Isolation and identification of microbial flora from EUS infected singhi *Heteropneustes fossilis*

**C.Muthu Ramakrishnan, M.A.Haniffa, P.Jeya Sheela**

**Abstract**

*Heteropneustes fossilis*, commonly called singhi is an obligatory air-breathing fish occurring shallow inland waters of India. For the past two decades this species is declining due to habitat loss, high fishing pressure and easy prone to Epizootic Ulcerative Syndrome (EUS). The infected individuals showing external symptoms like unresponsiveness, wounds, superficial lesions, swelling, discoloration, and deep ulcerative hemorrhages were subjected to bacteriological examination and the pathogens associated with EUS were isolated. The highest microbial load $6.3 \pm 0.4 \times 10^7$ cfu g$^{-1}$ was observed in muscle followed by $5.7 \pm 0.6 \times 10^6$ cfu g$^{-1}$ in gills and $7.2 \pm 0.9 \times 10^5$ cfu g$^{-1}$ in liver whereas the lowest load $4.3 \pm 0.7 \times 10^4$ cfu g$^{-1}$ was found in intestine. Similarly, a higher percentage of *A. invadans* (15%) and *A. hydrophila* (12.5%) was determined from the muscle of the diseased *H. fossilis* whereas the same was found to be low in the intestine (6% and 5.5% respectively). One fungal species, *Aphanomyces invadans* and nineteen bacterial species were isolated and identified. Among the 20 isolates, *A. invadans* was the only fungus and *A. hydrophila* was dominant among the bacterial isolates from muscle, gill, liver and intestine.

**Keywords:** Heteropneustes fossilis, EUS, A. invadans, A. hydrophila

1. Introduction

Among the 142 species of catfish found in the Indian subcontinent, the freshwater catfish *Heteropneustes fossilis*, commonly called singhi is an obligatory air-breathing fish occurring shallow inland waters of India [1]. *H. fossilis* has been identified as vulnerable among the 327 threatened freshwater fish species in India [2]. It constitutes a major bulk of the production in India. *H. fossilis* is an omnivore, highly tolerant to oxygen depleted waters. It commands good consumer preference due to fewer intra muscular spines, tender flesh with delicious taste, high protein, iron and low fat contents [3] and is often recommended to convalescent people [4]. For the past two decades this species is declining due to habitat loss, high fishing pressure and easy prone to EUS disease [5]. At this moment, *H. fossilis* has to be conserved for the future population, since alien species viz: African catfish *Clarias gariepinus* and Thai catfish *Pangasius sutchi* pose a heavy threat to biodiversity of freshwater fishes of our country.

Environmental factors and poor water quality resulting from increased pollution due to effluents discharge and pathogen transfer appear to be as important underlying cause of epizootics. EUS is one of the most destructive diseases among freshwater as well as brackish water fish species in the Asian Pacific region [6, 7]. It is very common in both northern and southern India and has spread through rivers, reservoirs and paddy fields to neighboring states, causing considerable loss to fish farmers. A diverse group of biotic agents such as viruses, bacteria and cutaneous ectoparasites may initiate skin lesions which are subsequently colonized by *Aphanomyces invadans* and ultimately lead to EUS [8].

*A. invadans*, a highly invasive, specific slow growing fungus causes Epizootic Ulcerative Syndrome [9]. Different pathogenic organisms including bacteria [10, 11], fungi [12, 13] and virus [14] have been reported from naturally infected fish. Roberts et al. [15] have reported natural outbreaks of EUS in more than 100 fish species especially in air-breathing fishes. According to Das [7], studies of affected fishes carried out in different countries recorded a wide range of pathogenic fungi including *A. invadans* and the bacterium *A. hydrophila* as the most prevalent. A critical problem in the study of microbial pathogens of the fish is the correct identification of the causative agents. To facilitate the precise identification of microbes involved in diseases, the usual practice is to undertake biochemical tests and thus identifying the isolates.
by their reaction to standard tests following the Bergey’s manual [16]. Hence, the present study deals with the isolation and identification of pathogenic microbes from the EUS infected *H. fossilis*.

2. Materials and Methods

2.1 Sample collection

A total of 167 infected *H. fossilis* of average mean length 20 ± 3 cm and average weight 65 ± 2.5 g were collected from Thamiraparani River (8.44° N, 77.44°E) fed systems and also purchased from local fish market at Melapalayam, Tirunelveli, Tamil Nadu, India in the month of April 2006 (Figure 1). They were transported to CARE Aquafarm and acclimatized in stocking ponds for further analysis. All the infected individuals showed external symptoms like unresponsiveness, external wounds, superficial lesions, swelling, discoloration, and deep ulcer hemorrhages.

Fig 1: Fish collection site-Tirunelveli, Tamil Nadu, India [8.44°N, 77.44°E]

2.2 Enumeration of Total Heterotrophic Count (THC)

One gram of each samples from muscle, gills, liver and intestine were taken from the diseased fish with moderate lesions, wound and septicemia using a sterile scalpel under aseptic condition. A small piece of muscle (2 mm²) beneath the margin of the ulcer was scrapped thoroughly and fixed in Czapek Dox agar with Penicillin G (100 units/ml) and oxolinic acid (100 mg/ml) to observe fungal growth [17]. Remaining muscle was homogenized with sterile distilled water and centrifuged at 1000 rpm for 10 minutes. One ml of the supernatant was serially diluted up to 10⁻⁹ dilution. 1ml was taken from each dilution and pour plate technique was carried...
out for the enumeration of total heterotrophic bacterial count using sterile nutrient agar for bacterial and Czapek Dox agar for fungal growth. The bacterial plates were incubated at 37 °C for 24 - 48 h and fungal plates were incubated at 24 °C for 7 days. After incubation, the bacterial colonies and fungal hyphae were observed. After the quantitative analysis, the isolated fish pathogens were subjected to standard microbial techniques for identification.

2.3 Identification of bacterial pathogens
Colonies obtained from different selective plates were isolated and streaked on nutrient agar slants and incubated overnight at 37 °C. The following biochemical tests were conducted to identify the fish pathogens following Bergey’s manual of Bacteriological classification [16] and Surendran and Gopakumar [18]. Biochemical tests include Gram’s staining, Motility tests (Hanging drop method and Soft agar stabbing method), Kovacs oxidase test, Catalase test, Acid production test (Huge and Leifson synthetic medium), Starch hydrolysis, Gelatin hydrolysis, NaCl tolerance (0%, 5% and 7%), Methyl Red test, Voges-proskauer test, Citrate test, Urease test, Triple sugar iron test, ONPG test, 0/129 Sensitivity test and Amino acid decarboxylase test.

2.4 Isolation of fungus
For fungal isolation, the suspected colonies were inoculated in basic GP broth and one loop of colony was streaked on Czapek Dox agar and incubated at 27 °C for 72 hrs. After incubation, the fungal colonies were identified based on their morphology and the results were recorded. The identification of the fungus A. invadans was made on the basis of attachment to the surface, hyphae and sporangial morphology [19].

3. Results and Discussion
The isolation of microorganisms was based on the infected fish species, their disease status, clinical signs and biochemical diagnosis. The results of the quantitative estimation of microbial count in muscle, gill, liver and intestine of diseased H. fossilis are given in Table 1. The highest microbial load of 6.3 ± 0.4 × 10⁹ cfu g⁻¹ was observed in muscle followed by 5.7 ± 0.6 × 10⁵ cfu g⁻¹ in gills and 7.2 ± 0.9 × 10⁵ cfu g⁻¹ in liver whereas the lowest load of 4.3 ± 0.7 × 10⁴ cfu g⁻¹ was found in intestine. The percentage distributions of mycotic and bacterial isolates are shown in Table 2. A higher percentage of A. invadans (15%) and A. hydrophila (12.5%) was determined from the muscle of the diseased H. fossilis whereas the same was found to be low in the intestine (6% and 5.5% respectively). One fungal species and nineteen bacterial species were isolated and identified. Among the 20 isolates, Aphanomyces invadans was the only fungus and A. hydrophila was dominant among the bacterial isolates. Other bacteria isolated from muscle, gill, liver and intestine include Pseudomonas sp, Vibrio sp, Serratia sp, Enterobacter sp, Edwardsiella sp, Flavobacterium sp, Yersinia sp, Klebsiella sp, Haemophilus sp, Staphylococcus sp, Alcaligenes sp and V. parahaemolyticus.

Table 1: Total heterotrophic bacterial count in muscle, gill, liver and intestine of diseased H. fossilis (values are mean ± SD)

<table>
<thead>
<tr>
<th>S.No</th>
<th>Sample (n=167)</th>
<th>Colony forming unit g⁻¹ (cfu g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Muscle</td>
<td>6.3 ± 0.4 × 10⁹</td>
</tr>
<tr>
<td>2</td>
<td>Gill</td>
<td>5.7 ± 0.6 × 10⁵</td>
</tr>
<tr>
<td>3</td>
<td>Liver</td>
<td>7.2 ± 0.9 × 10⁵</td>
</tr>
<tr>
<td>4</td>
<td>Intestine</td>
<td>4.3 ± 0.7 × 10⁴</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S.No</th>
<th>Sample (n=167)</th>
<th>Number of colonies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aphanomyces invadans</td>
<td>15 13.5 6.5 6 41</td>
</tr>
<tr>
<td>2</td>
<td>Aeromonas hydrophila</td>
<td>12.5 9.2 7.8 5.5 35.8</td>
</tr>
<tr>
<td>3</td>
<td>Pseudomonas sp</td>
<td>2 1 - 0.1 3.1</td>
</tr>
<tr>
<td>4</td>
<td>Vibrio sp</td>
<td>0.5 0.6 0.5 1.2 2.8</td>
</tr>
<tr>
<td>5</td>
<td>Serratia sp</td>
<td>0.9 0.5 0.1 0.6 2.1</td>
</tr>
<tr>
<td>6</td>
<td>Enterobacteria sp</td>
<td>0.6 0.4 0.4 - 1.4</td>
</tr>
<tr>
<td>7</td>
<td>Edwardsiella sp</td>
<td>0.7 0.2 0.4 0.4 1.7</td>
</tr>
<tr>
<td>8</td>
<td>Flavobacteria sp</td>
<td>0.4 0.1 0.3 0.1 0.9</td>
</tr>
<tr>
<td>9</td>
<td>Yersinia sp</td>
<td>0.2 - - - 0.5</td>
</tr>
<tr>
<td>10</td>
<td>Klebsiella sp</td>
<td>0.1 0.1 0.1 - - 0.3</td>
</tr>
<tr>
<td>11</td>
<td>Haemophilus sp</td>
<td>0.2 0.1 - 0.2 0.5</td>
</tr>
<tr>
<td>12</td>
<td>Staphylococcus sp</td>
<td>0.3 0.1 0.2 0.1 0.7</td>
</tr>
<tr>
<td>13</td>
<td>Alcaligenes sp</td>
<td>0.5 0.5 0.3 0.4 1.7</td>
</tr>
<tr>
<td>14</td>
<td>V. parahaemolyticus</td>
<td>1.2 0.3 0.4 0.4 2.8</td>
</tr>
<tr>
<td>15</td>
<td>A. salmonicida</td>
<td>0.9 0.5 0.6 - 2.2</td>
</tr>
<tr>
<td>16</td>
<td>Salmonella sp</td>
<td>0.7 0.6 0.4 0.1 1.8</td>
</tr>
<tr>
<td>17</td>
<td>Escherichia coli</td>
<td>0.6 0.1 0.1 0.4 1.2</td>
</tr>
<tr>
<td>18</td>
<td>Micrococcus</td>
<td>0.2 - - 0.3 0.5</td>
</tr>
<tr>
<td>19</td>
<td>Proteus rettgeri</td>
<td>0.6 0.3 0.2 0.2 1.3</td>
</tr>
<tr>
<td>20</td>
<td>Vibrio alginolyticus</td>
<td>0.4 0.4 0.5 0.4 1.7</td>
</tr>
</tbody>
</table>

A. invadans was found in the ulcerative tissue as macroscopic lesions in the muscles of the diseased H. fossilis. A. invadans was observed in Czapek dox agar incubated at 27 °C for 72 h. A. hydrophila is a motile gram negative bacterium, oxidase positive, catalase positive and produce H₂S. It never grows in NaCl and showed negative growth in 5% and 7% NaCl tolerance test. A. hydrophila was isolated and cultured using aeromonads isolation agar and the results are recorded (Table 3). The present study showed a high prevalence of motile aeromonad bacteria (35.8%) next to A. invadans (41%) in all lesions (n=167) from internal organs of muscle, gills, liver and intestine in ulcerated fish indicating systemic invasion. A good number of reports on fish diseases of temperate regions are available. Among the fish diseases Epizootic...
Ulcerative Syndrome is a dreadful disease predominantly affecting the fishes with *A. invadans* and *A. hydrophila* as the main pathogenic organisms. In the present study, the mean bacterial load was observed to be higher than fungal load. The mean bacterial load was found to be more in muscle (6.3 ± 0.4 × 10⁷ cfu/ml) followed by gills (5.7 ± 0.6 × 10⁶ cfu/ml). Similarly, Al-Harbi and Uddin [20] reported higher bacterial load in gills (8.7 ± 1.1x10⁶ cfu g⁻¹) followed by intestine (5.8 ± 0.4 x 10⁷ cfu g⁻¹) of hybrid tilapia. Hazen et al. [21] and Duenci and Candan [22] have stated that *Aeromonas* sp was the predominant microorganism isolated from the skin, gills and intestine of some diseased freshwater fishes. In the present study, 19 bacteria and one fungus were isolated from infected *H. fossilis*. Our results are supported by Katoch et al. [23] who have reported 25 bacterial and fungal species isolated and identified in freshwater carp at Himachal Pradesh, India and by Al-Harbi and Uddin [20] who have recorded 15 isolates of bacteria in hybrid tilapia from Saudi Arabia. Similarly, a total of 17 bacterial and mycotic species were isolated and identified in *C. striatus* in India with most of the isolates from muscle and gills [24].

The fungal species *A. invadans* was found in all the lesions of infected individuals in the present investigation. *Aphanomyces* attributed lesions in fish have been reported in the southeastern US, Australia, and Indo-Pacific region of Asia [25]. Atlantic menhaden are the estuarine fish species severely affected by ulcerative lesions characterized by solitary, typically perianal, focal, deep, granulomatous lesions containing oomycete hyphae, primarily those of *Aphanomyces* [26]. *A. invadans*, a highly invasive, specific, slow growing fungus, causes epizootic ulcerative syndrome [27]. This fungus *A. invadans* was identified by the attachment to the surface, hyphae and sporangial morphology. Hatai [28] identified the fungus based on morphological characteristics of the hyphae, zoosporangia, primary zoospore cysts and asexual reproduction. *A. invadans*, *Aspergillus flavus* and *A. fumigates* were the main fungi isolated from the Nigerian freshwater fishes [29].

The finding of the present study, *A. hydrophila* dominating the bacterial isolates in infected *H. fossilis* is supported by Thampuran et al. [30] who have reported dominance of *A. hydrophila* in the EUS affected *C. striatus*. Similarly, Manohar [31] has also reported the dominance of *A. hydrophila* in the infected *C. carpio*. Motile aeromonads have been associated from the surface of lesions in EUS affected fishes [32, 33]. The predominance of *A. hydrophila* in EUS affected fish has also been reported previously by Kumar et al. [34] in India, Tonguthai [35] in Thailand, Wong and Leong [36] in Malaysia, Dana [37] in Indonesia, Roberts et al. [38] in Myanmar and Balasurya [39] in Sri Lanka. Lio-Po et al. [32] reported that several species of bacteria and fungi were found to be associated with EUS affected snakehead *C. striatus* and that 89% of the total isolates were *A. hydrophila*.

Outbreaks of EUS and hemorrhagic septicaemia caused by *A. hydrophila* infection occur in both captured and cultured fishes such as snakehead (Channa striatus), walking catfish (*Clarias batrachus*), brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), chinook salmon (*Oncorhynchus tshawytscha*), Japanese eel (*Anguilla japonica*), American eel (*Anguilla rostrata*), goldfish (*Carassius auratus*), golden shiner (*Notemigonus crysoleucas*) and tilapia (*Oreochromis mossambicus*) [40, 41, 48].

Meanwhile, *Aeromonas* sp., *Pseudomonas fluorescens*, *Vibrio anguillarum*, *Flavobacterium columnare*, *Edwardsiella tarda*, *Streptococcus* sp. and *Enterococcus* sp. are also the common pathogens infecting fish species [42]. Under predisposing factors such as poor water quality, for instance high ammonia due to high stocking density and careless handling, stressful conditions and ectoparasites, the microorganism find a portal of entry into the fish host [43]. There are several studies on fish bacteria identification, infection and/or disease resistance [44, 20, 45]. Isolation and bacteriological examination from the ulcerated area of *C. punctatus*, *Puntius* sp. and *Mystus* sp. revealed the presence of 16 strains of bacteria belonging to the genus *Pseudomonas* sp, *Aeromonas* sp, *Micrococcus* sp, *Bacillus* sp, *Vibrio* sp and *Moraxella* sp., among which *Pseudomonas* sp and *Aeromonas* sp were the most common [46]. Reantaso [47] and Roberts et al. [12] reported that fungal and bacterial pathogens play an important role in EUS and *A. hydrophila* is the causative agent of motile aeromonad septicaemia, found in a wide variety of freshwater fish species [48]. *Aeromonas* sp are considered as autochronous inhabitant of aquatic environment [49]. They are opportunistic pathogens of many immune compromised poikilotherms and homeotherms [21, 49].

In the present investigation, Gram negative bacteria were found comprising about nearly 60%. Similarly Kumar et al. [34] have reported that Gram negative bacteria comprised major part (75%) among the isolated microorganisms. *A. hydrophila* can often be isolated from ulcers or internal organs of EUS-affected fish [10]. Some of these *A. hydrophila* strains have been characterized as virulent [50] or cytotoxic [51]. Yasmine et al. [52] have reported that *A. hydrophila* is one of the important pathogens of fish in freshwater and brackish water. In the present investigation, *Pseudomonas* sp, *Flavobacterium* sp, *Alcaligenes* sp and *Vibrio* sp were found in addition to *A. hydrophila* and *A. invadans*. Thampuran et al. [30] have also reported the presence of *Pseudomonas* sp, *Alcaligenes* sp *Micrococcus* sp and *E. coli* in infected *C. striatus*. This study revealed the dominance of *A. hydrophila* over other microbial species by the frequency of isolation from the samples. The role of *A. hydrophila* bacterium at the ulcerative stage of the disease cannot be ignored. Although the phenotypic diversity and variations in virulence suggest that the bacterial infection is secondary in nature, they are the main reason for the dreadful infections and ultimate mortality.

The introduction of alien fishes like African catfish, *Clarias gariepinus* and Thai catfish, *Pangasius sutchi* is a major cause of biodiversity decline in freshwater ecosystems. The impacts associated with the introduction of alien fishes are many, including; competition, habitat alteration, parasitism, predation, alteration of habitat quality and/or ecosystem function, host of pests or parasites. The alien species pose a severe threat to the already existing biota and in addition to this, if the native catfishes are allowed to succumb due to diseases like EUS, then there is no doubt that the native fishes will become extinct. The conservation of native catfishes is essential at this juncture, as in almost all ponds and lakes of our country, the alien catfishes we dominant.

### 4. Conflict of interest

We declare that there is no conflict of interest.

### 5. Acknowledgement

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