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Opportunistic pathogen *Klebsiella pneumoniae* isolated from Maldive's clown fish *Amphiprion nigripes* with hemorrhages at Agatti Island, Lakshadweep archipelago

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Abstract
Objective: To investigate the cause of mortality of Maldive's clownfish, *Amphiprion nigripes* during the culture period in ornamental fish hatchery, Agatti Island, Lakshadweep archipelago using molecular techniques.
Methods: During cultured period august 2010 the fishes were died with skin hemorrhages. Bacteria were isolated from the infected parts of liver, kidney and tissue of the fish using Nutrient agar and Zobell Marine Agar and the causative bacteria has been identified using 16S rDNA sequencing and confirmed with gene bank. The physico-chemical parameters were analyzed at the time of acclimatization and experimental period.
Results: The predominant bacterial morphology was identified as gram-negative rod-shaped, motile bacteria. Based on biochemical tests and sequence of 16S rDNA, the causative bacteria were identified as *K. pneumoniae* (Accession no. HQ589912). Bacterial cells were isolated from liver and kidney of all artificially infected moribund fish and confirmed as *K. pneumoniae* by morphological and biochemical characteristics. Fishes artificially infected by same causative agent, mortality was observed on 48th hour and no fish died within 24 hours of post exposure.
Conclusion: *K. pneumoniae* could be considered as an opportunistic pathogen and a symptom including hemorrhages, ulcers, and redness of the skin and it's actively causing disease when favorable conditions occurred like increasing of un-ionized ammonia in culture tank.

Keywords: *K. pneumoniae*, *A. nigripes*, abscess, 16s rDNA, infection, Lakshadweep archipelago

1. Introduction
Maldive's clown fish *A. nigripes* a small brightly coloured tropical fish that lives in close association with sea anemones. Many studies on ornamental fishes, particularly, larval rearing and nutrition have been performed [1], due to the increasing demand and their aesthetic appearance and eye-catching display in aquarium.
In commercial fish farms and hatchery, unfavorable environmental conditions (availability un-ionized ammonia, osmotic strength, oxygen levels, water quality) or poor management practices (inadequate nutrition, overcrowding, overfeeding) may stress the fish, causing a growth rate reduction and immunosuppression, making them more susceptible to disease outbreaks [2]. Opportunistic pathogen of *Klebsiella pneumoniae* is Gram-negative, aerobic; non-motile that is a common cause of infections in humans and animals. *Klebsiella* sp have been isolated from marine mammals from nonspecific abscess including poly-arthritis, meningoencephalitis and peritonitis [3]. In addition to this *Pseudomonas* sp., *Proteus vulgaris, Serratia* sp., *Staphylococcus* sp, *Salmonella* sp., are some of the normal opportunistic pathogens affecting both fresh and marine aquaculture causing tissue infections, hemorrhages in the internal organs, virulence and immunity reduction in host.
Lakshadweep archipelago is a part of the Chagos-Maldives-Lakshadweep archipelago which is the largest atoll system in the world [4]. The complex interaction of mega, meio and micro-fauna makes Lakshadweep as an example one of largest coral reef ecosystem in India [5, 6]. The Maldive's clownfish, *A. nigripes* is restricted to Maldives and Lakshadweep Islands, we established our field research station (Marine ornamental fish hatchery) at Agatti Island, Lakshadweep and we attained successful large scale production under difficult environmental conditions.

The present study was aimed to isolate and characterized syndromes of *K. pneumonia*e associated with skin hemorrhages and ulcer confirmed by 16s rDNA sequencing from infected Maldive's clown fish (*A. nigripes*). Experiments were carried out with *K. pneumonia*e to distinguish the sign of infection, histopathology and survival of the fishes.

Materials and methods

Isolation and characterization of pathogen

Moribund fishes of *A. nigripes* were collected from marine ornamental fish hatchery, Annamalai University Field Research Station (Lat: 10°50'43.16"N, Long: 72°11'15.76"E), Agatti Island, Lakshadweep during august 2010. Affected fish size ranged between 6-7 cm and 20±1g. In freshly dead fish liver and tissue abscess, hemorrhage was observed and aseptically excised. The gills and body surface was observed under microscope to check the presence of parasites. Liver, spleen, gill, and kidney of moribund fishes were aseptically removed and streaked on nutrient agar and zobell marine agar 2216 (Hi Media, Mumbai) with triplicate. After incubation at 28 ºC for 2 days, predominant bacterial colonies of clownfish pathogen (CP5) were sub-cultured on the nutrient medium to check the purity of the predominant isolates and it was subjected to Gram-staining, motility, flagella staining, physiological and biochemical tests. The biochemical characters of bacterial pathogen was characterized with biochemical kit-KB002 (Hi Media) which includes indole, methyl red, voges-proskaur, oxidase, catalase, citrate utilization, lysine decarboxylase, ornithine decarboxylase, urease, deamination, nitrate reduction, H_2S production and sugar utilization tests (sucrose, lactose, arabinose, glucose and sorbitol).

DNA extraction and 16S rDNA gene amplification

The strain CP5 was centrifuged at 10,000 rpm for 10 min and the pellet was dissolved in 567 µl TE buffer, 30 µl of 10% sodium dodecyl sulfate, and 3 µl Proteinase K (60 µg). After 1 h incubation at 37 °C, 100 µl of 5 M NaCl was added and mixed thoroughly. The 16s rDNA sequences were amplified by polymerase chain reaction (PCR) using universal primers of 8f (5′-AGAGTTTGATCCTGTGCTCAG 3′) and 1490r (5′-GACTTACCAGGGTATCTAATCC-3′). The 50 µl reaction mixture was prepared which consist of 5 µl of 10x buffer, 5 µl of 2.5 µM $MgCl_2$, 8 µl of dNTP mix (2.5 µM each), 1 µl of each primer 2 µl of template DNA, 0.5 µl of Taq DNA polymerase (5 U/µl) (Genei, Bangalore) and 29.5 µl of Milli Q1. The reaction was performed in a thermal cycler with an initial de-naturation at 94 °C for 5 minutes, followed by 30 cycles at 94 °C for 1 min annealing at 50 °C for 1 min and 72 °C for 90 seconds and final extension at 72 °C for 7 min by the method [7].

PCR Product Purification

The PCR product was purified to remove the excess primer, primer-dimers and low molecular weight DNA fragments generated by nonspecific amplification. Five volume of binding buffer was mixed with one volume of PCR product and loaded into the purification column. The sample was centrifuged and flow through was discarded. 500µl of the wash buffer was added and centrifuged at 11,000 rpm for 2 minutes. Once again the column was washed with the diluted wash buffer 2. Followed, the purified PCR product was eluted by adding the elution buffer at the center of the column and centrifuged at 11,000 rpm for 1 minute. The purified product was stored at (-) 20 ºC until analysis.

Sequencing and phylogenetic analysis

Nearly full length of sequences of the amplified 16S rDNA genes (541 bp) were obtained by automated sequencer (Bioserve Biotechnologies pvt. Limited, India). The sequences were edited by using Clustal X mega software and a BLAST search was executed in the National Center for Biotechnology Information (NCBI) database to find out the nearest neighbor of the amplified sequence. Phylogenetic trees were inferred using the neighbor-joining method.

Experimental infection

Healthy adults of Maldives clown fish (*A. nigripes*) (4-5 cm in length, 13±1 g in weight) were collected from grow-out culture facility of same station. The animals (n = 50) were maintained in four 200 liter FRP tanks supplied with aerated and UV - treated seawater. The water used for both the experimental and control tanks were from a single source and confirmed its physico-chemical parameters as same at the time of introduction. Two fish from each tank were confirmed as free from bacterial infection by plating in NA and ZMA and fishes were fed thrice a day with naturally available clam, tuna egg and fish meat feed during 30 days of the acclimatization period and the experimental periods and water exchange was not done. Twenty fish in the two experimental tanks were starved and exposed to a 24 h culture of *K. pneumonia*e with final concentration of 3×10^6 CFU/ ml^{-1} in 100^{-1} tank with 100 l UV-sterilized sea water under aseptic conditions. Ten control fish from the same source were exposed to the same amount of nutrient broth, which did not contain *K. pneumonia*e. Once the fish had been exposed to *K. pneumonia*e they were observed twice a day. 15 out of 20 dead fish were necropsied and the spleen, liver and kidney were aseptically streaked on NA and ZMA 2216 agar. After incubation at 28 ºC for 48 h, bacteria were identified the above methodology. The experimental period was one month to analyze the rate of increasing physico-chemical parameters; dissolved oxygen, temperature, pH, unionized ammonia, nitrite and nitrate in the water were determined.

Histopathology

Ten fish from each tank control and physically infected on the way out (languid and disoriented) fish was removed from the tank, sedated with MS- 222 and then the second gill arch, liver and kidney were removed and fixed in 10% formalin and processed for paraffin embedding. Sections were cut and stained with hematoxylin and eosin [8].

Results

Isolation and characterization

In the present study we isolated 5 different types of bacterial colonies from immune organs. Based upon the colony morphology (shape, size and colour) predominant bacterial colonies (CP5) was selected and sub cultured to check the purity of the predominant isolates and frequently streaked in Zobell marine agar plates and acquired pure colonies of the bacterial pathogen. Biochemical characteristics of the pathogen CP5 was showed gram –Ve rods, non- motile, Catalase, Capsule, Voges–Proskauer, Citrate, Urease, Nitrate were positive and MR, Indole, H_2S, were negative. Sugars were utilized by the isolated bacteria such as Glucose, Arabinose, Cellobiose, Glycerol, Lactose, Maltose, Mannitol, Mannose, Sucrose, Xylose, Raffinose, Sorbitol and Raffinose, Sorbitol shows negative.

Sequenced and phylogenetic analysis

In the present study bacterial pathogen isolated from the diseased *A. nigripes* were confirmed as *K. pneumoniae* prior to bacterial challenge by 16s rDNA sequence analysis and after the artificially infected the immune organs were analyzed by histological methods and stated the immune cells in the particular organs. In the present study, the strain CP5 was identified by using the PCR amplification of 16S rRNA gene followed by the sequencing. The genomic DNA of the bacterial strain CP5 was isolated and confirmed by agarose gel electrophoresis. The 16S rRNA gene was amplified from the genomic DNA of the strain CP5 under the most favorable conditions using universal primers. The amplified product was analyzed by resolving it in the 1% agarose gel. The gel shows a clear band corresponding to the marker resembling that the DNA was amplified. Molecular identification of the 16S rDNA CP5 shows 100% identity at 100% coverage with *K. pneumoniae*. The sequenced strain of CP5 was identified as is 541 bp in length, and exhibited 100% similarity with the 16S rDNA of *K. pneumoniae* strain E29 from GenBank database. According to the database similarity, a phylogenetic tree was generated (Fig. 1). The partially complete (*541 nt) 16S rDNA sequences of *K. pneumoniae* have been deposited in the GenBank database under accession number HQ589912.

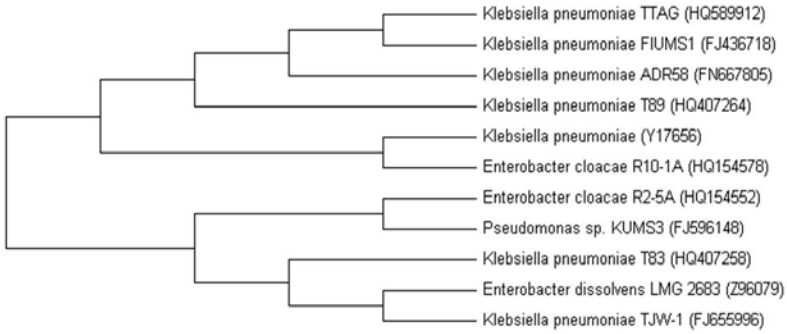

Fig 1: Phylogenetic tree constructed through Kimura 2-parameter model using the Neighborhood- joining method. Bar scale 0.02. Pseudomonas KUMS3 was used as an out group. Queried sequence: *Klebsiella pneumoniae* TTAG (HQ589912)

Histological observation

The gills, liver, kidney and spleen with similar white exudates were observed. The skins were often severely redness in caudal fin and surface of whole body. In these experimental animals, no other gross lesions were observed. Swellings were observed in the liver, gills, spleen and kidney of infected fishes. The liver is a largest organ with hematopoietic tissue and macrophage aggregates. After infection with *K. pneumoniae*, focal necrosis and irregularly shaped nuclei of hepatocytes, eosinophilic granulocytes and erythrocytes were observed. The trunk kidney of fish had enlarged sinusoids. In this study *K. pneumoniae* infected spleen showed densely packed melanomacrophage centers and red and white pulp when compared with control fish spleen.

Discussion

Generally *K. pneumoniae* are ubiquitous in nature [9], and the other being the mucosal surfaces of mammals such as humans, horses, or swine, on which they colonize. *K. pneumoniae* are also isolated from fish, where they produce histamine [10-12]. As high level intakes of histamine can cause seafood poisoning, *K. pneumoniae* are hence recognized as a risk in food safety. *K. pneumoniae* is not only producing histamine in seafood and the present study reported as ornamental fish pathogen in the marine environment. The isolated causative agent from the infected parts of clown fish have confirmed as *K. pneumoniae* by 16s rDNA sequence analysis. Interestingly, the clinical presentation was similar in first observation and artificially infected, with a severe mortality. Isolates were obtained from tissue, liver and kidney from all cases sampled. These tissues were sampled, although they become visibility normal, to investigate the potential route of entry of this bacteria.

A few reports available of *K. pneumonia*e described as pathogen in marine environment and none of the works particularly in culture systems. *Klebsiella* are also opportunistic pathogens to human and can give rise to severe diseases such as septicemia, pneumonia, urinary tract infections, and soft tissue infection, especially in hospitalized, immunocompromised patients [13]. Analysis of physico-chemical parameters of the experimental tank, quantitative of ammonia was high and it may be encourage the infectious disease in candidate species. High ammonia level in captivity can also cause severe stress, whereas slightly elevated levels can contribute to chronic stress. Unionized ammonia affects gills and restricts the respiration and made a bridge to invade the bacterial pathogens as a secondary intruder. In the acclimatization period none of the fishes died due to stress or poor management practices and no mortality were observed in the control tank during the experiment. After 24 hours of exposure, artificially infected fish in the experimental tank shows lethargic movement, stay away from the sea anemone and came to surface of the water and fishes died at 36 hours were observed. Finally all the infected fishes were died in the experimental tank within 72 hours. Survival rate of *A. nigripes* after infection of *K. pneumoniae* and mortality rate was calculated as number of fish for every 12 hours and mortality did not occur within 24 hours of infection (Fig.2). Infected fishes by *K. pneumoniae* were analyzed and recorded hemorrhages, ulcer and redness in the skin after 48 hours of injection. Similar bacterial colonies were isolated from tissue, liver, spleen and kidney of artificially infected moribund fishes and confirmed as *K. pneumoniae* by biochemical characteristics and 16s rDNA sequence analysis. The physico-chemical parameters of experimental tank at acclimatization period were analyzed (Table. 1). Since, this is first research station have confirmed the cause of mortality in the ornamental fishes with hemorrhages by *K. pneumoniae*. Nevertheless, until now, infections in ornamental fish caused by *K. pneumoniae* have never been reported. This *K. pneumoniae* appears to be emerging as a primary pathogen of coastal marine fishes. Further study of this bacterium from marine fishes and their associated animals with specific signs is necessary to improve of the epidemiology and pathogenesis of this emerging ornamental fish pathogen.

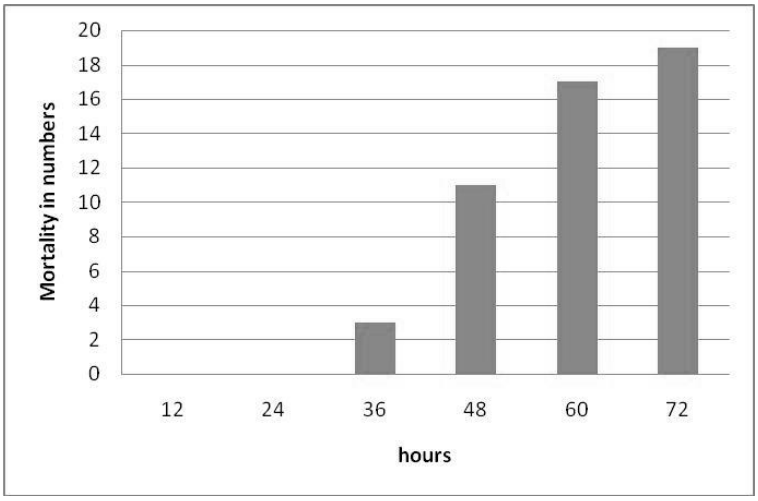

Fig 2: Survival rate of *A. nigripes* after infection of *K. pneumoniae*. Mortality rate was calculated as number of fish for every 12 hours and mortality did not occur within 24 hours of infection.

Table 1: Physico-chemical parameters of experimental tank

Parameters	2/9/2010	9/9/2010	17/9/2010	24/9/2010	30/9/2010
Temperature	27	26	26	26	27
Salinity (psu)	34	34	34	34.2	34.5
P^H	7.2	7.5	7.9	8.2	8.4
DO (mg/l)	5.8	6.2	6.3	6.4	6.3
TSS(mg/l)	15.4	14.7	14.4	14.12	13.97
Nitrite(µg/1)	0.23	0.36	0.47	0.52	0.66
Nitrate(µg/1)	0.48	0.57	0.69	0.77	0.87
Phosphate(µg/1)	0.37	0.39	0.41	0.42	0.44
Silicate(µg/1)	0.21	0.22	0.25	0.27	0.28
Ammonia(mg/1)	0.44	0.85	1.22	1.56	1.72

Conflict of interest statement

We declare that we have no conflict of interest.

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