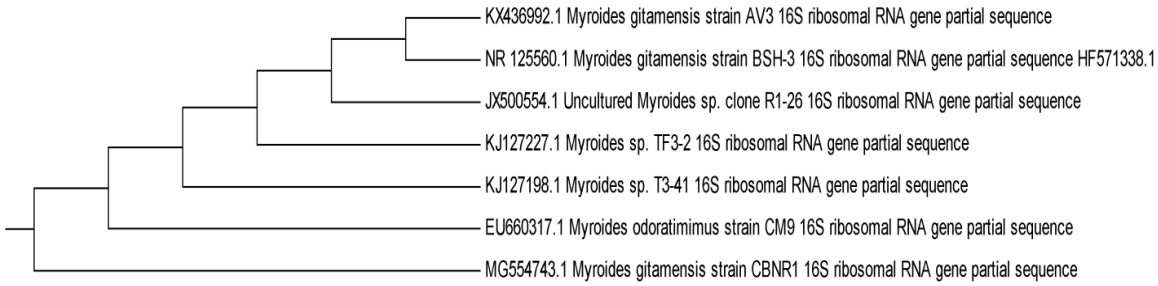

Figure 2. Phylogenetic tree representation for *Myroides gitamensis*

Myroides Spp. is a rare opportunistic pathogen,16S rRNA sequencing is a standardized bacterial strain identification method, even though it has been failing to provide any information on the status and mechanisms of antibiotic resistance in *Myroides* spp. [23]. [24] Investigated, the 4.29-Mb draft genome sequence of *Myroides* spp.N17-2, a new bacterium isolated from radiation-polluted soils in Xinjiang, Uyghur Autonomous Region, China. The acquisition of its genome will provide valuable information to reveal the relationship between radiation and multidrug resistance. [25], *Myroides gitamensis* spp L-asparaginase producing bacteria isolated from slaughter house soil sample in Visakhapatnam, India, thus the organism confirmed by phylogenetic analysis based on 16S rRNA gene sequence revealed that the strain BSH-3T belongs to the genus *Myroides* and is closely related to *Myroides odoratimimus* (98.4 %).

3.3. Antibiotic Susceptibility Test

In this study, the antibiogram profile revealed that the isolate (M*yroides gitamensis*- MG554743) is resistant to all the antibiotics. Totally 10 different types of antibiotics were used and the bacteria are fully resistant to 5 antibiotic which is resulting no zone of inhibition, ie; for Ofloxacin, Ciprofloxacin, Ampicillin, Ceftazidime, Norfloxacin, so the organism is highly resistant to these antibiotics. Ceftriaxone and Levofloxacin showing 4mm, Chloramphenicol 5mm,Gatifloxacinand Cefotaxime showing7mm, this is also resistantto the *Myroides gitamensis*, and the results shown in table (2) and figure (3).

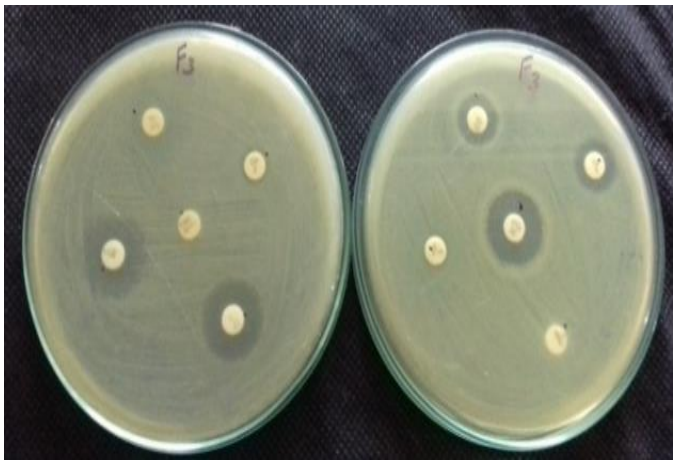

Figure 3. Antibiotic susceptibility test

Table 2: Antibiotic susceptibility test

Name of the Antibiotics	Zone of inhibition (mm)
Ofloxacin (OF 2mcg)	Nil(Resistant)
Ciprofloxacin (CIP 5mcg)	Nil (Resistant)
Ampicillin (AMP 2mcg)	Nil (Resistant)
Ceftazidime (CAC 30mcg)	Nil (Resistant)
Norfloxacin (NX 10mcg)	Nil (Resistant)
Ceftriaxone (CI 10mcg)	3mm(Resistant)
Levofloxacin (LE 5mcg)	4mm(Resistant)
Chloramphenicol (C 30mcg)	5mm(Resistant)
Gatifloxacin (GF 5mcg)	7mm(Resistant)
Cefotaxime (CTX 30mcg)	7mm(Resistant)

Our study results were similar to the results of [26], the antibiotic sensitivity testing of *M. odoratimimus* strain PR63039 was found to be resistant to ampicillin, amoxicillin, clavulanate, amikacin, aztreonam, chloramphenicol, cephalosporin, imipenem, gentamycin, levofloxacin, meropenem, shubatan, sulfamethoxazole, tetracycline, ciprofloxacin, and tazobactam. In their research antibiotics such as cefazolinoxime, amikacin, tetracycline, moxifloxacin, ciprofloxacin, and nitrofurantoin, were administered to the patient for 47 days, the infection was not cured. [27] Investigated antibiotic sensitivity of plasmid-containing *M. odoratimimus* SKS05-GRD was correlated with the plasmid or was chromosomally-mediated. They revealed that resistance to kanamycin, amikacin, and gentamicin was plasmid-mediated, and that resistance to ampicillin, cefadroxil, cefoperazone, ceftazidine, ceftriaxone, and netillin was chromosomally-mediated.

3.4. Bio preservatives used as a remedial measure

The United States Food and Drug Administration guidelines for storing seafood recommend that all seafood, including fish, should use within two days of purchase. Fish lasts for a shorter period of time if it is not stored properly. Some common traits of bad fish are a slimy, milky flesh (a thick, slippery coating) and a fishy smell. This is hard because the fish is smelly and slimy by nature, but these traits become much more pronounced when the fish has gone bad fresh fillets should glisten like they came out of the water. In this study the supernatants used as a bio preservative for increasing the shelf life of fish, in the efficacy study its shown 7mm of zone of inhibition and 4mm against the pathogens, compared to the antibiotic the results was effective. The bacteriocin was poured on to the fish, and observed for 3 days in room temperature, its structure, odor and texture shown as such for 1 day, besides, thus it didn't spoil for 3 days or produced a foul smell after 65hours. How should food producers manage to develop such products with a sufficiently long shelf-life and at a competitive price. Some of the most promising tools to this end are the so-called bio

preservatives, which are various types of products derived from lactic acid bacteria and other suitable microorganisms, namely bacteriocins and other antimicrobials, fermentates, bioprotective cultures, and bacteriophages [28]. [20] Reported, molecular study on multidrug resistant (MDR) pathogen *Pseudomonas* spp. strain TS-397 isolated from egg shell and used plant extracts as bio preservatives, plant extracts of *Androgrphis paniculate* and *Illicium vernum* were used as a bio preservative for increasing the shelf life of egg.

4. CONCLUSION

In the present investigation, the strain of bacteria isolated from marine fish shown resistant to most of the commercially available antibiotics. The multidrug resistant marine bacteria isolated from fish poses a potential threat to environmental and public health. *Myroides* spp. is a rare opportunistic pathogen. The genus *Myroides*, proposed as belonging to the family *Flavobacteriaceae,* was established with the reclassification of the species *Flavobacterium odoratum*. *Myroides gitamensis* isolated from soil samples are mainly reported, but not from the fish samples, so based upon the review articles, it's a different observation and our results are highlighting the remedial measure against pathogens (*Myroides gitamensis* - MG554743.1) based upon the results what we observed from antibiotic susceptibility test.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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