Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Suppl 2, 2013

Research Article

VIBRIOSIS IN POND RARED *LABEO ROHITHA* AND SUSCEPTIBILITY STUDIES

M. RADHA KRISHNA REDDY[1] and S.A MASTAN[2]

[1]Department of Biotechnology, Krishna University, Machilipatnam-521001, [2]P.G Department of Biotechnology, PG Courses, Research Center, DNR College, Bhimavaram- 534202, Andhrapradesh, India. Email: radhakrishnamicro@gmail.com.

Received: 19 Mar 2013, Revised and Accepted: 05 May 2013

ABSTRACT

Aim: This communication provides an insight in to the emerging infection "Vibriosis" in *Labeo rohitha.* Objective: The present study aimed to identify the causative agents involved in Vibriosis infection of *Labeo rohitha.* Methods& Results: The pathogen was isolated from diseased *Labeo rohitha* and characterized by morphological, biochemical and molecular approach, which includes 16s r DNA gene sequencing. PCR amplified 16s r DNA product (at variable V3 region using universal primers 27 F and 1492 R) was analyzed on agarose gel electrophoresis, to identify the pathogen. Identified virulent bacterial strain (*V. cholerae* LD_{50} $10^{8.28}$*),* initiated re infection in experimentally infected *L. rohitha* juveniles. The anti bio gram was made with different chemotherapeutic drugs against *V. cholerae*, results revealed that it become multi drug resistant. Conclusion: This communication provided the evidence of *Vibrio cholerae,* which was true causative agent of Vibriosis disease in *L. rohitha*. Artificial challenge study reveals that the isolate can become pathogenic to *L. rohitha*.

Keywords: *Labeo rohitha,* $LD_{50,}$ Vibriosis, 16s r RNA sequencing, Anti bio gram.

INTRODUCTION

Fish culture involves controlled cultivation and harvesting of fishes in either fresh, marine or lagoon waters. The aquaculture farmers of Janardhanapuram, Nandivada Md, Krishna Dt, AP, polyculture Indian Major Carp fishes as intensive type. Proper pond and water quality management is essential for successful and quality fish production. Maintaining a good culture environment through use of proper management practices will reduce the risk of disease and increase production, fish quality and marketability. Uneaten feed or fish excreta deposit continually to the bottom of the tank, when ever re cycling not done perfectly by bottom dwellers, laid down stone for disease problems. Nutrient rich waters with high ammonia, nitrite stresses the fish enormously and culture waters with high organic matter pollute not only the tank, but also surroundings, and support the growth of many pathogenic organisms. Due to lack of quality and quantity of water, water sanitizers have got pivot importance. Application of sanitizers to reduce the microbial load, regular water exchange, and usage of probiotics to reduce the bottom load, minimizes the disease out breaks. Due to lack of water resources the farmers of above said area get waters from budameru drain. The budameru receives domestic waste from nearby towns Vijayavada and Gudivada, the waters may enrich with diversed group of pathogenic organisms. *V. cholerae* is associated with live fishes, all they from part of the indigenous micro flora of the fresh water aqua ponds, because culture ponds are have stressful environment due to high organic matter and dissolved oxygen fluctuations which effect the general population under normal conditions, elevate the growth of pathogens like *V. cholerae* [8].

Vibrio cholerae is gram negative, curved rod, non spore forming, motile with single polar flagellum, 1-3µm long, 0.5 – 0.8µm width, fallow either fermentative or respiratory mode of metabolism, belongs to gamma sub division of the proteo bacteria and placed in vibrionaceae family [1]. These are of 2 bio types classical and E1 Tor, causing water and food borne infections to human beings. Growth of pathogenic Vibrio cholerae occurs optimally at temperature 37ºc, pH 7.5- to 8.5-, high temperature and high salinity conditions prevail the growth of such bacteria [10]. V. cholerae is able to infect oysters, blue crabs, clamps, shrimps, mollusk's and fish respectively [4]. Infections by these pathogens are acquired by consumption of aqua culture foods or sea foods or exposure to contaminated water [3]. Vibrio contaminated aqua culture products cause food poisoning and diarrhoea in humans [2, 5] Vibrionaceae members show wide range of niche specialization from free living forms to those attached to biotic components like water hyacinth and duck weed and attached to abiotic piezophiles in marine sediments [12-13]. In aqua culture settings copepods and chironomids considered as natural reservoirs [9] and are dispersed by migratory birds. The authors [6] reported association of V. cholerae with fresh water amoebae. The authors from Japan [7] reported V. cholerae non- 01 was isolated from ayu fish in Japan. Vibrio member's causes systemic infection in fishes after attached to epithelial papillosum of carp, grunt fin tissue, and fat head minnow epithelial cell and then internalize in to fish and cause infection [11]. Fishes affected by classical V. cholerae, show typical sings of a generalized septicemia, with hemorrhages on the base of the fins, exophthalmia, and corneal opacity. Moribund fishes are frequently anorexic with pale gills which are reflects a severe anemic, oedomatous lesions observed on hypodermis [11]. The present study aimed to isolate the pathogens and characterized by morphological, biochemical, and molecular approach which include 16s rRNA gene sequencing and to be checked their pathogenicity, and to prove them as true pathogens.

MATERIAL AND METHODS

Collection of water and diseased fish samples

Diseased moribund *Labeo rohitha* samples were collected from above said locality, (10) brought to the laboratory, observed for disease symptoms, all fishes showed grossly fin hemorrhages, pale gills, white coloured tiny nodules on visceral organs like liver, kidney, heart irrespective of size and sex of the fishes. 3 water samples were collected from sequential days of 15 for 45 days, to be checked the parameters like water temperature, pH, ammonia, nitrite, calcium, magnesium, alkalinity, hardness, chlorides, TDS, conductivity DO, BOD and COD [15].

Isolation and identification of bacteria

A loop full of sample was collected with the help of inoculation loop from diseased fish collected sample was transferred on to Brain heart infusion agar medium Hi media, Bombay, incubate the plates at 37ºc for 24 hours. Isolated colonies from primary cultures were re inoculated on to BHIA medium plates, plates were incubated for another 24 hrs at 37ºC to get pure culture. The pure culture colonies were sub cultured on to agar slants with mineral oil and used them in re infection studies. To differentiate organisms as gram positive or gram negative, differential staining was done. To identify bacteria further, cultures were tested for biochemical characteristics with the enterobacteriaceae kit, Himedia, Bombay. These tests were performed according to the manufacturer instructions. 16s rRNA gene sequencing studies was done for identification of bacteria in molecular level, which was involved in vibriosis disease.

DNA extraction

DNA from saturated bacteria liquid cultures was extracted by CTAB method, includes collection of bacterial cell pellet by centrifugation, lysis of cell pellet were attained by suspending in TE buffer with 100µg of proteinase K and 0.5% SDS final concentrations. After one hour of incubation at 37ºC the lysate was treated with 80µl of 5M Nacl and 100µl of 10% CTAB solution. Cell lysate was incubated at 60ºC for 10min. Degraded Proteins from the cell lysate were removed by precipitation with phenol, phenol\ chloroform and chloroform treatment respectively. Followed by protein precipitation bacterial genomic DNA was recovered from the resulting supernatant by iso-propanol precipitation. Precipitated DNA pellet was washed with 70% alcohol for removal of salts. The DNA pellet was allowed for air drying and re suspended in 50µl of de ionized water with 1µl 10mg ml-L RNaseA enzyme for the removal of RNA. Quality of the isolated DNA was analyzed by resolving on 1% Agarose gel electrophoresis with 1X TAE buffer.

Quantification of DNA

Isolated DNA was quantified by measuring absorbance at 260 and at 280nm. Ratio of absorbance 260/280 was used to determine the quality of isolated DNA. The concentration was calculated using the following formula.

Concentration of the DNA = OD260 X 50 X dilution factor = mg of DNA/mL.

PCR amplification

The variable V3 region of 16s r DNA was amplified by PCR with primers to conserved regions of the 16s r RNA genes. The nucleotide sequences of the primers were as fallows: RDF 27 - 5'- AGAGTTTGATCCTGGCTCAG –3' and RDR1492 –5'- GGTTACCTTGTTACGACT T–3'. Both primers have shown to anneal with a majority of bacterial sequences in the ribosomal data base. All the PCR amplifications were conducted in 50µl volume containing 2µl of total DNA having 54ng µl-L concentration, 200µM each of the four deoxy nucleotide tri phosphates, 1.5 µl Mgcl2 (50 m mol l-L), 5 µl of individual primers (10 µmol l-L) and 0.1 µl (1U µl Taq polymerase). An automated thermal cycler (Biorad) was used for PCR amplification, which was programmed for an initial denaturation at 95ºc for 3 min, 39 cycles of denaturation (1min at 95 ºc) , annealing (30s at 56 ºc), and extension (1min at 72ºc) and a final extension 72 ºc for 10 min. Finally, the amplified PCR product was stored at 4ºc. The samples were analyzed on 1% agarose gel (Lonza, USA) to know ribo print pattern (fig3). The separated bands were excised from the gel by using surgical blade for recovering DNA. Protocol used for the elution of DNA from agarose gel was taken from the user manual of Real Biotech DNA/PCR purification kit (CAT NP 36105).

DNA sequence and phylogenetic analysis

For sequencing analysis, amplified PCR products were sent to EUROFIN Company. All the 16s r DNA partial sequence were aligned with those of the reference micro organisms in the same region of the closet relative strains available in the Gen Bank data base by using the BLAST N facility (http:// www.ncbi.nlm.nih.gov\BLAST) and were also tested for possible chimera formation with the CHECK CHIMERA program(http://www.35.8.164.52\cgis\chimera.cgi? Su: SSU). Their sequences were further aligned with the closest matches found in the Gen Bank Data Base with the Clustal X function of (MEGA VERSION 5.1). Neighbor- joining phylogenetic tree was constructed with the molecular evolutionary genetic analysis package MEGA VERSION [14] 5.1. A boot strap analysis with 500 replicates was carried out to check the robustness of the tree. Boot strap re sampling analysis, for the replicates was performed to estimate the confidence of the tree topologies (fig 5).

Nucleotide sequence accession numbers

Sequence was submitted to the Nucleotide sequence data base to the Gen Bank Public data base under the accession number KC 679075.

Artificial challenge studies

Apparently healthy *L rohitha* juvenile's 60g kg-1 body weight taken from the hatchery of kaikalur, AP, India; the fish which had no history of disease or abnormality were stocked in 500L ferro cement tanks with aerated fresh water in a laboratory and acclimatized for 15 days before starting the experiment. They were fed with standard diet in 2 divided doses daily during the experiment. Water in the tanks was exchanged partially daily to remove waste feed and fecal matter. Mean lethal dose LD_{50} of these isolates estimated according to [16]. Five groups (group1- 5) with 6 fish in each were challenged with a series of dilutions of isolates. The bacterial suspension prepared in phosphate buffered saline (0.15M, pH 7.4) was injected to each fish intraperitoneally with 0.1ml of different dilutions of bacteria. The final concentration of the bacteria injected to each dose group of fishes was 10^5 - 10^8 CFU mL (table 4). A control fish was injected with 0.1mL phosphate buffered saline. Mortality in groups was observed till 10 days, and the cause of mortality was re confirmed by re isolating the bacteria from the liver, kidney, heart.

Kirby- bauer disc diffusion

Antibiotic susceptibility testing was performed for the antibiotics, ceftriaxome (30µg), co tri maxazole (25µg), cefuroxime (30µg), ciprofloxacin (5µg), ticarcillin (75µg), cefoperazone (75µg), amikacin (30µg), cefaclor (30µg), doxycyclin (30µg), gentamycin (10µg), nitro furontoin (300µg), chloramphenicol (30µg), tetracycline (30µg), tobramycin (10µg), ceftazidime (30µg), nalidictic acid (30µg), ofloxacin (5µg), cefotaxime (30µg), amoxyclav (30µg), netillin (30µg), cefixime (5µg) Hi media, Bombay by Kirby bauer disc diffusion method by with the isolate identified. Bacterial suspension matching 0.5 Mc Farland turbidity standards were inoculated on BHIA. Isolates show visible growth after 24 hr's incubate at 37°c (table3).

Table 1: Show results mean values of physico- chemical parameters

S. No.	Parameter	Mean Value	Mean + SD
1	Water temperature	28.5	0.5 ºC
2	pH	8.2	0.458
3	Ammonia	1	0.416 ppm
4	Nitrite	0.8	0.2 ppm
5	Calcium	54.6	7.02 mg L
6	Magnesium	91.3	6.42 mg L
7	Alkalinity	473.3	30.55 mg L
8	Hardness	155	5.56 mg L
9	Chlorides	175.6	4.50 mg L
10	TDS	1710	36.05 mg L
11	Conductivity	1.1457	0.024ms\cm^2
12	Dissolved Oxygen	1.76	0.251 mg L
13	BOD	2.03	0.05 mg L
14	COD	1.2	0.2 mg L

Table 2: Shows biochemical reactions of *V. cholerae*

S. No.	Test	Result
1	ONPG	Positive
2	Lysine	Positive
3	Ornithine	Positive
4	Urease	Negative
5	Phenyl alanine	Negative
	Nitrate reduction	Negative
6	H2S	Positive
7	Citrate	Positive
8	VP	Positive
9	MR	Negative
10	Indole	Negative
11	Melonate	Variable (Bluish Green)
12	Esculin	Negative(NC)
13	Arabinose	Variable(Pink)
14	Xylose	Variable(Pink)
15	Adonitol	Variable(Pink)
16	Rhamnose	Variable(Pink)
17	Cellobiose	Variable(Pink)
18	Melibiose	Variable(Pink)
19	Saccharose	Negative(NC)
20	Raffinose	Variable(Pink)
21	Trehalose	Negative(NC)
22	Glucose	Variable(Pink)
23	Lactose	Variable(Pink)

Table 3: Anti bio gram report of *V. cholerae*

Antibiotic	Inhibition in mm
Ceftriaxome	0
co tri maxazole	6
cefuroxime	1
ciprofloxacin	11
Ticarcillin	0
cefoperazone	0
Amikacin	10
Cefaclor	0
Doxy cycline	0
Gentamycin	11
Nitrofurontoin	10
Chloromphenicol	2
Tetracycline	0
Tobramycin	9
Ceftazidime	6
Nalidictic acid	2
Ofloxacin	10
Cefotaxime	0
Amoxyclav	5
Netillin	10
Cefixime	1

Table 4: LD 50 of *V. cholerae*

Group	Log dose	Death	Survived	Death	Cumulative survival	Total	Mortality Ratio	Mortality%
Control PBS 0.1ml	0	0	6	0	15	15	0/15	0
CFU 10^8 1.4	0.1461	2	4	2	7	9	2/9	29
CFU10^7 2.2	0.3424	3	3	3	5	8	3/8	38
CFU$10^6$3.9	0.5910	4	2	4	2	6	4/6	67
CFU10^5 5.0	0.6989	6	0	6	0	6	6/6	100

From the table it seen that 50% mortality lies between 10^5 and 10^6

Proportionate difference of the 50% point: 50% - Next lowest

Next highest% - Next lowest%: 50%- 38%: 12: 0.413

67% - 38% 29

Then 0.5910-0.3424 = 0.2486

0.2486×0.413 = 0.10267

LD50: 0.10267 + 0.3424 = 0.44507

LD50= 10^8 28 cfu/ml.

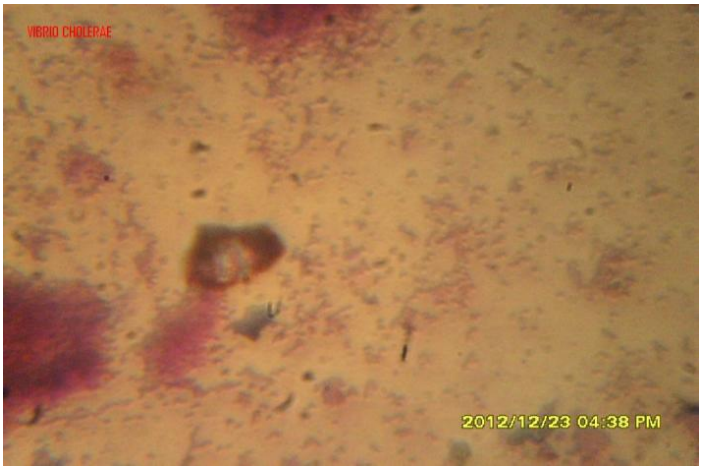

Fig. 2: gram report of isolate

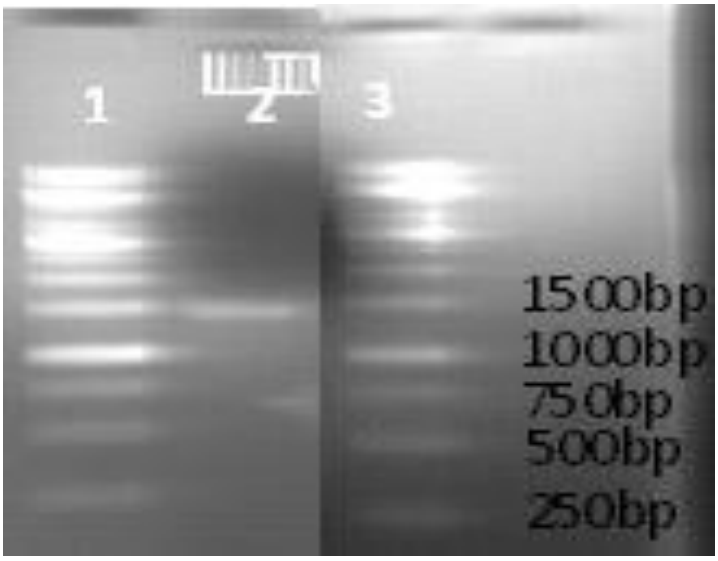

Fig. 3: Gel elution report of isolate

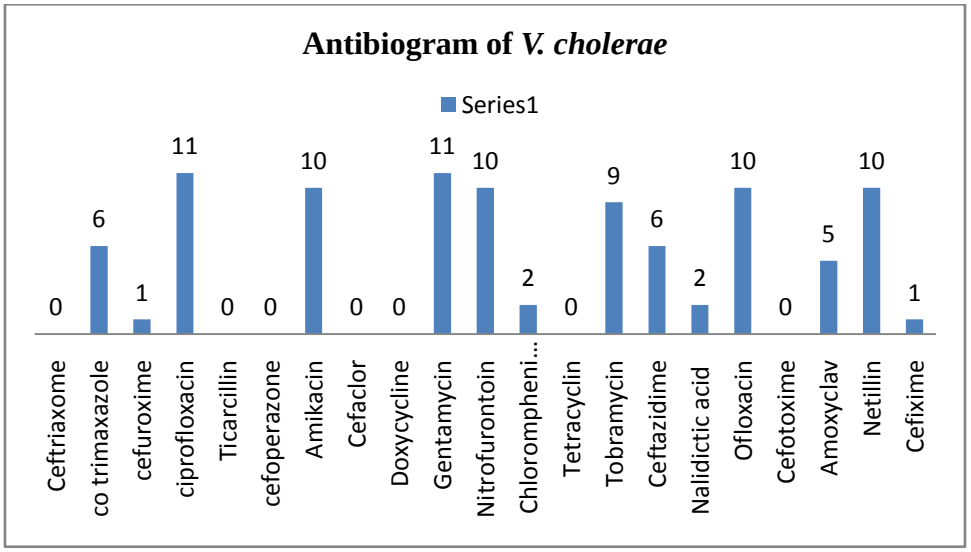

Fig. 4: Anti bio gram graph of *V. cholerae*

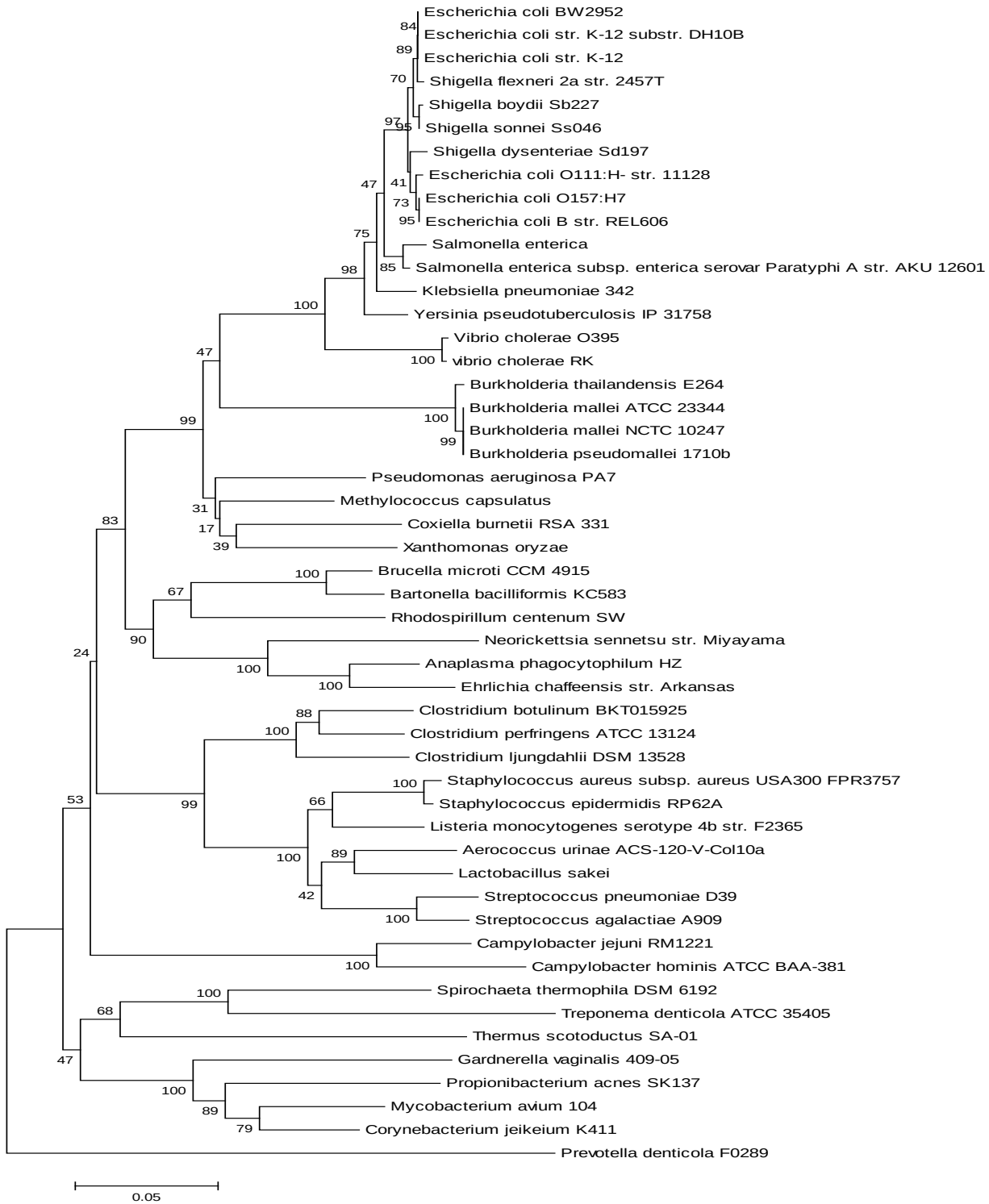

Fig. 5: Neighor- joining trees constructed using Mega 5.1 showing phylogenetic relation ships of 16S r DNA sequences from diseased fish to closely related sequences from Gene Bank.

RESULTS

The result of physico- chemical parameters of waters were presented in (table1). Water chemistry results indicated that variation in ranges of pH, ammonia, nitrite and total dissolved solids, alkalinity, BOD, COD. These altered parameters show great impact on aquatic biota including fish. Inoculated fish isolates on BHIA medium resulted in white creamy colonies are circular, convex, smooth and slightly opaque with entire margins. (figure1). Purity of the colony was tested by gram staining method, isolate is gram negative (figure2). The white creamy colonies are positive for ONPG, lysine, ornithine, citrate, H_2S, voges proskaver tests. Biochemical characterization of isolate results was given in the (table2). Comparison of the biochemical characterization with existing literature microorganism was identified as *Vibrio cholerae.* Quantification of DNA results as fallows *Vibrio cholerae* 185µg ml^{-1} concentration. The partial 16s r RNA sequence *Vibrio cholerae* 1465 submitted to Gene Bank public data base under KC 679075. The

isolated strain represented in phylogenetic tree as Vibrio cholerae RK. Artificial challenge study resulted that isolate can become pathogenic to *L. rohitha*. *V. cholerae* 10^5 dilution bacterial suspension killed all fishes in group 5 within 60 hours of post infection studies. The antibiogram report revealed that the isolated pathogen sensitive to very few chemotherapeutics ciprofloxacin, amikacin, gentamycin, nitrofurontoin, tobramycin, ofloxacin, and netillin respectively.

DISCUSSION

For effective cultivation of the fish good quality water is needed, due to lack of sustainable management practices in water quality, fishes prone to stress and susceptible to different diseases. All living organisms have optimum range of pH where the growth is best. Water with high alkalinity not more buffered and the degree of pH fluctuation is high. Alkalinity changes can affect the primary productivity in cultured ponds. DO fluctuations was high from time to time, these changes permit the stressful conditions, and growth of pathogens. Elevated levels of ammonia causes gill damage and reduce the growth of fishes. Water temperature show direct impact on metabolism, feeding rates, respiratory rates of aquatic biota, and influence the solubility of oxygen. Nitrite results from feed can disrupt the oxygen transport in live fishes. Hardness of culture waters is depending on levels of calcium and magnesium. High TDS value directly indicates the presence of organic matter in culture waters. Culture water with high organic matter not only pollutes the tank, but also surrounding areas and support growth of different pathogens like causative agents of Vibriosis. After observing the gross symptoms of fish we postulated emerging of new bacterial member's involvement in disease. Isolation and identification of pathogens were done in invitro by cultural, biochemical, and molecular methods for molecular level identification of pathogens. The identified organisms from diseased fish are challenged against healthy juveniles had no pre history of disease, to know LD_{50}. 6 doses can be chosen for determination of LD_{50} starting from no death to 100% mortality. *V. cholerae* $10^{5.50}$, $10^{6.39}$, $10^{7.22}$, $10^{8.14}$, 10^{1} killed the fishes in the rate of 100%, 67%, 38%, 29%, and 0% respectively. This communication provided the evidence of *Vibrio cholerae* which is true causative agent of Vibriosis disease in *L. rohitha*. The isolated member showed resistance to ceftriaxome, cefuroxime, ticarcillin, cefoperazone, cefaclor, doxycyclin, chloramphenicol, tetracycline, nalidictic acid, cefotaxime, cefixime, and show moderate sensitivity to co tri maxazole, and netillin respectively.

CONCLUSION

Good manufacturing practices should always be observed by the trade to minimize the risk of cholerae and *vibrio* food poisoning associated with the consumption of aqua food products. Hygienic quality of fish tank water in particular source water for keeping live fish food is also important.

REFERENCES

1. Hervio heath D., Colwell RR., Derrien AA., Fournier JM., and Pommepuy M. "occurrence of pathogenic *vibrios i*n coastal areas of France", *J appl, Microbiol* 2002; 92: 1123- 1135.
2. Wafaa MKB, Walaa AW, and Amani FA. Detection of *salmonella* and *Vibrio* species in some sea food in Alexandria. *J. American Sci* 2011; 7(9): 663-668.
3. Okonto I O, Ogun AA, Adejoye OD, Ogunjobi AA, Nkang AO, and Adebayo Tayo BC. Hazards analysis critical control points (HACCP) and Microbiology qualities of sea foods as affected by Handler's hygiene in Ibadan and Lagos, Nigeria. *African journal of food sciences* 2009; 3: 35- 50.
4. Thompson FL., Iida T and Swings J, Biodiversity of *vibrios*. *Microbial. Mol. Biol. Rev* 2004; 68: 403-431.
5. Cowell RR. Global climate and infectious disease: the cholera paradigm. *Science,* 1996; 20: 2025- 2031.
6. Susan Thom D Warthurst, and Drasar BA Association of Vibrio cholerae with fresh water amoebae *J. Med. Microbial* 1992; 3: 6303- 306.
7. Ciira kiiyukia A, Nakajima T, Nakai K, Muroga H, Kawakami and Hashimoto*. Vibrio cholerae non- 01* isolated from Ayu fish in Japan, *Applied and Env Microbiol* 1992; 58: 3078-3802.
8. Pfeffer CS, Hite FM, Oliver JD. Ecology of *Vibrio vulnificus* in estuarine waters of eastern north Carolina *Applied and Env Microbiol* 2003; 69: 3526- 3531.
9. Senderovich, Yigal, Izhaki, Ido, Halpern, Malka. Fish as Reservoirs and Vectors of *Vibrio cholerae PLOS ONE* January 2010; Vol 5: Issue 1, 1-
10. Maria del Refugio, Castañeda Chávez, Violeta Pardio Sedas, Erasmo Orrantia Borunda, Fabiola Lango Reynoso. Influence of water temperature and salinity on seasonal occurrences of *Vibrio cholerae* and enteric bacteria in oyster-producing areas of Veracruz, México *Marine Pollution Bulletin* December 2005; Volume 50: Issue 12, 1641–1648.
11. Wang XH and Leung KY. Biochemical characterization of different types of adherence of *Vibrio* species to fish epithelial cells *Microbiology* 2000; 146: Pt 4 989-998
12. Jerry reen F, Salvador Almagro-Moreno, David Ussery & Fidelma Boyd E. The genomic code inferring Vibrionaceae niche specialization *Nature Reviews Microbiology* September 2006; 4: 697-704.
13. Pieter TJ, Johnson, Sara H, Paull. The ecology and emergence of diseases in fresh waters *journal of fresh water biology* April 2011; Volume 56: Issue 4, 638–657.
14. Tamura, Koichiro, Daniel, Peterson, Nicholas, Peterson, Glen, Stretcher, Masatoshi, Nei, Sudhir Kumar. MEGA5 Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods *Mol. Biol. Evol* 2011; 28: (10), 2731–2739.
15. APHA Standard methods for the examination of water and waste-water analysis. 20th (Ed) *Washington D.C.* 1998; *12-13 pp.*
16. Reed LJ, and Muench HA. Simple method of estimating fifty percent end points. *Am. J hyg* 1938; 27: 493-497.