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Microbiology

MOLECULAR CHARACTERIZATION OF FISH PATHOGENS & FACTORS INFLUENCING PATHOGENICITY

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ABSTRACT Fish farming, is an fast growing business in aquaculture relies on mass culturing of fishes for their increasing demand on fish products, but forced confinement of farmed fish may in some circumstances make them more prone to be affected by environmental stressors leading to infectious disease causing heavy losses to fish farmers. Environmental stressors include adverse chemical condition of the water. Fish become susceptible to bacterial pathogens during stressed condition due to changes in ammonia ,potassium concentration ,pH & temperature fluctuation, created an unbalanced host, pathogen & water environment .The present study is on diseased fresh water fish , *Tilapia* , interplay of physiological factors contributing towards pathogenicity of bacteria responsible for infection. Infected fish was identified with hemorrhagic skin near gills ,skin peel off ,pop out eye, slight discoloration. From various parts of the infected fish the biopsy were taken. Skin scraping, gills, intestine, kidney, serially diluted & inoculated to nutrient to nutrient broth & stained , all the bacterial isolates are grown on chemically defined media to check for their nutritional requirement ,further to determine the pathogenicity of bacterial isolates , growth is analyzed ai different concentration of ammonia ,potassium with pH & temperature gradient .As a standard , auxenic cultures of *Aeromonas hydrophila* is studied .Finally molecular characterization of two unknown isolates studies for various physiological parameter were identified to be as *Flavobacterium Columnare & Streptococcus agalacitace.*

KEYWORDS : Tilapia,Bacterial isolates,Pathogenicity,Auxenic culture

1.Introduction:

In the last few years, due increasing demand for aquaculture products, there has been global increase in the production of aquatic animals & farmed species. In India species of fish that are commonly cultured are Major carp, Exotic carp, Catfish, Tilapia, Trout. Animals when reared in restricted condition mainly in artificial culture system poses stress, which either lower immune status of host or enhance pathogenecity of pathogen.Recently there have been many reports in outbreaks of Tilapia disease in Japan, Thailand & other Asian countries .Bacterial pathogens are the most serious disease problem in Tilapia production causing 80% of fish mortalities(Plumb, 2011; Woo & Bruro 2011). Diseases are the set of interaction among the host, pathogen & environment. Host factors include species of fish, size, age, immune status & general physiological condition. Pathogen factors include concentration of infectious agent, their physiological property & virulence. Difference between apparent health & disease typically depends on balance between the pathogen, host & the balance is greatly influenced by the environmental factors such as temperature & water chemistry.

Disease associated with cultured fish can be divided into infectious & noninfectious. Noninfectious occurs due to hereditary, nutritional deficiency & poor management practice(Lucas & Southgate 2003),infectious disease are caused by viral ,bacterial, fungal pathogens(Lucas & Southgate 2003). Infectious & noninfectious disease pose serious threat to the fish in terms of their survival & growth rates .Effective water management in fish holding facility is one of the important factors contributing to the success of fish culture(Hossain 2007).Ahmed et al (2009)found that seasonal variation in pH ,temperature & dissolved oxygen play an important role in the multiplication of pathogens leading to disease in fish .Slow & rapid changes in water temperature ,rapid or prolonged depression of pH,low alkalinity & low Do are seasonal aggregation of fish disease (Liley et al 1992). Therefore only multidisciplinary studies involving the characteristics of potential pathogenic micro-organisms for fish aspects of biology of the fish host as well as better understanding of environment factors affecting such cultures will allow the application of adequate measures to prevent & control the main diseases ,limiting the production of fish.

2.Material & Method

2.1 Samples

The research was carried out on fresh water Tilapia commonly cultured in Dakshina Kannada, the diseased fish, collected in sealed polythene bags & brought to the lab. Auxenic cultures of Aeromonas hydrophila is procured from Fishery college ,Mangalore & maintained through sub culturing on nutrient agar media .

2.2 Isolation & Identification of micro-organisms from fish tissue

Nutrient Agar media with the composition peptone 5g, beef extract 3g,sodium chloride 3g , distilled water 1000ml ,agar 15g was used for isolation. Micro-organisms are isolated from different tissue skin scarping, Kidney, Gills, Intestine, serially diluted & inoculated on to the nutrient agar medium. Colonized micro-organisms from 24 Hrs incubation on nutrient agar plates are identified through gram staining.

2.3 Growth analysis of isolates on Chemically defined medium

Chemically defined media with the following composition is used Sucrose,Potassium Di hydrogen phosphate(0.02M),Dipotassium Hydrogenphosphate(0.015M),Ammoniumnitrate(0.0125M),magnesi um sulphate hepta hydrate,Ferrous sulphate hepta hydrate ,Manganous sulphate hepta hydrate,water, Agar & growth is assessed by checking OD at 660 nm.

2.5.1 Effect of Ammonium nitrate concentration on Growth analysis of isolates

Chemically defined media with different Ammonium nitrate concentration(0.01M,0.001M & 0.02M) are used ,in each case growth of isolates is assessed by checking OD at 660 nm

2.5.2 Effect of Potassium Di hydrogen phosphate concentration on Growth analysis of isolates

Chemically defined media with different Potassium Di hydrogen phosphate concentration(0.01M,0.001M & 0.025M) are used ,in each case growth of isolates is assessed by checking OD at 660 nm.

2.5.3 Effect of Dipotassium hydrogen phosphate concentration on Growth analysis of isolates

Chemically defined media with different Dipotassium hydrogen phosphate concentration(0.01M,0.001 M & 0.02M) are used ,in each case growth of isolates is assessed by checking OD at 660 nm.

2.6 Effect of temperature on isolates

The isolates are inoculated on to the nutrient agar medium for different temperature gradient 100c,370c,600c, ,in each case growth of isolates is assessed by checking OD at 660 nm.

2.7 Effect of alkalinity on isolates

The isolates are inoculated on to the nutrient agar medium for differentsalt concentration 10% ,15%,20%,in each case growth of isolates is assessed by checking OD at 660 nm.

2.8 Molecular characterization of isolates

The two isolates namely gram negative rod & gram positive cocci responded to varied physiological parameters were subjected to molecular characterization through polymerase chain reaction

technique. Genomic DNA was isolated from the bacterial culture by standard protocol(Hoffman and Winston ,1987). Single colony was inoculated in nutrient broth and grown forovernight at 370C .Cell were harvested from 5ml of the culture and to this 100µl of lysozyme was added and incubated at room temperature for 30 min, followed by the addition of 700 µl of cell lysis buffer (Guanidium isothiocyanate, SDS,Tris-EDTA).The contents were mixed by inverting the vial for 5 min with gentle mixing till the suspension looked transparent.700µl of isopropanol was added on top of the solution. The two layers were mixed gently till white strands of DNA were seen.

The DNA extracted from aqueous layer was ethanol precipitated. The DNA pellet was dried and dissolved in 50µl of 1X TE buffer. The quality of the DNA was checked by running on 0.8% agarose gel stained with ethidium bromide(0.5µg/µl). A single intense band with slight smearing was noted. The extracted genomic DNA was used as a template DNA for amplification of the 16S rRNA gene.

PCR amplification of 16S rRNA gene:

PCR reaction was performed on MJ-Research Thermo Cycler (PTC-200, USA), the universal primers 27F & 1492 R were used for amplification. The reaction mixture of 30µl consisted of 10ng of genomic DNA,0.4 Units of 0.3 µl Taq DNA polymerase,3µl of amplification buffer 10X (100 mM Tris- HCl, pH 8.3, 20 mM MgCl2, 500 mMKCl), 0.6 µl of 200 µM of dNTPs (dATP, dTTP, dGTP, dCTP), 12 pmol of 2 µl each of forward and reverse primers .Amplification was done by initial denaturation at 94ºC for 5 min, followed by 35 cycles with each cycle consisting of 94ºC for 30 sec, annealing temperature of 55ºC for 30 sec and extension at 72ºC for 1 min. The final extension was set to 72 ºC for 10 min.

Primer Type	Target	Target gene	Sequence(5'-3')	Length in bp	Tm^0 C	Product length
27F	Universal bacteria	16S rRNA	AGAGTTTGATCCTGGCTCAG	20	50	1464
1492R			TACGG(C/T)TACCTTGTTACGACTT	22	62-64	1478

Agarose Gel Electrophoresis

10 µl of the reaction mixture was then analyzed by submarine gel electrophoresis using 1.0% agarose with ethidium bromide(0.5 µg/µl)as per standard protocols(Sambrook et al., 2001) at 80v/cm and the reaction product was visualized under Gel documentation system(Alpha Innotech)

Purification of PCR Product

The PCR product is subjected to purification by using Exosap-IT, it is a mixture of Exonuclease I and Shrimp Alkaline Phosphatase that removes left over primers and free nucleotides from the PCR reaction. To 5 µl of PCR product add 2 µl of Exosap. Further incubated at 370C for 15 mins to allow degradation of primers and free nucleotides . Tube was transferred to 800C water bath and incubated for 15 mins to inactivate the Exosap-IT enzyme. The sample is ready for sequencing reaction.

DNA sequencing of 16S rRNA gene

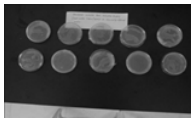
The 16S rRNA purified PCR product(100ng concentration) was subjected for the sequencing using ABI DNA 3730 XL sequencer (Applied Biosystem Inc). Sequencing of 16s rRNA genee of the bacterial isolate was done from both directions. The sequence obtained was subjected to BLAST search and the bacterial isolates were determined.

3. Result & Discussion

The present study is carried out to understand the role of ecological factors influencing the pathogenicity of fresh water fishes. From our observation ,the two isolates namely G+C,G-R & pure cultures of AH were not fastidious as they exhibited growth on chemically defined media, also these three different micro-organisms were tested varying concentration of different salts of chemically defined media where they showed variable result. G-R,G+C showed maximum growth at 0.02M ammoniumnitrate concentration, where as AH at 0.01M.All three micro-organisms showed maximum growth at 0.01M concentration of Potassium Dihydrogen phosphate. G+C showed maximum growth at 0.02M concentration of Dipotassium hydrogen phosphate, where as G-R,AH showed maximu growth at 0.001M concentration. All the three micro-organisms were grown on 3% NaCl on Nutrient broth where G-R showed best growth. Further the effect of alkalinity is checked by taking different concentration of NaCl 10%,15%,20% G-R had no effect growing in all concentration, where as G+C showed best growth at 15 & 20% respectively, AH showed growth at 10% .Since the pathogen infect poikilotherms, the effect of temperature is studied at different gradients 100C,37oC & 550C ,All the micro-organisms showed growth at 100C, but best growth is seen at 37oC probably indicating mesophillic nature, however moderate growth seen at 550C

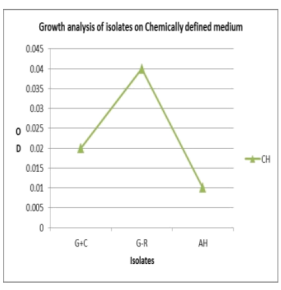
The bacterial isolate which showed growth at various physiological parameters were subjected to comparison of 16S rRNA gene, that has emerged as a preferred genetic technique. The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny), but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level (Sacchi et al., 2002). The use of 16S rRNA gene sequences to study bacterial phylogeny (Amann et al., 1995)and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel, J. B. 2001).The rRNA based analysis is a central method in microbiology used not only to explore microbial diversity but also to identify new strains. The present study has been carried out to identify the bacterial strain isolated from diseased fresh water fish namely Tilapia.Total of Forty five isolates obtained, from that two isolate were used for further analysis. Colonies were isolated from mixed population on nutrient agar plates were characterized and grams reaction was identified to be Gram positive cocci & Gram negative rod res, and the isolated further confirmation was done using molecular approach. Bacterial genomic DNA was isolated as per the standard protocol (Hoffman and Winston, 1987). The presence of bacterial genomic DNA isolated was confirmed on 0.8% agarose gel stained with ethidium bromide. An intense single band was seen along with the DNA marker. The extracted DNA was used as template for amplification of 16S rRNA gene. The universal primers 27F and 1429R were used for the amplification and sequencing of the 16S rRNA gene fragment. The optimum annealing temperature was found to be 55ºC. An intense single band was visible on 1% agarose gel stained with ethidium bromide (Fig.2).The PCR product was subjected to sequencing using BDT V3.1 cycle sequencing kit on ABI 3730 XL genetic analyzer from both forward and reverse directions. The sequences (Fig.3) obtained were compared with the NCBI gene bank database using BLAST search program (http;// www.ncbi.nlm.nih.gov) (Marchler–Bauer et al., 2000; Pruitt et al., 2005). The percentages of sequence matching were also analyzed.The homology search made using BLAST showed *Streptococcus agalacitace* has 96% identity with the isolate with a e-value of 3e-10 which is said to be acceptable value than compared to the other pathogenic organism for Gram positive cocci & for gram negative rod sequence similarity result showed *Flavobacterium columnare strain* has 96% identity with the other strains.

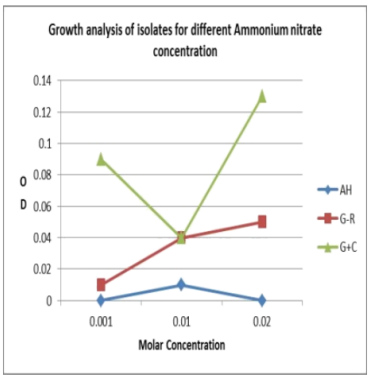
Legend 1: Infected Fish Sample

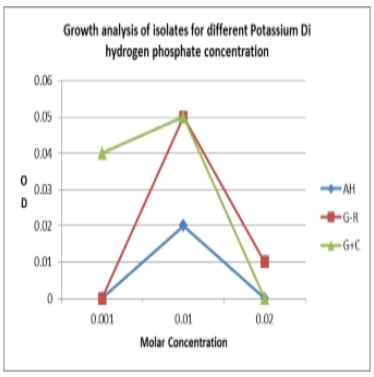


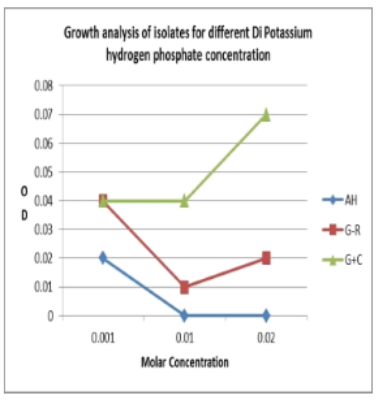
Legend 2: Growth on chemically defined media

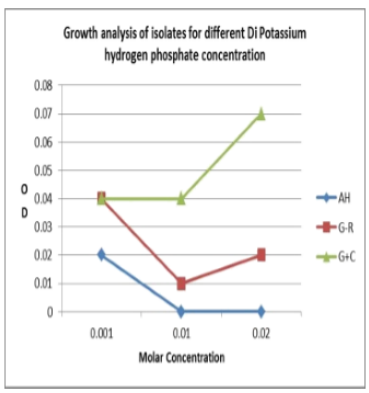
Legend 3: Growth of isolates exhibited at various physiological parameters

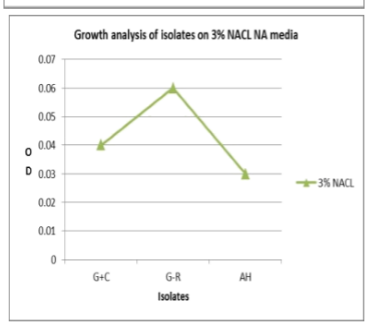


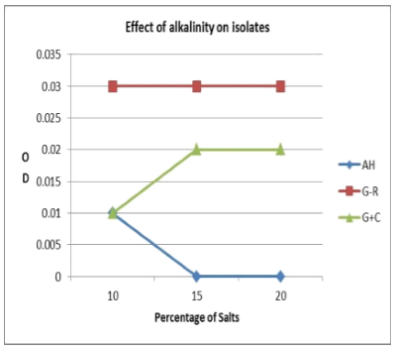


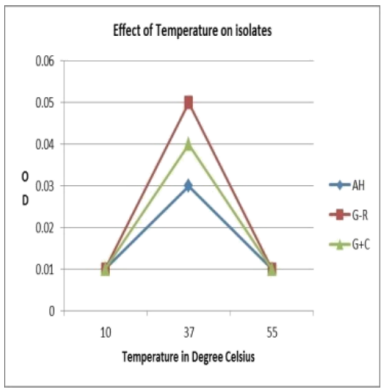


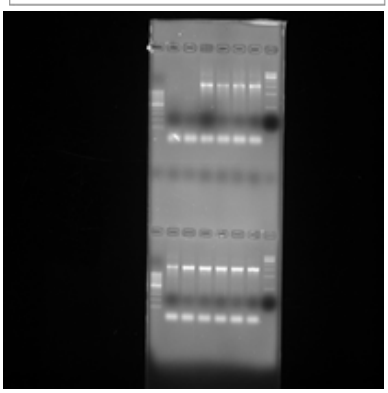












Legend 4: PCR amplified products
(First lane from left hand side added with 100bp nucleotides marker followed by positive-Listeria monocytogenus, negative-master mix, B1,B2,B3,B4&B5 and 500bp nucleotides marker)

Sequencing analysis of the Gram positive cocci was given in FASTA format

CTGGTCGGGGGGCCTTTTCTTGCAAGTCGAGCGATGCGTT
A A C A G C T T G C T G T T A
TGCAAGTAGCGGCGGACGGGAAAGTAACACGTGGGTAAC
C T G C C C A T A A G A C T G
GGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTT
T G A A C C G C A G G G G T
TCGAAATTGAAAGGCGGCTTCGGCTGTCCCTTATGGATGGA
C C C G C G T C G C A T T A
TCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACTATGCAT
A A C C G A C C T G A G A
GGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACT
C C T A C G G G A G G C A G
CAATAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAG
C A A C G C C G C G T G A G
TGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGA
A G A A C A A G T G C T A A T
TGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCC
A C G G C T A A C T A C G T
GCCACCAGCCGCGGTAATACTTAGGTGGCAAGCGTTATCCG
G A A T T A T T G G G C G T
AAAGCGCACGCAGGTGGTTTCTTAAGTCTGATGTGAAAGC
C C A C G G C T C A A C C G

TGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAG
A G G A A A G T G G A A T T C
CATGTGTAGCGGTGAAAGCATAGAGATATGGAGGAACACC
A G T G G C G A A T G C G A
CTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGG
G G A G C A C A C A G G A T
TAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAA
G T G T T A G A T G G T T T
CCGCCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGC
C T G G G G A G T A C G G C
CGGATGCTGAAACTCAAGGAATTGACGGGGGGCCCGCACA
G C G G T G G A G C A T G T
GCTTAATTCTAGCAACGCGAGAACCTTACCAAGTCTGACA
C C T C T G A C A T C C T A G
AATAGGGCTTCTCTTCGGGAGCAGAGTGACAGGTGTGCAT
G G T T G T C G T C A G C T C
GGGTCATGAAATGTGGGGTCAGTCGCAACGAGCGCACCTT
G G A T C G T A G T G G C A
TCATTAGGTCGGCACACTAAGGTGACTGGCGGGTGACAAC
CGGAAGAAGTGGGG GCTTACTGCA

BLAST sequence similarity

Organism		Maximum Score	Total score	Query cover	Identity	Accession I D
Aeromonas		*632*	*632*	*90%*	*89%*	*KRO63151.1*
hydrophila						
Flavobacterium		1404	1404	94%	89%	KF051086.1
columnare	*Strain*					
CFCRVB43						
Pseudomonas		1644	1644	97%	88%	FM173664.1
*sp.*CL3.1						
Psycrobacter		1644	1644	97%	94%	HQ202844.1
pulmonis strain T-151						
Streptococcus		1029	1149	96%	98%	EUO750701
agalacitace strain 15-						
92MP						
Caulobacter sp. Strain	699	699	93%	75%	KF051086.1	
THG-AG3.4						

The above blast sequence showed that Streptococcus agalacitace has 96% identity with the isolate with a e-value of 3e-10 which is said to be acceptable value than compared to the other pathogenic organism

Sequencing analysis of the gram negative rod was given in FASTA format

GGAGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGAT
AACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGA
ACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCAC
TTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTA
ACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAG
G G T G A T C G G C C A C A C T G G G A C T G A
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAA
TCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCG
TGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAG
GGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACG
GTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAG
C C G C G G T A A T A C G T A G G T G G C A A G
CGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGT
TTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGA
GGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGA
AAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATAT
GGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAA
CTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAA
GTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCA
TTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAA
CTCAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GGTGTTTATTCGAAGCAACGCGAAGAACCTTACCAGTCTT
GAACATCTCTGAACAACCTAGAGAAAGGGCTTCTCCTTCG
GAGCAGAGTGACAGGTGGTGCATGTTGTCGTCAGCTCTGT
CGTGAATGTTGATTAGTCGCAACGACGCACCCTGGATCTTA
TGTGCCATCATTAGTTGGCATCTAGAGGTAC

BLAST sequence similarity

Organism	Maximum score	Total score	Query cover	Identity	Accession I D
Aeromonas	691	691	84%	78%	KX692886.1
Hydrophila					
Flavobacterium	1443	1443	83%	96%	KF051086.1
columnare strain					
CFCRVB43					
Pseudomonas	1460	1460	98%	92%	KR708860.1
syringae HGPY-1					
Psycrobacter	1377	1377	98%	90%	FJ613605.1
pulmonis strain T-151					
Streptococcus	1100	965	89%	75%	KT545678.1
agalacitace strain					
5A111					

Blast sequence showed that Flavobacterium columnare strain has 96% identity with the other strain.

4. Conclusion

Bacterial species have at least one copy of the 16S rRNA gene containing highly conserved regions together with hyper variable regions. The use of 16S rRNA gene sequences to identify new strains bacteria is gaining momentum in recent years. We showed the use of 16S rRNA gene sequence to characterize the bacterial isolate from the diseased fresh water fish Tilapia tannery two isolates studied for varied physiological parameters were found to be Streptococcus agalacitace with 96%and Flavobacterium columnare strain has 96% .Thus, the genotyping method using 16S rRNA gene sequence is both simple and effective in strain identification.

REFERENCES

1. Abdel Rahman AM, Nobi G and Moustafa A, et al. (2002). Studies on Pseudomonas septicemia among tilapia fish in Abassa fish farms Vet .J., 50: 25-34
2. Ahmed SA, Marzouk MS, Megid RM, Moustafa A and Gado M, et al. (1995)Vet.Med.J., 43: 389-396.
3. Pathiratne A. & Jayasinge R.P.P.K.(2000). Environmental influence on occurrence of epizootic ulcerative syndrome (EUS) in fresh water fish in Bellanwani- Attidiya wetlands,Sri lanka.Journal of Applied Ichthyology.(in press)
4. Austin B. & Austin D.A .(1989).Methods for microbial examination of fish and shellfish pp.317.Ellis Horwood Limited.
5. Shoots E.M .& Bullock G.L .(1975).Bacterial diseases of fishes : diagnostic procedures for gram-negative pathogens.Journal of Fisheries Research Board Canada 32:1243-1247
6. Hoffman and Winston (1987). Genomic DNA extraction. Gene. 57, 267-272.
7. Sambrook J; Fritsch, E. F. and Maniatis, T. 1989, Molecular Cloning, A laboratory manual, 2rd Edn (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
8. Marchler-Bauer, A; Panchenko, A. R: Shoemakar,B . A;Thiessen , P.A;Geer, L.Y. and Bryant, S .H.(2000). CDD: A database of conserved domain alignments with links to domain three-dimensional structure.J. Nucleic Acids Res.30,281-283
9. Patel,J.B.2001,16SrRNA gene sequencing for bacterial pathogen identification in clinical laboratory.Mol.Diagn.6,313-321
10. Amann ,R.I;Ludwig,W. and Scleifer,K.H., 1995,Phylogenetic identification and insitu detection of individual microbial cells without cultivation.Microbiol.Rev.59,143-169
11. Sacchi,C.T;Whitney,A.M;Mayer,L.W;Morey,R. & Steigerwalt,A.(2002),Sequencing of 16S rRNA gene:A Rapid Tool for identification of Bacillus anthracis. Emerg.Infect.Dis.8,1117-1123.
12. Plumb,J.A and Hanson,L.A.2011.Health maintance and pricibal microbial diseases of cultured fish. Third ed. Wiley-Blackwell, lowa,USA. 244ppdoi; 10.1002/ 9780470958353.chl1
13. Woo,P.T.K and Bruno,D.W (2011).Fish disease and disorders; viral, bacterial and fungal infections volume 3.CABI Publishing,Newyork,USA
14. Lucas,J,S.,Southgate P.C(2003).Aquaculture: farming aquatic animals and plants. Blackwell publishing.
15. Hossain DM Kabil M,Rehman M(2006). water quality parameters and incidence of fish disease in some water bodies in nature Journal of life science & earth science foundation .9(1&2)125-13
16. Lilley J H,Philips MJ,Tonguthai K.(1992) A review of epizootic ulcerative syndrome(EUS) in Asia.Aquatic Annual Health Research Institute and Newyork of Aquaculture Centres in Asia-Pacific Bangkok