Annexure I

Final Project Report

Title

Studies on preventive and control measures using vaccines and Indian medicinal plants against ulcerative disease caused by *Aeromonas caviae* in farm reared Indian catfish, *Clarias batrachus*.

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Introduction

Aquaculture is a type of agriculture and human-controlled cultivation and harvest of freshwater and marine plants and animals. Other terms used include fish farming, fish culture, mariculture, fish breeding and ocean ranching. Throughout the world, aquaculture operations constitute an integral part of fisheries and aquatic resource management. Organisms as varied as trout, carp and tuna (i.e., finfish), shrimps, oysters (i.e., shellfish) and seaweed are grown using ponds, tanks or nets in salt, brackish and fresh waters. *Clarias batrachus* is a freshwater air breathing catfish found primarily in Southeast Asia and commonly called as walking catfish because its ability to "walk" across dry land, to find food or suitable environments. While the technology of induced breeding and seed production of this catfish species has been perfected; its large-scale production is yet to be taken up. With more or less similar pond management measures as that of carp culture practices and stocking with 20,000-50,000 fingerlings/ha, production levels of 3-5 tonnes/ha is achieved in grow-out culture of magur, which attain a weight of 200-300 g in 6-8 months. This catfish can be suitably cultured both in monoculture and polyculture systems. In spite of the availability of huge potential resources in the form of swamps and derelict waters that could be effectively used for commercial farming and huge market demand of this species, large scale culture of catfish yet to receive due attention. Development of balanced supplementary feed owing to its carnivorous feeding habits and availability of desired quantity of seed of right size are the two critical aspects, which have to be addressed.

Disease is one of the most important factors influencing the success of catfish culture. Channel catfish virus disease (CCVD) was first recognized as a disease problem during the early days of commercial catfish farming. During the late 1960s, high mortalities were reported in channel catfish (*Ictalurus punctatus*) fingerlings and fry shortly after transfer from

the hatchery to nursery ponds. The causal agent was identified as a herpesvirus in 1971. The disease is specific to channel catfish and brood stock and is believed to be the major source of infection to young fish. The disease is strongly influenced by environmental stressors. There are no effective preventive or treatment measures, but the effect of the disease can be minimized through optimal mangement practices. CCVD occurs in fry and fingerlings less than a year old and less than 6 inches long, both in the hatchery and in ponds. The first sign is a slowing of feeding activity. Fish may be seen swimming erratically, often in an aimless spiral pattern. Ultimately, large numbers of fish may congregate along the sides of hatching troughs or ponds and hang motionless in a head-up, tail down position (Camus, 2004). Acute CCV epizootics in populations of fry and fingerling channel catfish can result in mortalities as high as 95%within one week (Plumb, 1978). CCV causes an acute haemorrhagic disease in channel catfish fry and fingerlings (Buck, 1990; Plumb, 1977). This herpesvirus infects by entrance through the gills and causes lytic destruction in most tissues.

Motile species of *Aeromonas* such as *A. hydrophila, A. caviae* and *A. sorbia* are associated with hemorrhagic septicemia in fresh water fish and are pathogenic to fish. There are some reports that high mortality of fish is associated with the presence of *A. caviae* (Munro *et al.,* 1993; Ringo and Vadstein, 1998). Munro *et al.,* (1993) noted low survival of fish larvae associated with high proportion of *A. caviae*. High colonization of *A. caviae* in the gut of turbot fish larvae caused 100% mortality in fish larvae (Ringo and Vadstein, 1998). *A. caviae* is frequently isolated from both healthy and unhealthy turbot larvae and may be associated with high mortality in larval stages of fish (Munro *et al.,* 1993, 1994) There are lot of reports on isolation of *A. caviae* from other fishes like carp, gold fish, catfish, mullet, tilapia (Sugita *et al.,* 1994, 1995) and responsible for health-related problems in human being. Siri *et al.* (2008) have reported the infection caused by *A. caviae* in catfish and treatment of this infection by plant extract.

Recently, a disease namely ulcerative disease (UD) has been encountered in catfish farms located in different parts of the country including local catfish farms in Vellore, Vilupuram and Thiruvannamalai districts. The loss has been estimated about Rs. 20 to 30 lakhs in local catfish farms every year due to this ulcerative disease. The clinical signs include ulcerative lesions on the body surface and base of the fins of infected fish. Our preliminary studies revealed the involvement of a bacterium in causing ulcerative disease and the bacterial pathogen has been identified as *Aeromonas caviae.* The UD has been reproduced in healthy catfish using *A. caviae* in laboratory to satisfy Koch's postulate. The extensive use of antibiotics in aquaculture system has been banned. Vaccines and use of medicinal plants

are the possible alternatives to antibiotics to prevent and control the UD in catfish farms. In this context, the present work was carried out using UGC grant to develop vaccines, medicinal plants and actinomycetes extract to control the infection caused by *A. caviae* for the benefit of catfish farming of local farmers.

Materials and Methods

Preparation of whole bacterial cell vaccine

Whole bacterial vaccines were prepared following the methodology described by Benedicte *et al.* (1999). Heat and formalin killed whole bacterial cells were used to vaccinate the fish. For heat killed bacterial cells, *A. caviae* was inoculated into 50 ml sterile nutrient broth and incubated for 24 hrs at 28-30°C. After 24 hrs of incubation, the bacterial cells were centrifuged and the supernatant was discarded. The bacterial pellet was washed three times in saline. After suspending in sterile saline, the bacterial cells was inactivated in water bath at 70°C for 1 hr. After inactivation, they were checked for their sterility by plating on to nutrient agar.

For formalin killed bacterial vaccine, the bacterial cells in broth were mixed with 1% formalin and kept for 24 hrs. After formalin exposure, the killed bacteria were harvested by centrifugation at 5000 rpm for 10 min. The formalin killed bacterial cells were washed thoroughly for three times in PBS by repeated centrifugation and washing. Finally, the bacterial pellet was suspended in PBS and checked the sterility by plating on to nutrient agar.

Preparation of recombinant vaccine

Genomic DNA from *Aeromonas caviae* was extracted and purified according to the method described by Sambrook et al. (1989). The porin gene was identified by PCR approach using genomic DNA as a template and gene specific primers with appropriate restriction sites for convenient cloning of the amplified product. The gene was cloned in pRSET vector as per the standardized protocol being followed in our lab [We have already cloned several WSSV genes of white spot syndrome virus of shrimp and porin gene of *V. anguillarum* with a high degree of success (Sathish et al., 2004; Yoganandhan et al., 2004; Rajeshkumar et al., 2007)]. The recombinant gene was confirmed by restriction analysis and PCR using gene specific forward and T7 reverse. The plasmid with recombinant gene was transformed into *Escherichia coli* (BL21DE3). Expression of the protein was done by IPTG induction and analysed by SDS PAGE and commassie brilliant blue staining, and confirmed by Western blot using anti-histidine MAb (Amersham Pharmacia Biotech). The recombinant protein was purified using affinity chromatography.

Immunization of fish

Fish were immunized using whole bacterial cell vaccine or recombinant porin protein by intramuscular injection according to the standard procedures described by different workers (Boesen et al., 1997; Lonnstrom et al., 2001; Bowden et al., 2002; Vervarcke et al., 2004). The immunized fish were challenged by intramuscular injection with *A. caviae*

Evaluation of various plant extracts against *A. caviae*

Collection and identification of terrestrial medicinal plants

Indian medicinal plants with known medicinal properties particularly antimicrobial activity were selected and tested for their efficacy to inhibit the growth of *A. caviae* and control the bacterial infection in catfish. The plants were collected from different localities in Tamil Nadu and identified using identification manual (Warrier, 1996).

Processing the plant materials

The parts of selected plants (leaves, bulbs, rhizomes or whole plant) were removed, cut into pieces and washed thoroughly in tap water and shade dried at room temperature (28 ± 2°C) for 30 to 40 days. The air-dried materials were powdered separately using commercial electrical stainless steel blender. About 100 gm of powdered plant materials were extracted in a Soxhelt apparatus first with petroleum ether (Qualigens), then benzene (Reachem), diethyl ether (Nice), chloroform (Qualigens), ethyl acetate (SRL), methanol (Qualigens), ethanol (Hayman) and finally with distilled water. All the extracts were concentrated through distillation at a temperature of 5ºC above the boiling point. The extracts were dried at 60ºC for 24 h, and then weighed for yield per gram of dry sample. The plant residue after each solvent extraction was completely air-dried before the next solvent extraction (De *et al*., 1997). The crude extracts (1 mg) from different terrestrial plants with various solvents were first dissolved in 0.2 ml of acetone (SRL) and then mixed with 0.8 ml of distilled water to obtain the concentration of 1000 ppm. Polysorbate 80 (Tween 80, Qualigens) was used as an emulsifier at the concentration of 0.05% in the final test solution. The control was set up with acetone and polysorbate 80 (Saxena and Yadav, 1983).

For aqueous extract, the dry plant material (1 mg) was dissolved in 1 ml of distilled water to obtain the concentration of 1000 ppm. The control was set up with sterile distilled water alone.

Antibacterial activity of plant extracts

Muller Hinton Agar medium was prepared and autoclaved following manufacturer's instruction. The test organism was transferred from the stock into a glass test tube containing

5 ml of sterile nutrient broth with the help of a wire loop. The inoculated broth was incubated at 30ºC for 10-12 hrs to obtain moderate turbidity.

The agar well diffusion method (Perez *et al.,* 1990) was followed to determine the antibacterial activity of plant compounds against *A. caviae*. The antibacterial activity was evaluated by measuring the zone of inhibition around the well. The MIC of the plant extract against *A. caviae* will be determined by the tube dilution technique.

Actinomycetes isolates

An attempt was made to screen the actinomycetes isolates isolated from different sources for antibacterial activity against *A. caviae*.

Collection of Actinomycetes isolates

In the present study, actinomycetes isolates were obtained from National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kochi (47 isolates) to determine the antimicrobial activity against viral and bacterial pathogens of shrimp, prawn and fish. All these isolates were isolated from different environments like marine, soil and plants using standard isolating procedures and recommended culture media. Standard protocols were followed for sub-culturing and storing of these actinomycetes isolates in the laboratory as per the instruction given by the supplier. All the potential isolates are available in our laboratory for third party validation and scientific community for research.

Extraction of metabolites

Actinomycetes isolates were grown separately in different media such as starch casein broth (SCB) and production medium-I (PM-I), PM-II, and PM-III to select the most suitable medium. The cultures were incubated at 28ºC in a rotary shaker. The cultures were harvested after 7 days and filtered through Whatman No.1 filter paper. The culture filtrates were centrifuged at 10,000 rpm at 4ºC and the cell free supernatants were used for the extractions of secondary metabolites. Each culture supernatant was extracted three times by manual shaking with equal volume of ethyl acetate (1:1) in a separating funnel. The solvent layer was collected and concentrated using a rotary evaporator (Buchi, Switzerland) to obtain crude metabolites. The antiviral and antibacterial activity of ethyl acetate actinomycetes extract against viral and bacterial pathogens of aquatic animals was determined using standard protocols.

Preliminary Screening of antibacterial activity

The antibacterial activity of actinomycetes extract against *A. caviae* was tested by agar diffusion assay and cross streak method (Monisha et al., 2011). MIC of bioactive compounds was determined for this bacterial pathogen by tube microdilution method (Monisha et al.,

2011). During the primary screening, actinomycetes isolates were screened against *A. caviae* by using perpendicular streak method (Peela, et al., 2005). In this method, nutrient agar medium was used and each plate was streaked with individual actinomycetes isolate at the center of the plate and incubated at 28°C for 7 days. Later, 24 h fresh sub-cultured test bacterial pathogen was prepared and streaked perpendicular to the actinomycetes isolates, but not touching each other and incubated at 28 °C for 24 h. After the incubation, the antibacterial activity was assessed by determining the distance of inhibition zone between target bacteria and actinomycetes colony margins. Those isolates which showed maximum inhibition zone were selected for secondary screening. Plates with the same medium without inoculation of actinomycetes isolates but with simultaneous streaking of bacterial pathogens were maintained as control.

Secondary screening of antibacterial activity

Isolates that showed activity against bacterial pathogens in the preliminary screening by cross streak method were selected for secondary screening. The secondary screening was carried out by agar well diffusion method (Grammer 1976; Thirumurugan and Vijayakumar, 2015). The inoculum of bacterial pathogens was prepared by inoculating the respective pathogen into 9 mL of sterile nutrient broth and allowed to grow for 12 h to obtain the turbidity of 0.6 OD at 530 nm. The sterile swab was dipped into properly adjusted inoculum and excess liquid was removed by gentle rotation of the cotton swab against the inner surface of the test tube. To obtain even growth, the entire Mueller Hinton agar (MHA) surface was swabbed uniformly with the cotton swab. The inoculated plates were left at room temperature for 3-5 minutes to allow for any surface moisture to be absorbed before applying the extract. The ethyl acetate extract of actinomycetes isolates was prepared as described above. The well was prepared in the plate by using sterile cork borer (6 mm in diameter). A volume of 50 µL having 500 µg of crude extract was carefully dispensed into each well and allowed to diffuse for 2 h and incubated at 28°C for 24 h. The well with sterile broth was used as a negative control. After 24 h of incubation, zone of inhibition around each well was measured. The experiment was repeated three times (Waheeda and Shyam, 2017).

Treatment of infected fish with plant extract

Healthy catfish were infected by *A. caviae* using standard infection protocol (Sahul Hameed et al. 2006). The infected fish with clinical sign of ulcer were removed and reared separately in filtered freshwater. The infected fish were treated with plant extract.

Field trials

Field trials were conducted using plant extract to avoid ulcerative disease in catfish farms using standard protocols in Sekar Aqua Farm located at Walajah, Near our College.

Results and Discussion

Pathogenicity of *A. caviae* in healthy catfish *C. batrachus* by immersion methods, oral and intramuscular routes

The susceptibility of *C. batrachus* to *A. caviae* was tested by immersion challenge, oral route and intramuscular injection. The results are given in Tables 1 and 2. The accumulated percentage mortality of fingerlings exposed to *A. caviae* by immersion challenge was given in (Table 1). The highest concentration of *A. caviae* (39×10^6 CFU ml^{-1} of rearing medium) caused 20, 46.6, 53.3, 56.6 and 63.33% mortality in immersion route after 24, 48, 72, 96 and 120 hours of exposure, respectively. But this highest concentration failed to produce 100% mortality even after 10 days of post infection. The LC_{50} value of *A. caviae* for immersion route was determined at different time intervals. The LC_{50} value was found to be 3.82×10^5, 3.96×10^5 and 4.82×10^6 CFU ml^{-1} of rearing medium, respectively, after 24, 48 and 72 hours of post infection.

The highest concentration, 55×10^5 and 55×10^6 viable cells of *A. caviae* per animal caused 100% mortality within 120 and 96 hours of post inoculation, respectively, when the animals were injected intramuscularly. Whereas the lower concentrations of 55×10^3 and 55×10^4 viable cells of *A. caviae* per animal caused 43.3 and 66.6 % mortality after 120 hours of post injection (Table). The LD_{50} value of the *A. caviae* for intramuscular route was determined at different time intervals and it was found to be 2.21×10^6 and 1.12×10^7 CFU per animal after 72 and 96 hours of post injection, respectively. The results are given in (Table 2). *A. caviae* did not affect the catfish when the bacterium was given through oral route.

The clinical signs of ulcerative lesions were observed in experimentally infected catfish. The signs observed in the experimentally infected catfish were lethargy and foul smell at the time of mortality.

Clinical manifestations in catfish injected with *A. caviae* were monitored. Catfish which were injected with fewer bacteria and which lived longer exhibited clinical signs such

as ulcerative lesions on the body surface (Fig. 1) and foul smell being observed in the dead fish.

Confirmation of pathogenicity

The pathogenicity of *A. caviae* was confirmed by satisfying Koch's postulates. *A. caviae* was isolated from moribund and dead animals subjected to experiments. It was isolated from the liver, spleen and ulcerative lesions of experimentally infected catfish (*C. batrachus*). The characters of these re-isolates resembled the original isolates isolated from naturally infected catfish.

Immunological Parameters

Total erythrocyte and leucocyte counts

The total leucocyte count in the normal catfish was found to be 21 $x10^3$ cells/mm^3. The Total erythrocyte count in normal catfish was found to be 38.8 $x10^6$ cells/mm^3. However the total leucocyte count at 24 hrs p.i was found to be 20 $x10^3$ cells/mm^3 and 18 $x10^3$ cells/mm^3 at 96 hrs p.i. The total erythrocyte count at 24 hrs p.i was found to be 23 $x10^6$ cells/mm^3 and 20 $x10^6$ cells/mm^3 at 96 hrs p.i. The results are given in (Table 3)

Serum Lysozyme activity

The results of the serum lysozyme activity suggest that there was significant reduction at 24 hrs p.i . The serum lysozyme activity was found to be 510 units/ml at 24 hrs p.i and 420 units/ml at 96 hrs p.i when compared to the normal which was found to be 650 units/ml. The results are presented in (Table 3)

Reactive Oxygen species production assay (ROS)

The ROS production suggests that there was significant increase in the ROS level when compared to the normal catfish. The ROS level was found to be 1.8 at OD 650 nm at 24 hrs p.i and 2.0 at OD 650 nm at 96 hrs p.i . The results are given in (Table 3)

Antibody titration by bacterial agglutination assay

No agglutination was observed in sera of control fish and 0 day sera of experimental fish. Immunised groups developed high levels of antibodies. Peak antibody titers appeared on the 14th day after the fish were challenged with heat killed bacteria.

Evaluation of various antimicrobial agents against *A. caviae*

Antibiotic Sensitivity Test

The sensitivity of *A. caviae* against the sixteen antibiotics was tested (Table 4). Among the sixteen antibiotics tested, *A. caviae* showed sensitivity to 9 antibiotics namely gentamicin, oxytetracycline, chloramphenicol, ciprofloxacin, tetracycline, oxacillin,

trimethoprim, norfloxacin and sulfamethoxazole. The highest inhibition zone was found in the case of ciprofloxacin, oxacillin, cholramphenicol, tetracycline and norfloxacin. The results showed that the isolate was sensitive to ciprofloxacin, oxacillin, trimethorium, norfloxacin, tetracycline, cholramphenicol, and resistant to penicillin, bacitracin and ampicillin.

The minimum inhibitory concentrations of Norfloxacin, and Tetracycline against *A. caviae* is summarised in (Table 5). The MIC of Norfloxacin, and Tetracycline against *A. caviae* was determined by the tube dilution technique. Tetracycline was found to be the most effective antibiotic in controlling the *A. caviae* at 20 ppm level. Significant growth of *A. caviae* was observed in the medium treated with tetracycline up to 10 ppm, poor growth was observed at 15 ppm and the growth was completely inhibited at 20 ppm level of tetracycline. In the case of norflaxicin, appreciable growth of *A. caviae* was observed in the medium treated with norflaxin up to 20 ppm level and the growth was completely inhibited by norfloxacin from 50 ppm level onwards (Table 5).

Prevention of bacterial infection by vaccination

Heat Killed Vaccine

After inactivating the bacterial culture in water bath, they were checked for their sterility by plating on to nutrient agar. The plate was incubated at 28-30° C for 24 hrs. No bacterial growth was observed in the nutrient agar plate after heat inactivation.

Catfish (*C. batrachus*) immunized with heat killed vaccine was assessed for protection against *A. caviae* by intramuscular challenge. Mortality following exposure to the bacteria was lower in fish vaccinated with Heat Killed vaccine compared to the fish without vaccination. The cumulative percentage of mortality was also calculated and the results are presented in (Table 6). The heat killed vaccine protected the fish from the bacterium with 93.4% of relative percentage survival (RPS) when compared to that of the non-vaccinated fish with 92% cumulative percent mortality (CPM).

Formalin Killed vaccine

After inactivating the bacterial culture by 1% formalin, they were checked for their sterility by plating on to nutrient agar. The plate was incubated at 28-30° C for 24 hrs. No bacterial growth was observed in the nutrient agar plate.

Catfish (*C. batrachus*) immunized with formalin killed vaccine was assessed for protection against *A. caviaei* by intramuscular challenge. Mortalities following exposure to the bacteria were lower in fish vaccinated with formalin killed vaccine when compared to non-vaccinated fish. The cumulative percentage of mortality was also calculated and the

results are presented in (Table 7). The Formalin killed vaccine protected the fish from the bacterium with 89.1 % of relative percentage survival (RPS) when compared to that of the non-vaccinated fish with 92% cumulative percent mortality (CPM).

Evaluation of various plant extracts against *A. caviae*

Twelve Indian medicinal plants with known medicinal properties particularly antimicrobial activity belonging to different families were selected and tested for their efficacy to inhibit the growth of *A. caviae* to control the bacterial infection in catfish. The results revealed that among the twelve plants selected the aqueous extract of *Citrus aurantifolia* was found to have antibacterial activity.

Well diffusion method

Well diffusion method was followed to evaluate the antibacterial activity against *A. caviae*. The results revealed that the aqueous extract of *C. aurantifolia* had a zone of inhibition of 28 mm. The results are given in (Table 8)

Comparative study of selected plant extract with commercial antibiotic disc

The extract loaded disc was compared with the commercial antibiotics. The results revealed that the extract loaded disc had a zone of inhibition of 30 mm when compared to the commercial antibiotic Ciprofloxacin which had a zone of inhibition of 40 mm. Minimum inhibitory concentration of the plant extract was found to be 20 ppm. These results are presented in (Tables 9 and 10)

Field trials

The field trials carried out using the aqueous extract of C. *aurantifolia* was found to be effective in controlling the ulcerative disease in catfish. The farmers have started using it for treating the disease in catfish. The experiments were conducted in cement tanks in which catfish are being reared. The results revealed that there was about 90% survival when treated with aqueous extract of *C. aurantifolia.*

The studies carried out revealed that the antibacterial plant compound had an impact on the bacteria which is responsible for causing the ulcerative disease. It could inhibit *A. caviae* which is responsible for the ulcerative disease. The fish was healthy and no signs of the ulcerative disease were noted. In general, the plant compound was useful in treating the disease and 90% survival was noted.

Antibacterial activity of actinomycetes isolates against bacterial pathogens

Actinomycetes isolates obtained from different sources were screened for antibacterial activity against *A. caviae*. Eighty-nine isolates were screened for antibacterial activity by cross-streak and well diffusion methods, and the results are shown in Table 11. Among these

isolates obtained by cross-streak method, 19 isolates showed antagonistic activity against *Aeromonas caviae*. The zone of inhibition caused by the actinobacterial isolates for *A. caviae* ranged from 0 to 36 mm (ciprofloxacin - 31 mm) (Fig. 2, Table 12).

Vaccination of fish is the alternative to the antimicrobials for controlling infectious diseases. It is becoming an increasingly important part of aquaculture, aimed at preventing and controlling infectious diseases in fish farms and at reducing the use of antimicrobial drugs. In the recent years, fish vaccination has greatly benefited from the better understanding of the immune system of fish, and the identification of the virulence factors of the main fish pathogens (Gudding *et al*., 1999). Live, attenuated vaccines should potentially have many advantages in preventing fish infection by stimulating the cellular immunity, and by an effective dissemination of the antigen in the population (Benmansour and de Kinkelin, 1997). However, the use of live attenuated vaccines in aquaculture is highly controversial, and most authorities consider live vaccines to be undesirable because of the risk of uncontrolled spread in the aquatic environment. Commercial vaccines are available against different bacteria (*Yersinia ruckeri, Aeromonas salmonicida, Vibrio anguillarum, V. ordalii, V. salmonicida, Edwarsiella ictaluri*). These consist of formalin-killed broth cultured (bacterines) vaccines , and they frequently include an adjuvant and therefore have to be administered by injection. But some vaccines can also be delivered by other routes, such as immersion, spray, and oral. Administration strategy depends on the size and species of fish, the bacterial species, and the type of vaccine available.

Catfish (*C. batrachus*) immunized with heat killed vaccine was assessed for protection against *A. caviae* by intramuscular challenge. Mortalities following exposure to the bacteria were lower in fish vaccinated with Heat Killed vaccine compared to those of the control group injected with bacteria. The heat killed vaccine protected the fish from the bacterium with 93.4 % of relative percentage survival (RPS) when compared to that of the control fish with 92% cumulative percent mortality (CPM). Mortalities following exposure to the bacteria were lower in fish vaccinated with formalin killed vaccine when compared to those of the control group injected with bacteria. The Formalin killed vaccine protected the fish from the bacterium with 89.1 % of relative percentage survival (RPS) when compared to that of the control fish with 92% cumulative percent mortality (CPM).

Kozinska *et al.* (2003) vaccinated juveniles of the common carp (*Cyprinus carpio*) Linn with oil emulsified experimental vaccines of *Aeromonas bestiarum* containing formalin killed whole cells, and formalin killed whole culture or crude lipopolysaccharide. Significant

protection in vaccinated fish for 30 days was noticed. Our results also revealed that there was significant protection in the fish immunized with formalin killed vaccine.

Vaccination of fish for the prevention of specific bacterial diseases affecting commercially reared fish species has a significant impact. The first vaccines to be successfully commercialized were those against *V. anguillurutn, V. ordalii***,** and *Yersiniu ruckeri* in the late 1970s. Vaccines against *V. sulmonicidu***,** a pathogen of salmonids, *A. salmonicida,* a pathogen of salmonids and carp, and *Edwardsiella ictaluri***,** a pathogen of channel catfish have also been commercialized and are in widespread use.

Virulent isolates of *Renibacterium salmoninarum*, killed either by heat or formaldehyde, have demonstrated a wide range of prophylactic efficacies in rainbow trout (Pascho *et al.*, 1997; McCarth *et al.,* 1984) a species that is relatively refractory to bacterial kidney disease (Starliper *et al*., 1997; Kawamura *et al.,* 1977; Sakai *et al.,* 1991; Sanders *et al.*, 1978). Several vaccination methods have been adopted for preparation of vaccine to vaccinate fish including the use of chemicals like chloroform, formalin and phenol, in addition to heat, sonication and lysis with sodium hydroxide. The heat killed cells of *E. tarda*, *V. anguillarum* and *V. ordalii* fared better than their formalin inactivated cells (Austin, 1984). Our results also revealed that heat killed vaccine protected the fish from the bacterium with 93.4 % of relative percentage survival (RPS) better than the formalin killed vaccine which protected the fish from the bacterium with 89.1 % of relative percentage survival (RPS).

The large-scale settings of aquatic animal husbandry have resulted in an increased antibiotic resistance in bacteria potentially pathogenic to fish and related environment (Smith *et al*., 1994; Alderman and Hastings, 1998; Petersen *et al*., 2002; Alcaide *et al*., 2005; Cabello, 2006). The continuous use of antimicrobial agents in aquaculture has resulted in more resistant bacterial strains in the aquatic environment. Continuous use of synthetic antibiotics reveals the threats to consumers and non target organism in the environment (Muniruzzaman and Chowdhury, 2004; Abutbul *et al*., 2005). Treatment of bacterial diseases with various herbs has been safely employed widely in organic agriculture, veterinary and human medicine (Direkbusarakom, 2004). Since ancient times, medicinal plants have been used for the treatment of common infectious diseases (Rios and Recio, 2005) and treatments with plants having antibacterial activity are a potentially beneficial alternative in aquaculture (Abutbul *et al*., 2005). Medicinal plants as the alternative agents are effective to treat the infectious diseases and mitigate many of the side effects that are associated with synthetic antimicrobials (Punitha *et al*., 2008).

Previous work on the antibacterial activities of plants provides evidence of the importance of some plants against *A. caviae*. In the present work, among twelve plants tested, *C. aurantifolia* was found to exhibit potent antibacterial activity against *A. caviae*. The aqueous extracts of the leaf of *C. aurantifolia* were found to be the most effective against *A. caviae* and was used to control the infection in *C. batrachus* . The same plant has been reported for its antimicrobial activity against some Gram positive, Gram Negative organisms and fungi (Ibukun Aibinu *et al*., 2007). Oboh *et al*. (1992) and Oboh and Abulu (1997) have also reported the antibacterial effect of lime leaves on some bacterial isolates. The result of this study agrees with the previous reports of the antimicrobial activity of lime leaves by previous workers.

Garlic has been known to possess dietary and medicinal properties (Ross *et al*, 2001). Several studies have proved that garlic has antimicrobial effects (Rees *et al,* 1993; Reuter *et al*, 1996; Lawson, 1998; Martin and Ernst, 2003) and inhibits the growth of both Gram-negative and Gam-positive bacteria, molds and yeasts (Pai and Platt, 1992; Ross *et al.*, 2001). However, the present study revealed that garlic had no effect in controlling *A. caviae* infection in catfish.

Heavy antibiotic use in aquaculture needs to be reduced and replaced with alternative processes for treating fish diseases to avoid the emergence of antibiotic resistance in pathogenic and environmental bacteria (Sørum and L'Abée-Lund, 2002; Cabello,2006). Natural substances like thyme oil, clove oil and pine oil were used as alternative bio-herbicides and bio-pesticides in ecological agriculture (Verschwele, 2005; Perez and Lewis, 2006). Similarly, the herbal plants may be used as potential and promising source of pharmaceutical agents against fish pathogens in the aquaculture. Our field trials with the aqueous extract of the leaf of *C. aurantifolia* and encouraging results obtained indicate the possible of controlling the ulcerative disease syndrome caused by *A. caviae* in field.

In recent years, Gas Chromatography–Mass Spectrometry (GC–MS) has been applied to identify the structures of different phytoconstituents in plant extracts and biological samples with great success (Prasain *et al.,* 2004). GC–MS is a reliable technique to identify the constituents of volatile matter, long-chain branched hydrocarbons, alcohols acids and esters (Anjali *et al.,* 2009). This identification was done by comparison of their mass spectra on both columns with phytochemical and ethnobotanical databases libraries or with mass spectra from the literature (Adams, 2001; Jennings and Shibamoto, 1980) and home-made library. Eight phyto components were identified in *C. aurantifolia* in aqueous leaf extract. They were (I) Propanoic acid, 2,2-dimethyl-, 2-ethylhexyl ester, (II) Undecanoic acid, 2-

methyl, (III) Tridecanoic acid, methyl ester, (IV) Didodecyl phthalate, (V) á-D-Glucopyranose, (VI) Nonanoic acid, (VII) 1,2-Benzenedicarboxylic acid butyl octyl ester and (VIII) Hexanedioic acid, bis(2-ethylhexyl) ester. Of these eight compounds, propanoic acid 2,2-dimethyl-, 2-ethylhexyl ester, Didodecyl phthalate and 1,2-Benzenedicarboxylic acid butyl octyl ester are reported to have antimicrobial/ antifouling activities. It is well known that essential oils from *Citrus* spp. have pronounced antimicrobial effect against both bacteria and fungi (Lanciotti *et al*., 2004; Caccioni *et al*., 1998; Dabbah *et al*., 1970).

Most studies have focused on essential oils from subtropical citrus. The essential oils from two cultivars of tropical citrus, including *Citrus hystrix* DC., and *Citrus aurantifolia* exhibited antimicrobial activity against *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella typhi* (Chaisawadi *et al*., 2003).

Executive Summary

Clarias batrachus (Linn) is a species of catfish of the family claridae. A survey was carried out to study the prevalence of bacterial ulcerative disease in *C. batrachus*, reared in a farm located at Walajapet, Vellore Dist., Tamil Nadu, India. To reproduce the infection in healthy catfish, organs such as liver, spleen and tissue were used to prepare the inoculum in NTE buffer. The inoculum was injected into healthy fingerlings through intramuscular injection and immersion challenge. The tissue supernatant from infected catfish was screened for isolation and identification of the causative organism responsible for ulcerative syndrome in catfish. Based on biological, biochemical, morphological and physiological characters the isolate isolated from infected catfish was tentatively identified as *Aeromonas caviae*-like bacterium. It was further confirmed by the analysis of 16S rRNA gene of *A. caviae* using PCR. The heat killed vaccine protected the fish from the bacterium with 93.4% of relative percentage survival (RPS) when compared to that of the non-vaccinated fish with 92% cumulative percent mortality (CPM). The Formalin killed vaccine protected the fish from the bacterium with 89.1 % of relative percentage survival (RPS) when compared to that of the non-vaccinated fish with 92% cumulative percent mortality (CPM). Twelve Indian medicinal plants with known medicinal properties particularly antimicrobial activity belonging to different families were selected and tested for their efficacy to inhibit the growth of *A. caviae* to control the bacterial infection in catfish. The results revealed that among the twelve plants selected the aqueous extract of *Citrus aurantifolia* was found to have antibacterial activity. Twelve Indian medicinal plants with known medicinal properties particularly antimicrobial activity belonging to different families were selected and tested for their efficacy to inhibit the growth of *A. caviae* to control the bacterial infection in catfish. The results revealed that among the twelve plants selected the aqueous extract of *Citrus aurantifolia* was found to have antibacterial activity. Actinomycetes isolates obtained from different sources were screened for antibacterial activity against *A. caviae*. Eighty-nine isolates were screened for antibacterial activity by cross-streak and well diffusion methods. Among these isolates obtained by cross-streak method, 19 isolates showed antagonistic activity against *Aeromonas caviae*. The zone of inhibition caused by the actinobacterial isolates for *A. caviae* ranged from 0 to 36 mm (ciprofloxacin - 31 mm).

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Studies on ulcerative disease caused by *Aeromonas caviae*-like bacterium in Indian catfish, *Clarias batrachus* (Linn)

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ABSTRACT

Clarias batrachus (Linn) is a species of catfish of the family claridae. A survey was carried out for a period of one year from May 2008 to June 2009 to study the prevalence of bacterial ulcerative disease in *C. batrachus*, reared in a farm located at Walajapet, Vellore Dist., Tamil Nadu, India. To reproduce the infection in healthy catfish, organs such as liver, spleen and tissue were used to prepare the inoculum in NTE buffer. The inoculum was injected into healthy fingerlings through intramuscular injection and immersion challenge. The tissue supernatant from infected catfish was screened for isolation and identification of the causative organism responsible for ulcerative syndrome in catfish. Based on biological, biochemical, morphological and physiological characters the isolate isolated from infected catfish was tentatively identified as *Aeromonas caviae*-like bacterium. It was further confirmed by the analysis of 16S rRNA gene of *A. caviae* using PCR.

1. Introduction

Indian walking catfish, *Clarias batrachus* (Linn), is an economically important fish species in south and south-east Asian countries, including India, because it is suitable for monoculture or polyculture with other catfish species. It is euryhaline across its native range, inhabiting fresh and brackish water as well as muddy marshes (Sen, 1985). Infectious diseases caused by viruses and bacteria are responsible for substantial financial loss to catfish farmers worldwide. Channel catfish virus (CCV) is an important fish virus causing economic losses in channel catfish *Ictalurus punctatus*. CCV is a cytopathic herpes virus that can cause an acute, hemorrhagic and lethal disease in the channel catfish (Fijan, 1968). This virus also causes an acute hemorrhagic disease in channel catfish fry and fingerlings (Buck, 1990; Plumb, 1977). Crumlish et al. (2003) challenged hybrid catfish (*Clarias gariepinus*×*C. batrachus*) with *Aeromonas hydrophila* under stressful conditions and reported high mortality of fish in high temperature stress. Chattopadhyay et al. (1992) isolated fifteen strains of *A. hydrophila* from three species of fish including *C. batrachus* with typical ulcers on the surface of the body. In the present study, high mortality of young *C. batrachus* with clinical sign of ulcers on the body surface was observed in a hatchery and grow out ponds. An attempt was made to isolate and identify the causative organism responsible for high mortality of catfish fry. Experimental pathogenicity was conducted to reproduce the infection in normal fish using the bacterial isolate isolated from naturally infected fish. Chauhan (2012) carried out a study on certain fungal diseases in culturable and non-culturable species of fishes of Upper Lake, Bhopal. *Achlya* and *Saprolegnia* were found to be the most virulent genera. Out of 2066 fishes examined, the most affected species was *C. batrachus* (24.6%). Sarkar and Rashid (2012) investigated pathogenicity of the bacterial isolate *Aeromonas hydrophilla* to catfishes, carps and perch. They reported a mortality of 60–100% in all the six species of catfishes studied at a water temperature of 30 °C.

2. Materials and methods

2.1. Collection and maintenance of naturally infected fish

The young ones (fingerlings) of Indian catfish (*C. batrachus*) weighing approximately 3–5 g with clinical signs of ulcerative lesions having a depth of approximately 2 mm on the body surface and base of the fins were collected from a farm located at Walajapet, Tamil Nadu and transported within half an hour at 28 °C in live condition to the laboratory in a container with continuous aeration. In the laboratory, the infected animals were maintained in 100-l aquarium tanks containing ground spring water at room temperature (27–30 °C). The animals were fed with commercial fish feed.

2.2. Physico-chemical parameters

The physico-chemical parameters of the pond water such as pH, temperature and dissolved oxygen (DO) were measured following standard procedures. The dissolved oxygen was estimated by the Winkler method (Strickland and Parson, 1968).

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2.3. Collection and maintenance of experimental animals

Healthy catfish, *C. batrachus* were collected for experimental purpose from different farms with no record of ulcerative syndrome. The fish were transported in live condition and maintained in 100-l fiberglass tanks with continuous aeration at room temperature (27–30 °C) in fresh water. The animals were fed twice a day with commercial fish feed.

2.4. Reproduction of infection

100 mg of ulcerative skin from naturally infected fish was cut and placed in a homogenizer along with sterile PBS buffer, and homogenized. The homogenized sample was centrifuged at 1000 ×*g* for 10 min at 4 °C. Healthy fish were injected intramuscularly with 25 μl of suspension and maintained for a period of 10 days. The control fish were injected intramuscularly with 25 μl of sterile PBS. Experimental fish were examined twice a day for clinical signs of disease and mortality.

2.5. Viral pathogens

The infected fish were screened for viral pathogens, by inoculating the homogenized organ samples prepared from infected fish into the catfish fin cell line (Babu et al., 2011) following the method of Inaba et al. (2007) with modifications. The cells of catfish fin cell line were grown in Leibovitz's L-15 medium, supplemented with 15% fetal bovine serum, at 28 °C in a tissue culture flask. The organs (liver, kidney, gills, spleen, ulcerative skin and heart) were homogenized separately using L-15 medium. The homogenate was centrifuged at 5000 ×*g* at 4 °C for 10 min. The supernatant was collected, freeze thawed three times, and filtered through 0.45 μm filter. For virus isolation, the cells of catfish fin were seeded in the cell culture flask and incubated for 12 to 24 h at 28 °C to give a confluence of 60–70%. After removal of the medium, 100 μl of tissue filtrate was inoculated into the cell culture flask, and allowed to adsorb for 1 h. 5 ml of maintenance medium containing 5% FBS was then added. The cells were incubated at 28 °C and examined daily for the appearance of cytopathic effect (CPE) for 15 days. Five blind passages were carried out as mentioned above to confirm the infection.

2.6. Bacteriological analysis

The organs (liver, kidney, gills, spleen, ulcerative skin and heart) from naturally infected fish were dissected out and placed in a homogenizer along with sterile PBS buffer. The samples were homogenized separately and ten-fold serial dilutions were made to avoid overgrowth of bacteria as described by Bullock (1971). The diluted samples were inoculated onto nutrient agar, *Aeromonas* agar, Pseudomonas agar, trypticase soy agar, and thiosulfate citrate bile salt (TCBS agar) by the spread plate technique (Collins and Lyne, 1976). After inoculation, plates were incubated at 28 °C for 24–48 h. Bacterial colonies were examined carefully and counted. Morphologically similar and dominant bacterial colonies were selected and streaked onto nutrient agar plates in order to obtain pure cultures. These pure cultures were maintained on nutrient agar for further study. Bacterial isolates were identified according to the taxonomic schemes of Buchanan and Gibbons (1984) and West and Colwell (1984). The gene encoding the 16S ribosomal RNA (16S rRNA) of *A. caviae* was amplified by polymerase chain reaction (PCR) with specific primer set which was designed based on the nucleotide sequence of *A. caviae* (Gen Bank Accession No. *AF198380*). The sequence of the primers is: **5′ TCG TTG GGT TGG GAT GTG 3′** (forward) and **5′ TGT TAC CGC CGT GAA AGG 3′** (reverse). The size of the DNA amplicon was 157 bp. Reference strains such as *A. hydrophila* (1739), *A. caviae* (6832) and *A. salmonicida* (1945) were obtained from MTCC, Chandigarh, India for comparison.

The use of 16S rRNA gene sequences to study bacterial phylogeny 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 (1500 bp) is large enough for informatics purposes (Patel, 2001).

2.7. Experimental pathogenicity of bacterial isolate in healthy catfish

Pathogenicity of the new bacterial isolate isolated from naturally infected fish was studied in healthy fish. All the three routes of infection namely immersion, intramuscular injection and oral route were followed to determine the pathogenicity of bacterial isolate in healthy catfish based on the protocols described by Ducklow et al. (1980) and Egidius (1987).

2.8. Preparation of bacterial inoculum

The bacterial isolate isolated from naturally infected fish was grown on nutrient agar for use in pathogenicity experiments. The pathogenicity of the bacterial isolate was tested by bath exposure, intramuscular injection and oral administration. The bacterial count was determined by standard dilution and plating methods as described by Ducklow et al. (1980).

2.9. Experimental infection by bath exposure

Healthy fish (10 fish per tank) were reared in aquarium tanks of 50 l capacity containing sterilized freshwater with continuous aeration. Air stones and air tubes were sterilized by immersing them in 2.6% sodium hypochlorite and washing them thoroughly with sterilized tap water before use. The tanks were covered to prevent contamination. Aseptic techniques were used throughout the experiment. Fish were fed with commercial fish feed. For the experimentally induced infection, the fish were exposed to different concentrations of bacterial cells (10^3,10^4, 10^5, 10^6 and 10^7 CFU ml^{-1}). The control consisted of fish exposed to freshwater only.

2.10. Infection via intramuscular injection

Fish (10 per dosage and tank) were maintained in fiberglass tanks (100 l) containing freshwater at 28 °C. The bacterial isolate was inoculated into healthy fish by intramuscular injection, near the dorsal fin at doses of 10^3,10^4, 10^5, 10^6 or 10^7 CFU $animal^{-1}$. Control fish received only sterile PBS buffer.

2.11. Oral infection

Fish were individually isolated in the aquarium tanks and starved for 24 h. Each of them was then fed with a piece of fish meat which was injected with 1 ml of bacterial suspension (10^{12} CFU). The fish were fed three times, with an interval of 8 h. After the last feeding with infected meat, the animals were fed with non-infected meat for a week.

In the control group, fish were fed only with non-infected meat. In all experiments, animals were examined twice a day for clinical signs of disease and mortality.

2.12. Confirmation of pathogenicity

The specific action of the bacterial isolate isolated from naturally infected fish as a pathogen was confirmed by re-isolating the bacterium

from the spleen, liver and ulcerative tissues of moribund fish to satisfy Koch's postulates. The samples were inoculated on nutrient agar and TSA agar plates by spread plate technique for isolation of bacterial pathogen. The isolated bacteria were identified using the procedure described above.

3. Results

The physicochemical characteristics of pond water were determined. Temperature, pH, and dissolved oxygen ranged from 26 to 31 °C, 7.2 to 7.4 and 4.6 to 6.0 mg/l, respectively, during the collection of infected fish. A high temperature of 31 °C was recorded during summer. Mortality of fish due to infection reached 95–97% within 3–5 days after the appearance of ulcer on the surface of infected fish. The clinical signs of infected fish include ulcerative lesions on the body surface, bases of the fins and around the anus. The ulcerative lesions are seen most often with reddish coloration on the surface.

The inoculum prepared from ulcerative tissue of infected fish produced clinical sign of ulcerative lesions in healthy fish under experimental conditions. The inoculum used for experimental infection was screened for viral pathogens using the catfish fin cell line. No cytopathic effect (CPE) was observed in the cells even after five blind passages (Fig. 1).

Bacteriological analysis was carried out on different organs obtained from infected catfish at each time of sampling. Two dominant bacterial isolates from each of the bacterial media were tested for their pathogenicity in the healthy catfish. One bacterial isolate from nutrient agar and Aeromonas agar caused mortality and reproduced the clinical sign of ulcerative lesions in healthy catfish. The other isolates from these media did not cause significant mortality and clinical signs. The bacterial isolates responsible for mortality and reproduction of clinical signs of ulcerative lesions in healthy catfish were selected and identified based on the colony morphology, biochemical and physiological characteristics.

Colonies which developed on nutrient agar were circular, 2–4 mm in diameter, entire, smooth, convex and cream colored. The bacterial cells were Gram-negative rods and motile. The selected characteristics such as oxidase reaction, fermentation of glucose, and production of acid with gas from glucose determine this bacterium to the genus *Aeromonas*. Based on the biological, biochemical, morphological and physiological characters, the isolate isolated from infected catfish was tentatively identified as *Aeromonas caviae*-like bacterium. It was

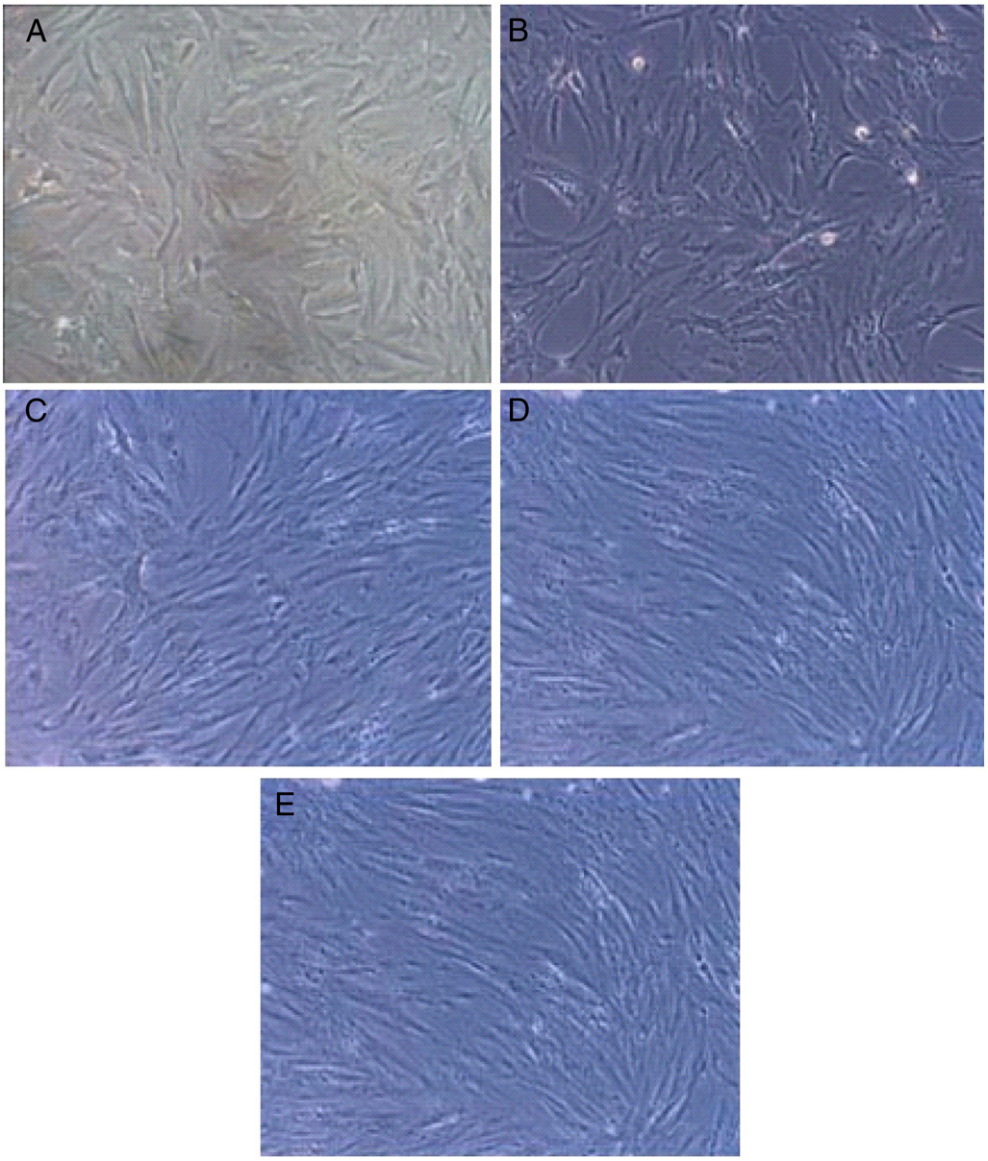

Fig. 1. ICF cells inoculated with samples prepared from different organs of naturally infected catfish with ulcerative lesions. No cytopathic effect was observed even after five blind passages. A — heart; B — liver; C — gills; D — spleen; E — kidney.

further confirmed by the analysis of 16S rRNA gene of *A. caviae* using PCR. The gene encoding the 16S ribosomal RNA (16S rRNA) of *A. caviae* was amplified by polymerase chain reaction (PCR) with specific primer sets and the results are shown in Fig. 2. The PCR was also carried out using the same set of primers with other reference bacterial species such as *A. hydrophila, A. salmonicida* and *V. angulliarum*, used in this study. No amplification was observed in them.

The sequencing of *A. caviae* revealed a homology of 95.3%. Therefore it is an *Aeromonas* sp. with characteristics of *Aeromonas caviae*.

The susceptibility of *C. batrachus* to *A. caviae*-like bacterium was tested by intramuscular injection, immersion challenge and oral route. The highest concentration, 55×10^5 and 55×10^6 viable cells of *A. caviae*-like bacterium per animal caused 100% mortality within 120 and 96 h of post inoculation, respectively, when the animals were injected intramuscularly, whereas the lower concentrations of 55×10^3 and 55×10^4 viable cells of *A. caviae*-like bacterium per animal caused 43.3 and 66.6% mortality, respectively after 120 h of post injection. The LD_{50} value of the *A. caviae*-like bacterium for intramuscular route was determined at different time intervals and it was found to be 2.21×10^6 and 1.12×10^7 CFU per animal after 72 and 96 h of post injection, respectively. No mortality was observed through oral route.

The highest concentration of *A. caviae*-like bacterium (39×10^6 CFU ml^{-1} of rearing medium) caused 20, 46.6, 53.3, 56.6 and 63.33% mortality in immersion route after 24, 48, 72, 96 and 120 h of exposure, respectively. But this highest concentration failed to produce 100% mortality even after 10 days of post infection. The LC_{50} value of *A. caviae*-like bacterium for immersion route was determined at different time intervals. The LC_{50} value was found to be 3.82×10^5, 3.96×0^5 and 4.82×10^6 CFU ml^{-1} of rearing medium, after 24, 48 and 72 h of post infection respectively. The clinical sign of ulcerative lesions was observed in experimentally infected catfish. The pathogenicity of *A. caviae*-like bacterium was confirmed by satisfying Koch's postulates. *A. caviae*-like bacterium was re-isolated from moribund and dead animals subjected to experimental infection.

4. Discussion

C. batrachus is considered as a potential aquaculture species in the Indian subcontinent. The population of catfish has been affected by many factors including diseases caused by viral and bacterial pathogens, even though it is a hardy species. High mortality of young *C. batrachus* was observed in nursery and grow-out ponds located in Tamil Nadu and Andhra Pradesh. Among the bacterial pathogens, motile *Aeromonas* caused high mortality in catfish (Lewis and Plumb, 1979; Meyer, 1975) and induced serious epidemics of ulcerative disease in fish in Southeast Asia and other regions of the world (Angka et al., 1988; Areerat, 1987; Llobrera and Gacutan, 1987; Roberts et al., 1992; Ruangpan et al., 1986). For the past two years, an ulcerative disease has been observed in nursery and grow-out ponds located in Tamil Nadu and Andhra Pradesh. The fish farmers in Walajapet, a town near our Institution have lost crops of Indian catfish regularly due to ulcerative disease of unknown cause.

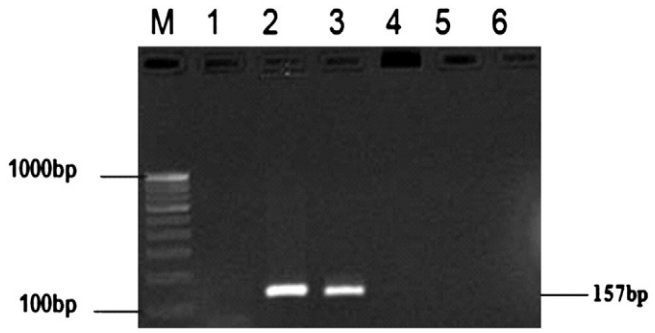

Fig. 2. Agarose gel showing the amplification of 16S rRNA gene of *A. caviae*. Lane M — marker; Lane 1 — negative control, Lane 2 — *A. caviae*-like bacterium (isolated from infected catfish), Lane 3 — *A. caviae* MTCC (6832), Lane 4 — *A. hydrophila* MTCC (1739) Lane 5 — *A. salmonacida* MTCC 1945, Lane 6 — *V. anguillarum*.

The clinical signs of infected catfish include ulcerative lesions on the body surface, bases of the fins and around the anus. Our observation on clinical signs of infected catfish agrees with the report of Lowry and Smith (2007) who reported similar clinical signs associated with other bacterial infections.

The infected fish samples collected were screened for viral, bacterial, fungal and protozoan pathogens using standard protocols. The samples were screened for viral pathogens by inoculating the homogenized organ samples prepared from infected fish into the catfish fin cell line following the method of Inaba et al. (2007). The homogenized samples from infected catfish inoculated in fin cell line did not develop any CPE even after five blind passages. This indicated that no viral pathogen was involved in ulcerative disease syndrome of catfish.

Bacteriological analysis was carried out on different organs such as liver, spleen and swab taken from ulcerative lesions from infected catfish using different bacterial culture media such as nutrient agar, Trypticase Soy Agar (TSA), TCBS agar, MacConkey agar, *Pseudomonas* agar, *Aeromonas* agar and brain heart infusion agar. From each bacterial culture medium, two morphologically different and dominant bacterial colonies were isolated and tested for re-infection in healthy catfish to confirm that the bacterial isolate isolated from infected catfish was a pathogen to satisfy Koch's postulates. One bacterial isolate from nutrient agar, *Aeromonas* agar, TSA and MacConkey agar caused mortality and reproduced the clinical sign of ulcerative lesions in healthy catfish, but the other isolates from these media did not cause significant mortality and clinical signs. Several workers have reported that among the bacterial pathogens motile *Aeromonas* has caused high mortality in catfish (Lewis and Plumb, 1979; Meyer, 1975) and induced serious epidemics of ulcerative disease in fish in Southeast Asia and other regions of the world (Angka et al., 1988; Areerat, 1987; Llobrera and Gacutan, 1987; Roberts et al., 1992; Ruangpan et al., 1986). Motile *Aeromonas* in Java was found to be the dominant pathogen in the outbreaks of ulcerative disease in walking catfish. In the present study also *Aeromonas* was found to be the dominant pathogen in the outbreaks of ulcerative disease in walking catfish.

The bacterial isolate isolated from infected catfish *C. batrachus* was identified as *A. caviae*-like bacterium according to colony morphology, biochemical and physiological characteristics. There are some reports that high mortality of fish is associated with the presence of *A. caviae* (Munro et al., 1993; Ringo and Vadstein, 1998). Munro et al. (1993) noted low survival of fish larvae associated with high proportion of *A. caviae*. High colonization of *A. caviae* in the gut of turbot fish larvae caused 100% mortality in fish larvae (Ringo and Vadstein, 1998). There are many reports on the isolation of *A. caviae* from other fishes like carp, gold fish, catfish, mullet and tilapia (Sugita et al., 1994). In the present study, the bacterial isolate isolated from infected catfish was identified as *A. caviae*-like bacterium and was confirmed as a pathogen to satisfy Koch's postulates in normal catfish. PCR was performed to identify the bacterial isolate at molecular level using primers specific to 16S rRNA gene of *A. caviae*.

Species identification definition using 16S rRNA gene sequence data was carried out. Unfortunately, no universal definition for species identification via 16S rRNA gene sequencing exists, and authors vary widely in their use of acceptable criteria for establishing a “species” match. In none of the studies does the definition of a species “match” ever exceed 99% similarity (Michael Janda and Sharon, 2007).

The susceptibility of catfish *C. batrachus* to *A. caviae*-like bacterium was tested by immersion challenge, oral route and intramuscular injection in fingerlings. From the results of the pathogenicity experiments, it may be concluded that the main portal of entry of the pathogen is by means of penetration of tissue at the site of lesions or

wounds. The mortality data in the pathogenicity experiments showed that the pathogenicity of the present isolate depends on the dosage and period of exposure.

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