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ISOLATION AND CHARACTERIZATION OF BACTERIAL PATHOGENS FROM INFECTED ORNAMENTAL FISHES IN MADURAI DISTRICT, TAMILNADU, INDIA

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ABSTRACT

The present research work were aimed for collection, screening and identification of bacterial species from infected ornamental fishes and estimate the biochemical characterization and DNA quantity of predominated microbes from experimental fishes. The various bacterial species were detected. The bacterial species such as *Pseudomonas aeruginosa, Pseudomonas fluorescence, Vibrio cholerae, Vibrio vulnificus, Salmonella sp. Enterobacter E. coli* and *Aeromonas sp.* were isolated from the experimental fishes. The results of this present work, concludes that the ornamental fishes from Madurai district indicate that the pathogenic bacteria species were abounded in the aquarium. The presence of bacteria of definite economic importance is also reported in this study on pathogens distribution in aqua farms.

KEYWORDS: aqua farm, ornamental fish, Maniatis method, Microbiota, pathogenic bacteria.

INTRODUCTION

The ancient Romans were the first to keep ornamental fishes as pets as home. Tropical aquarium keeping is a peaceful, educational and stress-free hobby, which cuts light through all artificially created farming and trade are gaining greater momentum. There are about 30,000 to 40,000 species of fishes differing from each other in shape, size and habitat. They live in all the seas, rivers, canals, lakes, dams, ponds and in almost every place where there is water.[1] Most of these belong to the super order Teleosteii. Among these, 600 species valued as ornamental fishes are grown for hobby and export. However, India's contribution to the international export market is alarmingly low earning about less than one crore rupees per annum.

As aquacultural production becomes more intensive, the incidence of disease including various infection diseases had increased as a result of it leading to significant economic losses disease on a crucial factor which inhibits the expansion of aquaculture. Various chemotherapeutics have been used for treatment or prevention of diseases. However, the use of anti-microbial agents in aquaculture has resulted in more resistant bacterial strain. These resistant bacterial strains could have a negative impact on the therapy of fish disease (or) human disease and the environment of the fish farm.[2]

The distribution of many fish disease agents, disease detection and identification procedures, fish pathology, and treatment methods were reported.[3,4,5,6] Transmission in natural outbreaks has not been definitively shown experimentally we were able to infect and kill fish by injecting spores. It is possible that the spores present in the water can enter through small wounds in the skin and through the gut epithelium where internal parasites have opened wounds.

The purpose of the study were collection, screening and identification of bacterial species from infected ornamental fishes and estimate the biochemical characterization and DNA quantity of predominated microbes from experimental fishes.

MATERIALS AND METHODS

Diseased infected ornamental fishes were collected from an aqua farm, located at Kadchanenthal, Madurai and were used for the study of the disease causing organism. The different fishes are used to the experiments are given as follows.

1. Kissing Gowrami - *Helostoma temmincki*
2. Golden fish - *Carassiur auratus*
3. Red sward tails - *Xiphophorous helleri helleri*
4. White shark - *Caracharadon caracharias*
5. Black shark - *Etmopterus carteri*
6. Black molly - *Molliensia sphenops*
7. Molly - *Poecilia sphenops*

Isolation and identification of microorganisms from experimental fishes

The microorganisms are isolated and identified from various experimental fishes. The following proceedings are adapted.

Primary culture technique for bacteria

The nutrient agar was prepared with bacteria isolation agar at approximately 50 µg / ml in aseptic condition. The various fishes mucus, caudal fin and gill region was carefully scraped form the dorsal body using a sterilized cotton buds, ventral skin mucus was not collected to avoid intestinal and sperm contamination. The sample was spread over the surface of the plates. The plates were incubated at 37˚C for 24 hours.

Pure culture technique

After incubation the result were observed the nutrient agar were prepared aseptically each microorganisms are streak the plates. The plates were incubated at room temperature for 24 hours. Prepare the slides view under the microscope. Identification of species based on standard biochemical test.

Preparation of chromosomal DNA from bacteria (Maniatis method)

Grow bacterial cells in 1.5 ml of Luria broth. Harvest the cells from 1.5 ml culture. Resuspend and lyse in 200µl of lysis buffer by vigorous pipetting. The add 66µl of 5M NaCl, mix well and leave at 20˚C for 10 minutes. Centrifuge the viscous mixture at 12K (12000) for 10 minutes at 4˚C. After transferring the supernatant to a fresh tube add equal volume of choloroform: isoamyl alcohol (24: 1). Mix well and centrifuge. Transfer the upper phase into a clean eppendrof tube and discard the lower phase. Precipitate the DNA with 100% ethanol (or) 1 volume isopropyl alcohol. Centrifuge at 12000 rpm for 10 minutes. Collect the DNA pellet and wash it with 70% ethanol and air dry the DNA dissolve the DNA pellet in 50µl of TE buffer. (Can be stored at -20˚C till use).

Agarose gel electrophoresis

Prepare DNA gel using buffer (1 x TAE buffer) in distilled water. Melt 0.7% agarose with 1 x TAE buffer in distilled water. Pour the molten and cooled (to 45-55˚C) agarose onto DNA gel platform already cleaned ends sealed with cellophane tape. Place the gel comb on to the gel in the proper place. Let it solidify in room temperature for 15-30 minutes. After solidification, remove the gel comb and cellotape and place the agarose gel on the gel running tank filled with 1 x TAE running buffer, so that the gel is immersed completely. Marker DNA is load the first well, remaining wells load the DNA samples into wells after mixing with 6 x DNA loading dye. Run the gel with 60-100 volts. After run, stain the agarose gel using (0.5 µl ml distilled water) ethidium bromide stain. Agarose gel is keep under the UV-transilluminator. The DNA will appear pinkish red discrete bands.

Quantification of DNA

1. Bacterial cells in 25 ml saline EDTA solution in a flask. Add 1 ml of Lysozyme solution to the above suspension and incubate with occational shaking at 37˚C for 30 minutes.
2. To facilitate complete cell lysis and add 2 ml of SDS solution and heat the mixture in a water bath at 60˚C for 10 minutes. And then cool it to temperature shaking at 37 ˚C for 30 minutes.
3. Add 7 ml of 5M sodium perchloride so that is final concentration in the above preparation in one molar.
4. Mix well but gently and then add an equal volume (35 ml) of chloroform isoamyl alcohol and slowly shake in a highly stoppered flask for 30 minutes at room temperature.
5. Centrifuge the above mixture at 10,000 rpm for 5 minutes at room temperature 3 layers will be formed. Collect the upper aqueous phase in a beaker.
6. Add 2 volumes of chilled 95% ethanol to this aqueous phase.
7. Gently stir the preparation and spool the thread like fibres on the glass rod. If DNA doesn't form fibrous thread like structure at 300 rpm for few minutes dissolve the pellet in 10 ml of saline EDTA solution and Precipitate with 2 volume of 95% ethanol.
8. Air dry the spooled DNA on the glass rod and dissolve it in a test tubes containing 10 ml of saline citrate. Store the solution at 0-4˚C.
9. The obtained DNA is quantitatively estimated.

RESULTS

In this experiment the contaminating microbiota were isolated and identified from various experimental fishes, such as *Helostoma temmincki, Carassium auratus auratus, Xiphophorous helleri helleri, Carcharadon carcharias, Etmopterus carteri and Molliensia sphenops* and there are highly similar for each fish. The various bacterial species were detected. The bacterial species such as *Pseudomonas aeruginosa, Pseudomonas fluorescence, Vibrio cholerae, Vibrio vulnificus, Salmonella sp. Enterobacter E. coli* and *Aeromonas sp.* were isolated from the experimental fishes and interpreted in the table.1 and 2.

The amount of DNA in *Enterobacter* sp. *Vibrio vulnificus*, *Salmonella* sp, and *Pseudomonas aeruginosa*, of *Helostoma temmincki* (Kissing Gowrami) was found to be 0.921, 0.928, 0.925, 0.939 mg/ml respectively. The quantity of DNA present in *E. coli, Enterobacter Pseudomonas aeruginosa, Vibrio cholerae*, of *Carassium auratus auratus* (Gold fish) was found in 0.927, 0.923, 0.933, 1.167. mg/ml, respectively. The quantity of DNA in *E. coli, Pseudomonas aeruginosa* of *Xiphophorous helleri helleri* (Red sward tail) were analyzed and the amount of DNA was found in 1.141, 0.947. mg/ml, respectively. Bacterial species such as *Enterobacter sp, Pseudomonas aeruginosa, Aeromonas sp.* of *Carcharadon carcharias* (White Shark) were analysed and the total DNA content was estimated 0.928, 0.934 and 1.145. mg/ml respectively. Total amount of DNA

present in the bacterial species such as *Enterobacter*, *Pseudomonas fluorescence*, *Pseudomonas aeruginosa and Aeromonas sp.* of *Etmopterus carteri* (Black Shark) was found in 0.926, 0.883, 0.932 and 1.144. mg/ml respectively. The DNA content present in the bacteria such as *Enterobacter sp*. *Pseudomonas aeruginosa, Aeromonas sp.* of the fish *Molliensia sphenops* (Black molly) was estimated and quantify was 0.928, 1.175, 0.934 mg/ml respectively. The whole amount of DNA in the bacterial species such as *Vibrio vulnificus Pseudomonas aeruginosa and Aeromonas sp.* of *Poecilia sphenops* (Molly) were analyzed and their value was found that 0.927, 0.935 and 0.931 mg/ ml respectively (Table 3).

Table 1: Shows identification of bacterial species present in the experimental fishes collected from Aquarium of Madurai district.

S. No	Experimental fishes	Bacterial species
1.	*Helostoma temmincki* (Kissing gowrami)	*Vibrio vulnificus* *Salmonella* sp. *Enterobacter* sp. *P. aeruginosa*
2.	*Carassius auratus auratus* (Gold fish)	*E.coli*, *P. aeruginosa*, *Enterobacter* sp.
3.	*Xiphophorous helleri helleri* (Red sward Tail)	*E.coli* *P. aeruginosa*
4.	*Caracharadon caracharias* (White shark)	*Enterobacter* sp. *P. aeruginosa* *Aeromonas* sp.
5.	*Etmopterus carteri* (Black shark)	*Enterobacter* sp. *P. aeruginosa* *P. fluorescence* *Aeromonas* sp.
6.	*Molliensia sphenops* (Black Molly)	*Enterobacter* sp. *P. aeruginosa* *Aeromonas* sp.
7.	*Poecilia sphenops* (Molly)	*Vibrio vulnificus* *P aeruginosa* *Aeromonas* sp.

Table 2: Identification based on biochemical test for specific bacterial sp. isolated from experimental fishes.

Bacterial species	Gram`s staining	Morphology	Motility	Indole Test	Methyl Red Test	Voges Proskauer	Citrate Test	H_2S	Glucose Test	Lactose Test	Sucrose Test	Catalase Test	Oxidase Test	Gelatin
Enterobactor sp.	-	R	+	-	-	+	+	-	+	+	+	+	-	-
Pseudomonas aeruginosa	-	R	+	+	-	-	+	-	+	-	-	+	+	+
Vibrio cholerae	-	R	+	+	+	+	+	-	-	-	+	-	+	+
Salmonella sp	-	R	+	-	+	-	+	+	+	-	-	+	-	-
Vibrio vulnificus	-	R	+	+	+	-	-	-	-	+	-	-	+	-
E.coli	-	R	+	+	+	-	-	-	+	+	+	+	-	-
Aeromonas sp	-	R	+	+	+	+	+	+	-	-	-	+	+	+
Pseudomonas fluorescens	-	R	+	+	-	+	-	-	+	-	-	+	+	-

Table 3: Shows quantitative analysis of total DNA content of bacterial species from different experimental fishes.

S.No	Experimental fishes	Bacterial species	Total DNA content mg/ml
1	*Helostoma temmincki*	*Enterobacter* sp.	0.921
		Vibrio vulnificus	0.928
		Salmonella sp	0.925
		Pseudomonas aeruginosa	0.939
2.	*Carassium auratus auratus*	*E. coli*,	0.927
		Enterobacter sp,	0.923
		Pseudomonas aeruginosa	0.933
		Vibrio cholera	1.167
3.	*Xiphophorous helleri helleri*	*E. coli*,	1.141
		Pseudomonas aeruginosa	0.947
4.	*Carcharadon carcharias*	*Enterobacter sp*,	0.928
		Pseudomonas aeruginosa,	0.934
		Aeromonas sp.	1.145
5.	*Etmopterus carteri*	*Enterobacter*,	0.926
		Pseudomonas fluorescence,	0.883
		Pseudomonas aeruginosa	0.932
		Aeromonas sp.	1.144
6.	*Molliensia sphenops*	*Enterobacter sp*.	0.928
		Pseudomonas aeruginosa,	1.175
		Aeromonas sp.	0.934
7.	*Poecilia sphenops*	*Vibrio vulnificus*	0.927
		Pseudomonas aeruginosa	0.935
		Aeromonas sp	0.931

DISCUSSION

Biological diversity is frequently used as an indicator of environmental health, there is a normal microbial diversity on aquatic animals in healthy environments. Because every aquatic organism harbors a normal bacterial flora on their skin, it is rational to presume that the presence and frequency of any given microbial species will indeed affect the overall frequency and total allotment of others species within a diverse microbial population.

The achievement of ornamental fish culture depends on the health condition of the candidate species.[7] Being aquatic, and secondarily being enforced to remain under packed circumstances, the ornamental fishes are subjected to different diseases of changeable nature.[8] Bacterial infections are well thought-out as the major source for diseases and mortality.[9] A complete understanding of the aetiological agent, the pathogenesis, antigenicity, epizootiology and the inter-relationship of stress-related and ecological factors is vital for flourishing management and control.

Ornamental fish may develop acute systemic and chronic granulomatous diseases, where the bacteria may cause damage to the fins and ulcerations of the skin and these infections may be caused by primary pathogens, but more commonly are due to stress induced secondary infections.[10]

The clinical signs include lethargy, erythema and petechiation of the skin and fins, along with ulcerations of the skin, and internal lesions and these lesions are most commonly associated with Gram-negative organisms such as *Aeromonas, Pseudomonas, Vibrio, Flavobacteria, Yersinia*, and *Edwardsiella* spp. The pathogenic bacterial isolates of fish such as *Pseudomonas aeruginosa* and *A. hydrophila* were tested for their pathogenicity.[7]

The present findings supported by the previous works revealed that several pathogenic bacterial genera- *Aeromonas*, *Micrococcus*, *Edwardsiella*, *Pseudomonas, Coryneformes*, *Flavobacterium*, *Enterobacteriaceae*, *Acinetobacter*, *Achoromobacter,* and *Alcaligenes*- were reported in farmed Thai pangas in Bangladesh.[11]

Total 33 microbial colonies were isolated from the rearing water, skin, gut and egg surface of *C.auratus*. The microbial genera such as *Bacillus, E.coli, Staphylococcus, Flavobacterium, Citrobacter* were recorded in rearing water. Likewise, in the skin surface *Bacillus, Streptococcus, Vibrio, Shigella, Pseudomonas* and *Klebsiella* were recorded.[12] In the present study clearly indicated that, occurrence and dominant nature of specific microbes in the host system may be influenced by the immediate environmental change and also its biological condition. Thus the variation in distribution of bacterial genera in *C.auratus* and its rearing environment were justified.

Two new pathogenic strains of *Aeromonas* spp. were isolated from blue gourami fish that showed symptoms of skin ulcers and from the water of the aquarium

wherein these fishes were cultured.[11] The *Pseudomonas* spp., *E. coli*, *Serretia* spp., *Shigella* spp, *Enterobacter* spp. and *Klebsiella* spp. isolated in this study are considered to be opportunistic pathogens, capable of producing infections in immunologically weakened fish or as secondary invaders in fish populations suffering from others diseases.[10,12,13]

CONCLUSION

In conclusion, this study provides evidence for the presence of specific external microbiota associated with particular fish species. The results of this present work, concludes that the ornamental fishes from Madurai district indicate that the pathogenic bacterial species were abounded in the aquarium. The presence of bacteria of definite economic importance is also reported in this study on pathogens distribution in aqua farms. The composition and structure of this microbiota are likely to be impacted by several cofounding variables including abiotic factors linked to geographic locality and season as well biotic factors related to the nutrient potential or antimicrobial components of fish mucus.

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