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Studies on association of various bacteria with epizootic ulcerative syndrome in freshwater fish species found in Dharmasagar Lake, Telangana, India

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Abstract
The Epizootic Ulcerative Syndrome is one of the serious infections noticed in the finfish of Asia-Pacific during the last three decades. Different bacterial agents were isolated from EUS infected *Channa striatus* and *Channa punctatus* fishes from Dharmasagar Lake of Warangal, Telangana, India. *Aeromonas hydrophila*, *Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella salmonicida* were most isolated groups. Isolates have been obtained and ability to induce dermal lesions in infectivity studies make bacteria is one of the likely primary aetiological agents that could be triggering the initiation to EUS. However, further investigations may be required to fully establish the role of these bacterial agents in the induction of EUS. Bacteria was observed as persistent infections in fishes evidenced by their presence in cell culture also require further investigations for their role in predisposing the freshwater fishes of *C. striatus* and *C. punctatus*. The environmental conditions were also found in fluctuation during the winter season the maximum EUS fishes affected. In the present study isolation of bacterial pathogens from infected fishes like *Aeromonas hydrophila*, *Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella salmonicida* had shown its presence in bacterial load on gills and pancreas throughout the year and except in the summer months.

Keywords: Epizootic ulcerative syndrome, bacterial pathogens, *Channa punctatus*

1. Introduction
All the animals and include fish suffers from environmental, nutritional and infectious diseases. Causative organisms such as protozoan parasites, trematode parasites, bacteria, fungi and viruses are pathogens capable of causing disease in cultured and wild populations of fish. Poor nutrition and water quality can also cause disease under the stressful conditions (Reena, 2009) [1]. Revealed EUS attack among different species of fishes the studies in the last decade (Kar *et al*., 1993 [2]; Kar *et al*., 2000 [3]. Showed that species like *C. striatus, C. punctatus, C. batrachus* and *A. testudineus* have been severely affected by EUS and the outbreak has been occurring during the period from November to March. Epizootic ulcerative syndrome (EUS) was a term coined to describe a severe cutaneous ulcerative epizootic condition affecting many species of wild and farmed freshwater and estuarine finfish (Roberts *et al*., 1994) [4]. Due to the consistent presence of the fungus in the infected fishes, EUS was later defined to include the presence of invasive *Aphanomyces* infection and necrotizing ulcerative lesions typically leading to a granulomatous response (Baldock *et al*., 2005) [5]. Now it is suggested to rename EUS as epizootic granulomatous aphanomycosis (Rijijohn *et al*., 2012) [6]. EUS is currently recognized as similar to Red Spot Disease (RSD) in Australia and ulcerative mycosis in Western Atlantic estuaries and Mycotic granulomatosis (MG) of cultured Ayu (*Plecoglossus altivelis*) in Japan (Lilley *et al*., 2012) [7]. EUS infected fish is characterized by the presence of single or multiple lesions of varying shapes with acute dermatitis, hyperaemia and oedema leading to the development of typically large shallow or deep ulcers with a hemorrhagic necrotic base anywhere in the body with muscular inflammation and multiple granuloma (OIE 2009) [8]. EUS is one of the listed diseases of fishes as per (Lilley *et al*., 1992) [9]. The disease has been found to infect more than 100 species of fishes and cause huge economic losses. It causes an annual loss of 0.7 million USD in Australia, while in Thailand, EUS caused a loss of 100million USD during the 10 year period from 1983 to 1993. Bangladesh suffered a revenue loss of 4.8 million USD during the 2-year period of 1988–89

due to EUS. The disease also caused a loss of 0.3 million USD loss in Pakistan in 1996, while in Indonesia the loss was to the tune of 0.235 million USD during 1980–87. Several bacterial and fungal pathogens were isolated from EUS infected *C. striatus* and *C. punctatus* fishes. The present study was attempted to cover the bacterial agents henceforth identified in association with EUS.

2. EUS affected species

A wide range of freshwater and brackish water wild and cultured fish involving over 100 species have been affected by EUS (Frerichs *et al*., 1989) [10]. Snakeheads were by far the most seriously affected species, even though species like *Puntius sp*., catfishes (*Heteropneustes fossilis, Clarias batrachus*), Indian major carps (*Catla catla, Cirrhinus mrigala, Labeo rohita*), climbing perch (*Anabas testudineus*), mullet (*Mugil cephalus*), gobies (*Glossogobius giurus*, *Oxyeleotrism armoratus*), spiny eel (*Mastacembelus armatus*), swamp eel (*Fluta alba*), gouramis (*Trichogaster pectoralis*) were also among those seriously affected. Among snakeheads, *Channa striatus* was the most severely affected while *C. marulius* and *C. punctata* were affected to a slightly lesser extent (Ahmed *et al*., 1995) [11]. A few commercially important freshwater and brackish water species including milkfish (*Chanos chanos*), tilapias, Chinese carps were found consistently resistant to EUS (Lio Po *et al*., 2003) [12]. Bacterial pathogens have been consistently found associated with EUS infected fishes. Infections from *Aeromonas hydrophila*, *Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella salmonicida* isolated from EUS infected fishes were found to induce EUS –like lesions in fish (Lio Po *et al*., 1998) [13]. Role of environmental factors like high rainfall, poor water quality and low pH in the induction of EUS have been documented (Choongo *et al*., 2009 [14]; Pathiratne *et al*., 2001 [15]. Low salinity and sudden rainfall were implicated in EUS induction in mullets in estuaries (Mohan *et al*., 1999) [16]. They noticed that an increase in salinity increases healing of ulcers. Snakehead of *C. striatus* and *C. punctatus* fishes infected with EUS showing ulceration in the head region and onset of the lesion on the side. Typically EUS infected snakehead fishes with deep ulcers and heavy loss of muscle tissue. The present study deals with the isolation and identification of pathogenic microbes and pathogenic influence on bacterial load on gills and pancreas of infected fishes of Dharmasagar Lake.

3. Materials and methods

3.1 Fish sample collection

A total of 220 infected *C. striatus* and *C. punctatus* of average mean length 20 ± 3 cm and average weight 120±2g were collected from Dharmasagar Lake (20.10" 32.460 N and 80.37" 22.810 E) and also purchased from local fish market at Warangal, Telangana, India (Figure 1 &2) during this period of (June 2012 to May 2013). Fishes were brought to the laboratory for further clinical examination. All these infected individuals showed external symptoms like unresponsiveness, external wounds, superficial lesions, swelling, discoloration, and deep ulcer hemorrhages. Monthly wise fish samples were collected and estimation of bacterial load from the Dharmasagar Lake fishes which have more infectious. During winter season maximum EUS affected fishes were reported. Pathogenic bacteria were also reported during these months in the water of these freshwater bodies. In the present study *Aeromonas hydrophila*, *Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella salmonicida* its presence throughout the year except the summer months in this lake. The microbial investigation was carried out in different infected parts were separated from fishes of *C. striatus* and *C. punctatus* namely, Gills and Pancreas.

3.2 Isolation and culture of bacteria

Tissue samples of internal organs of gills and pancreas was streaked on to Nutrient Agar and Trypticase Soy Agar and using suitable dilutions then incubated for 24-48 hrs at 25 ± 2 ºC. The growing colonies were harvested in pure form and re incubated in Trypticase Soy Agar (TSA) for further identification. Identification of isolates was done by cultural, morphological and biochemical characters according to (Quinn *et al*., 2002 [17]; Austin and Austin 2007 [18] and by API-20E (Biomerieux) for gram-negative fish pathogens.

4. Results

The isolation of microorganisms was based on the infected fish species, their disease status and clinical diagnosis. To know the aetiology of Epizootic Ulcerative Syndrome (EUS) infected fishes, a year survey of local market was carried out and it was found that the bottom dwelling fishes, *Channa striatus* and *Channa punctatus* were mostly EUS affected and they were brought by the fishermen to market from the lake of Dharmasagar. Then it was decided to study the behavioral aspects of infected fishes. They have also observed that expression of disease was eroded tail, torn fin, yellow-grayish lesions on the body surface and head region of fish (Figure 3&4) (Baura *et al*., 1991)[19]. Also mentioned that clinical signs of epizootic ulcerative syndrome (EUS) in small freshwater fishes of Bangladesh such as *Channa sp*., *Puntius sp Anabas sp* and *Clarias sp*. consisted of gray-cotton lesions and missing caudal region and fins (Ahmed *et al*., 1999)[20].

The total plate count on Plate count Agar on the gill of *Channa striatus* found at Dharmasagar lake were from $11x10^3$ - 32 $x10^3$ collectively in first and second phases of the year 2012-13. *Aeromonas hydrophila* was found from 38 $x10^3$ -51 $x10^3$, *Pseudomonas aeruginosa* were from $5x10^3$ -11 $x10^3$, *Salmonella salmonicida* were from $7x10^3$-12 $x10^3$ and *Staphylococcus aureus* were $5x10^3$ - $9x10^3$. (The results are tabulated in Table 1& Fig. 1).

The total plate count on Plate count Agar on the pancreas of *Channa striatus* found at Dharmasagar lake were from $15x10^3$ - 35 $x10^3$ collectively in first and second phases of the year 2012-13. *Aeromonas hydrophila* was found from 5 $x10^3$ -38 $x10^3$, *Pseudomonas aeruginosa* were from 12 $x10^3$ -22 $x10^3$, *Salmonella salmonicida* were from 6 $x10^3$-$11x10^3$ and *Staphylococcus aureus* were $23x10^3$ - $25x10^3$. (The results are tabulated in Table 2& Fig. 2).

The total plate count on Plate count Agar on the gill of *Channa punctatus* found at Dharmasagar lake were from $11x10^3$ - 32 $x10^3$ collectively in first and second phases of the year 2012-13. *Aeromonas hydrophila* was found from 38 $x10^3$ -51 $x10^3$, *Pseudomonas aeruginosa* were from 5 $x10^3$ - 11 $x10^3$, *Salmonella salmonicida* were from 7 $x10^3$ - 12 $x10^3$ and *Staphylococcus aureus* were $5x10^3$ -$9x10^3$. (The results are tabulated in Table 3& Fig. 3).

The total plate count on Plate count Agar on the pancreas of *Channa punctatus* found at Dharmasagar lake were from $11x10^3$ - 35 $x10^3$ collectively in first and second phases of the year 2012-13. *Aeromonas hydrophila* was found from 17 $x10^3$ -28 $x10^3$, *Pseudomonas aeruginosa* were from 5 $x10^3$ -7 $x10^3$, *Salmonella salmonicida* were from 3 $x10^3$ -8 $x10^3$ and

Staphylococcus aureus were $10x10^3$ -$14x10^3$. (The results are tabulated in Table 4 & Fig. 4).

The bacterial pathogens isolated from Dharmasagar Lake such as *Aeromonas hydrophila Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella salmonicida spp.* This microbial load estimated from gills and pancreas of *C. striatus* and *C. punctatus.*

Fig 1: Dharmasagar Lake

Fig 2: Satellite view of Dharmasagar Lake

Fig 3: *Channa striatus*

Fig 4: *Channa punctatus*

Table 1: Bacterial load in the Gill of *Channa striatus* fish from Dharamsagar Lake (Phase I) 2012-13.

Sl. No	Month	PCA	*Aeromonas hydrophila*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Salmonella salmonicida*
1	June	$15x10^3$	0	0	0	0
2	July	$11x10^3$	0	0	0	0
3	Aug	$17x10^3$	0	0	0	0
4	Sept	$13x10^3$	$38x10^3$	$5x10^3$	$7x10^3$	0
5	Oct	$19x10^3$	0	0	$9x10^3$	$12x10^3$
6	Nov	$22x10^3$	$45x10^3$	$7x10^3$	$11x10^3$	0
Bacterial load in the Gill of *Channa striatus* fish from Dharamsagar Lake (Phase II) 2012-13.						
7	Dec	$28x10^3$	$51x10^3$	$9x10^3$	$5x10^3$	$7x10^3$
8	Jan	$32x10^3$	$43x10^3$	0	0	0
9	Feb	$29x10^3$	0	$4x10^3$	0	0
10	Mar	$22x10^3$	0	0	0	0
11	Apr	$16x10^3$	0	0	0	0
12	May	$13x10^3$	0	0	0	0

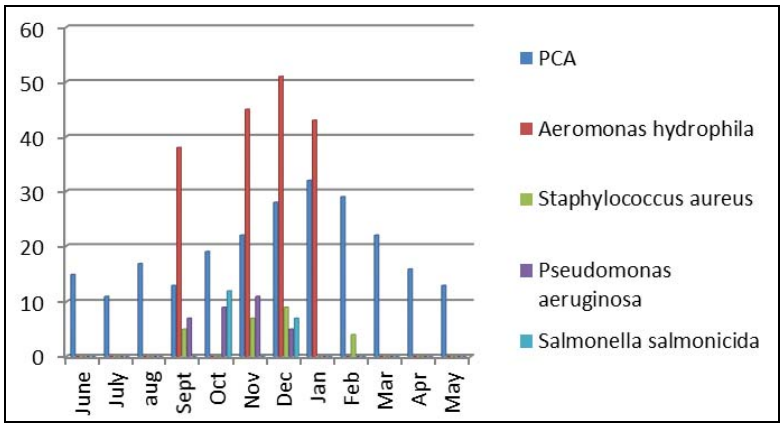

Fig 1: Bacterial load in the Gill of *Channa striatus* fish from Dharamsagar Lake 2012-13.

Table 2: Bacterial load in the Pancreas of *Channa striatus* fish from Dharamsagar Lake (Phase I) 2012-13.

Sl. No	Month	PCA	*Aeromonas hydrophila*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Salmonella salmonicida*
1	June	$34x10^3$	0	0	0	0
2	July	$25x10^3$	0	0	0	0
3	Aug	$25x10^3$	0	0	0	0
4	Sept	$25x10^3$	0	0	0	0
5	Oct	$25x10^3$	$38x10^3$	$23x10^3$	$12x10^3$	$8x10^3$
6	Nov	$25x10^3$	$28x10^3$	0	0	0
Bacterial load in the Pancreas of *Channa striatus* fish from Dharamsagar Lake (Phase II) 2012-13.						
7	Dec	$35x10^3$	$13x10^3$	0	$17x10^3$	$11x10^3$
8	Jan	$15x10^3$	$5x10^3$	$25x10^3$	$22x10^3$	$6x10^3$
9	Feb	$23x10^3$	0	0	0	0
10	Mar	$17x10^3$	0	0	0	0
11	Apr	$25x10^3$	0	0	0	0
12	May	$15x10^3$	0	0	0	0

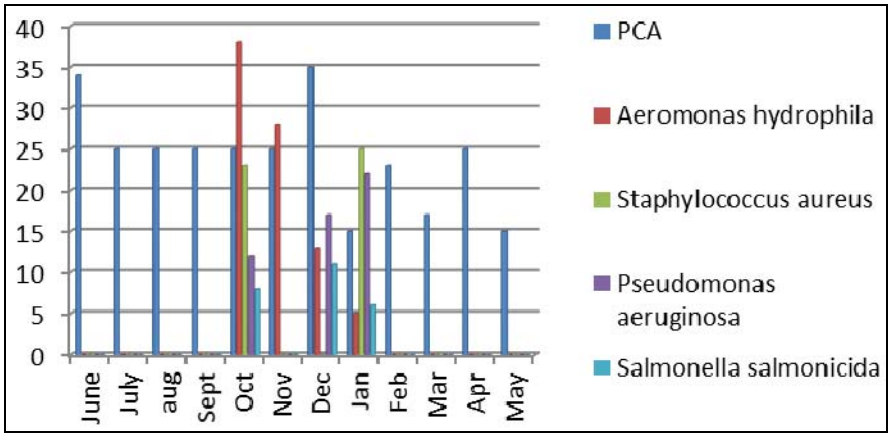

Fig 2: Bacterial load in the Pancreas of *Channa striatus* fish from Dharamsagar Lake2012-13.

Table 3: Bacterial load in the Gill of *Channa punctatus* fish from Dharamsagar Lake (Phase I) 2012-13**.**

Sl. No	Month	PCA	*Aeromonas hydrophila*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Salmonella salmonicida*
1	June	$15x10^3$	0	0	0	0
2	July	$11x10^3$	0	0	0	0
3	aug	$17x10^3$	0	0	0	0
4	Sept	$13x10^3$	0	$5x10^3$	$7x10^3$	0
5	Oct	$19x10^3$	$43x10^3$	0	$9x10^3$	$12x10^3$
6	Nov	$22x10^3$	$45x10^3$	$7x10^3$	$11x10^3$	0
Bacterial load in the Gill of *Channa punctatus* fish from Dharamsagar Lake (Phase II) 2012-13.						
7	Dec	$28x10^3$	$51x10^3$	$9x10^3$	$5x10^3$	$7x10^3$
8	Jan	$32x10^3$	0	0	0	0
9	Feb	$29x10^3$	$38x10^3$	$4x10^3$	0	0
10	Mar	$22x10^3$	0	0	0	0
11	Apr	$16x10^3$	0	0	0	0
12	May	$13x10^3$	0	0	0	0

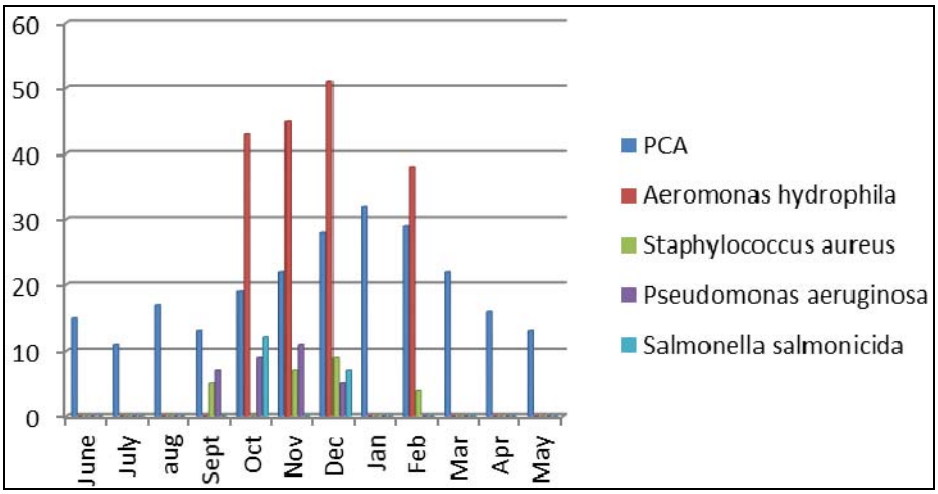

Fig 3: Bacterial load in the Gill of *Channa punctatus* fish from Dharamsagar Lake 2012-13.

Table 4: Bacterial load in the Pancreas of *Channa punctatus* fish from Dharamsagar Lake (Phase I) 2012-13.

Sl. No	Month	PCA	*Aeromonas hydrophila*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Salmonella salmonicida*
1	June	$18x10^3$	0	0	0	0
2	July	$11x10^3$	0	0	0	0
3	aug	$17x10^3$	0	0	0	0
4	Sept	$22x10^3$	0	0	0	0
5	Oct	$29x10^3$	$19x10^3$	$14x10^3$	$5x10^3$	$8x10^3$
6	Nov	$32x10^3$	$17x10^3$	$12x10^3$	$7x10^3$	$5x10^3$
Bacterial load in the Pancreas of *Channa punctatus* fish from Dharamsagar Lake (Phase II) 2012-13.						
7	Dec	$28x10^3$	$22x10^3$	$10x10^3$	0	$3x10^3$
8	Jan	$35x10^3$	$28x10^3$	0	$6x10^3$	0
9	Feb	$25x10^3$	0	0	0	0
10	Mar	$17x10^3$	0	0	0	0
11	Apr	$12x10^3$	0	0	0	0
12	May	$15x10^3$	0	0	0	0

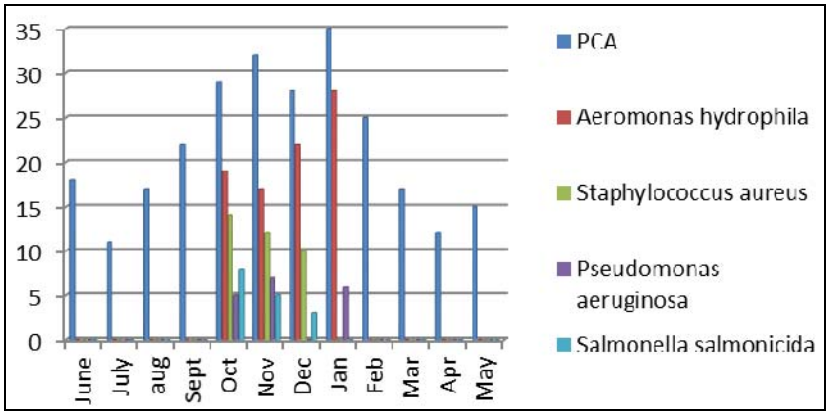

Fig 4: Bacterial load in the Pancreas of *Channa punctatus* fish from Dharamsagar Lake 2012-13.

5. Discussion

The predominance of *A. hydrophila* in EUS affected fish has also reported previously (Kumar *et al*., 1999) [21] in India (Tonguthai, 1985) [22]. In Thailand (Wong and Leong 1987) [23] (Dana, 1987) [24] in Indonesia (Roberts, 1997) [25] in Mayanamar and (Balasurya 1987) [26] in Srilanka (Lio- Po *et al*., 1992) [27]. Reported that bacterial and fungal pathogens related with EUS affected Snake head *C. striatus* and 80% of the total isolates were *A. hydrophila.* (Smith *et al*., 2012) [28]. Categorized the different groups of bacterial pathogens in the aquarium water where gold fish and Chinese algae eaters thrived using molecular appearances and identified the presence of 30 phyla of pathogenic microbes with Proteobacteria (52%), Bacteriodates (18%) and planctomycetes (60%), representing the top three phyla.

In the present study it was observed that the signs of red spot disease associated often fraying and reddening of skin and fins, accompanied by regular, variable sized areas of de-pigmentation as well as reddish pigmentation that can occur anywhere on the body surface. Infections can develop at any

age of fish, but losses are usually most severe in fry and fingerlings. Similar conditions of red spot disease were also reported in the rural carp culture by several authors (Amin, 2000; Mazid, 2001)[29,30]. Suggested some preventive measures at the beginning of the winter season which includes application of lime and salt, disinfecting of equipment, addition of water *etc*., in the present study, it was observed that many farmers responds to disease problems with application of chemicals, by little knowledge of the effectiveness of chemicals. But better results can be obtained with minor changes in management practices.

In the present study, red spot disease is recorded both first and second cycles of fish rearing during the year 2012- 13 in Dharmasagar Lake in Warangal district, Telangana, India. The red spot disease is high during second cycle than the first cycle. Low temperature, poor nutrients and poor oxygen were also noticed in the lakes and this clearly indicates the deterioration of water quality in the lakes. The water parameters might have induced the bacterial infections causing red spot disease as well as tail and fin rot in the fry and fingerlings of murrel fishes.

Present study had shown the occurance of four species of bacteria (*Aeromonas hydrophila, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella salmonicida*).In the Gills and Pancreas of freshwater *C. striatus* and *C. punctatus* were associated with bacterial load is causing red spot disease. In the Dharmasagar Lake during the year (2012-13) *Aeromonas hydrophila* is almost dominant in both cycles in gills, and pancreas of in two fishes of *C. striatus* and *C. punctatus*. The positivity of *Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella salmonicida* also exhibited more during the second cycle in gills and pancreas than the first cycle.

Finally the present study has clearly indicated that the concurrent infections of bacteria results in increasing the mortality. The infections of these bacteria in different tissues like gills and pancreas resulted in clinical symptoms sometimes and without any clinical symptoms also resulted in death of fishes. Fins and tails also observed during the present study apart from red sores on the external body. Factors like water quality also play a role in the incidence of diseases. Poor dissolved oxygen, increased levels of gases such as ammonia and nitrite are the contributory factors for the escalation of mortality rate that finally resulted in less realization of seed from the cultured lakes in Warangal district, Telangana state, India. Overcrowding the culture lakes, erratic feed regime, poor husbandry practices do the supplement onset of diseases.

6. Conclusion

The present study, showed the percentage contribution of different bacterial species *Aeromonas hydrophila, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella salmonicida* in different tissues of teleost fishes seems to be fluctuating owing to the potential hazard of *Aeromonas*. This indicates that there is need to put more emphasis on hygiene and sanitary conditions in the culture lakes of Indian murrels. Aqua farmers should be educated on how to observe clinical signs of fish disease and stress and control/ preventive measures to be taken as a remedial action. Aqua farmers also need to follow good management practices.

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