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# ISOLATION, IDENTIFICATION AND PATHOGENICITY STUDIES ON *Edwardsiella tarda* FROM DISEASED GOLD FISH *Carassius aurtus* IN KERALA

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## ABSTRACT

**Diseases are the one of the most common problem faced by the farmer who rears ornamental fishes and also these kinds of infections in fishes may extent to the public health by inappropriate care. Within the study, there are about five samples of diseased goldfishes were collected from local ornamental fish farm in Ernakulam, Kerala. The external symptoms of bacterial infection were recorded and they were dissected and inoculated into nutrient broth. Five bacterial isolates were obtained from the sample by spread plate technique followed by serial dilution. Thereafter biochemical test performed for the identification and the DNA of the isolates were extracted, followed by PCR amplification of 16S ribosomal RNA gene using 16S rRNA primers. The bacteria isolated from the sample contain strains of *Edwardsiella tarda, Micrococcus luteus, Bacillus safensis, Aeromonas caviae*, and *Staphylococcus aureus* were identified by sequencing by Sanger's method. The result suggests that high microbial load of particular pathogen was *Edwardsiella tarda*. It gives the first evidence to be a pathogen responsible for the infection. After then, challenge study performed for the confirmation. During this study, *Edwardsiella tarda* confirmed as the pathogen and for the treatment antibiotic sensitivity test is performed there by, any of the drug can be used for the treatment. This study also assessed the toxic activities of extracellular products (ECPs) from *Edwardsiella tarda* using in vitro assays on carp fin cell line.**

**KEYWORDS:** *Edwardsiella tarda*, Gold fish, Pathogen, Extracellular Products

One of the most significant bacterial pathogens to fish aquaculture is *Edwardsiella tarda,* among many other bacterial pathogens, such as *Vibrio spp., Aeromonas spp.,Streptococcus spp., etc. E. tarda* had been reported to cause disease in many economically important fish species worldwide. With the rapid developments in aquaculture, *E. tarda*had been recognized as one of the leading pathogens of freshwater and marine cultured fish worldwide. Infection by *E. tarda* often leads to the development of a systematic disease called Edwardsiellosis, characterized by ascites, hernia, exophthalmia, brain and severe lesions of internal organs. Fishes are highly susceptible to a wide variety of bacterial pathogens. Most of them are infectious and are saprophytic in nature. Bacteria become pathogenic when fishes are nutritionally deficient, physiologically unbalanced, or there are other stressors, i.e., overstocking, poor water quality, thereby even opportunistic bacterial infections may occur.When diseased fish is introduced to a new pond, the infected fish spread the disease to the normal ones by the consumption of infected dead fishes or feces of infected fishes. Sometime infected fishes show open ulcers thereby it act as a source of infection. Other than environmental stresses even primary pathogens can cause disease (Subasinghe and Barg, 1998).There are different types of fish diseases reported, each of them have their own symptoms such as, Ulcers, fin rot, Tail rot, Mouth rot, Dropsy, red mouth disease etc., and these diseases are the prime agent affecting fish mortality.

Human infections are mainly found in individuals having infected edible fish, but it can be also caused from ornamental fish during inappropriate handling. It may depend on the immune status of the individual. Symptoms produced from the human infections are usually self limiting in healthy people. Most common organism causing diseases in humans from fishes are *Streptococcus iniae, Mycobacterium spp., Vibrio alginolyticus, Photobacterium damselae, Erysipelothrix rhusiopathiae, Vibrio vulnificus, Vibrio parahaemolyticus* and other *vibrios, Vibrio cholerae, Aeromonas spp., Escherichia coli, Staphylococcus aureus, Plesiomonas shigelloides, Clostridium perfringens, Edwardsiella tarda, Listeria monocytogenes, Clostridium botulinum, Campylobacter jejuni, Delftia acidovorans, Legionella pneumophila, Shigella spp.* etc. People mostly get infected due to contact with diseased fish while handling, cleaning aquarium with bare hand, injuries from fishes such as fish bite etc. so this incidence to public can be reduced by taking appropriate treatment for fishes.

*Edwardsiella tarda*, a member of the family *Enterobacteriaceae*, is the causative agent of septicemia in a variety of fish species, being frequently isolated from Japanese flounder Paralichthys olivaceous (Miwa and Nobuhiro, 2000), eels Anguilla japonica, and catfish Ictalurus punctatus. In Brazil, *E. tarda* was isolated from farmed rainbow trout Oncorhynchus mykiss (Alexandrino et al., 1999). Although it is commonly classified as opportunistic, *E. tarda* is considered as a serious pathogen

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of fish and is also important due its zoonotic aspects. Infected fish processed for human consumption is a source of gastroenteritis and meningitis (Janda and Abbot, 1993; Inglis et al., 2001; Muratori et al. 2001).

## MATERIALS AND METHODS

About five samples of diseased goldfishes were collected from local ornamental fish farm in Ernakulam, Kerala. The external symptoms of bacterial infections were recorded and they were dissected and inoculated into nutrient broth. Five bacterial isolates were obtained from the samples by spread plate technique followed by serial dilution. Thereafter biochemical tests were performed for the identification and the DNA of isolates were extracted, followed by PCR amplification were performed. The bacteria isolated from the samples were identified by Sanger's method and challenge study was performed forthe confirmation of pathogen along with that, for treatment, antibiotic sensitivity test was also performed. The toxic activity of the extracellular products (ECPs) from pathogen were assessed byinvitro assay on carp fin cell line.

### Challenge Studies of the Bacterial Isolates

All fishes were maintained in 500 L capacity glass aquarium containing 400 L water. Continued aeration was provided with exchange of 50% of water daily, and temperature of water of the tanks recorded twice daily and ranged from 26 to 29 °C. Before challenge studies, the fishes were acclimatized for 15 d in the laboratory and inoculum were injected intra muscularly into healthy goldfishes (n=10, body weight, 15.5g ± 1.4g) for fulfilling Koch's postulates.

### Inoculum Preparation

A loopful of culture were inoculated into a test tube containing 9 mL nutrient broth and incubated at room temperature for 24 hrs. It was serially diluted and $10^{-5th}$ dilution was transferred to eppendorf tubes and centrifuged at 8000 rpm for 10 minutes.500 µl Phosphate Buffer Saline (PBS) were added after discarding the supernatant and mixed well.

### Inoculation

About 5 fishes were maintained on each five tanks and all of them were examined before inoculation to assure that it is disease free by observing on the gill portion. Each of the bacterial isolate prepared by above manner, was injected both intramuscularly and intra-peritoneally (100 µl each).

Muscular injection- Injection was made just below the dorsal fin base on each side and gently massage the area of injection (50µl each).After injection, fish was immediately transferred to the tank. The above procedure was repeated for control fish by giving PBS.

Intraperitoneal injection- Use a fish net to transfer the fish to the small tank for easy handling.For injections use a fine 1 ml needle. Make sure syringe is free of air-bubbles and load it with inoculum about 100µl Wear glove and Keep fingers in water for some time and gently lift the fish and turn it upside down. Injection is made between the pelvic fin bases. The needle is inserted at a shallow angle to avoid penetrating to the internal organs, until it is just past the body wall. After injection, immediately transfer the fish back to tank. The above procedure repeated for control fish by giving 100 µl PBS.

Observation- Clinical symptoms shown by the fish and death time noted, after then by using sterile forceps and scissors aseptically cut out each organ such as spleen, liver, kidney and gill and they were inoculated to broth separately and incubated at room temperature ($20^0$C-$25^0$C). Serially diluted the incubated broth and from $10^{-7th}$ and $10^{-5th}$ dilutions, about 100µl was added to fresh nutrient agar plate to perform spread plate technique. After incubation, the plates were examined for the bacterial colonies and counted. The isolates obtained from the sample was identified by biochemical tests and molecular methods of identification. Infected fin and muscle tissue of diseased fishes were examined and bacteria were re-isolated after the experimental period. All procedures were carried out aseptically.

### Cytotoxicity Test of Bacterial Extracellular Products-Protein extraction(Culture filtrate method)

Eppendorf tube containing 1000µl nutrient broth were inoculated to test organism and incubated at $28^0$C overnight in a shaking incubator. The broth culture was centrifuged at 8000 rpm for 15 minute at $4^0$C.The supernatant was collected and the pellet was discarded. The supernatant was centrifuged again at 8000 rpm for 10 minute at $4^0$C and pellet was discarded.The supernatant was filtered by using sterile 0.22 µm syringe filter and they were preserved at $-20^0$C.On the other hand, small amounts of these filtrated samples were inoculated into nutrient broth and incubated to examine the presence of live bacterial cells.

**Preparation of Goldfish cells on cover slip for cytotoxicity assay**

Cultures were viewed using an inverted microscope to assess the degree of confluency and to confirm the absence of bacterial and fungal contaminants. Removed spent medium and washed the cell monolayer with PBS without $Ca^{2+}$/ $Mg^{2+}$ using a volume equivalent to half the volume of culture medium. Repeat this wash step for 3-5 times. Pipetted trypsin/EDTA onto the washed cell monolayer using 1ml per 25 $cm^2$ of surface area. Rotated flask to cover the monolayer with trypsin. Decanted the excess trypsin and returned flask to the incubator and leave for 2-10 minutes. Examined the cells using an inverted microscope to ensure that all the cells are detached and floating. The side of the flasks may be gently tapped to release any remaining attached cells. Resuspended the cells in a small volume of fresh serum-containing medium to inactivate the trypsin. Removed 100-200 µl and perform a cell count. Place the sterile cover slip on each well of the multiwell plate by using sterile forceps.Transferred the required number of cells to a well plate, about 200µl and 100µl of protein extract of the sample and added on the surface of the cover slip.Rinsed the contents using a micropipette and 400µl serum was added to each of the well.The sample was kept in low temperature incubator and the plate was examined under inverted microscope on each day.

## RESULTS

**Table 1: Biochemical identification of each bacterial strain isolated from naturally infected fish as per Bergey's Manual of Determinative Bacteriology (1984)**

| CHARACTERS | TEST | GF-1 | GF-2 | GF-3 | GF-4 | GF-5 |
|---|---|---|---|---|---|---|
| **Morphological characteristics** | KOH mount | + | - | + | + | - |
| | Gram staining | Gram -ve bacilli | Gram +ve bacilli | Gram -ve bacilli | Gram -ve bacilli | Gram +ve bacilli |
| | Motility | Non motile | Non motile | motile | Motile | Motile |
| **IMViC reactions** | Indole | + | - | - | - | - |
| | MR | + | + | + | + | + |
| | VP | - | + | - | - | + |
| | Citrate | - | - | - | - | + |
| **Respiratory reaction** | Catalase | - | + | + | + | + |
| | Oxidase | - | + | + | + | - |
| | Urease | + | + | - | - | + |
| | Nitrate reduction | + | + | + | + | + |
| | TSI | K/AG | K/AG | K/AG | A/AG | K/A |
| | Lysine decarboxylase | + | + | - | - | - |
| | Ornithin decarboxylase | + | + | + | + | + |
| | Mannitol | - | - | + | + | + |
| **Fermentation reaction** | Glucose | + ve | AG | +ve | AG | AG |
| | Lactose | - ve | AG | -ve | +ve | -ve |
| | Sucrose | + ve | AG | -ve | +ve | AG |
| | Maltose | + ve | AG | +ve | -ve | AG |
| | Galactose | -ve | AG | -ve | -ve | AG |
| | Rhamnose | -ve | AG | -ve | -ve | -ve |
| | Sorbitol | - ve | AG | -ve | -ve | -ve |
| | Adonitol | +ve | AG | -ve | -ve | -ve |
| | Arabinose | -ve | AG | -ve | +ve | -ve |
| | Fructose | + ve | AG | +ve | +ve | -ve |
| | Raffinose | -ve | AG | -ve | -ve | -ve |
| | Mannose | -ve | AG | +ve | -ve | AG |
| | Salicin | +ve | AG | -ve | +ve | -ve |
| | Dulcitol | - ve | -ve | -ve | -ve | -ve |
| | Inulin | -ve | -ve | -ve | -ve | -ve |
| | Melibios | -ve | AG | -ve | -ve | -ve |
| | Inositol | -ve | AG | -ve | -ve | -ve |
| **Identification** | | ***Edwardsiella*** | ***Micrococcus*** | ***Bacillus*** | ***Aeromonas*** | ***S.aureus*** |

**Table 2: Molecular identification of each Bacterial strain (GF-1to GF-5) by using Basic local alignment search tool (BLAST).**

| Isolates | Organism |
|---|---|
| GF-1 | *Edwardsiella tarda* |
| GF-2 | *Micrococcus luteus* |
| GF-3 | *Bacillus safensis* |
| GF-4 | *Aeromonas caviae* |
| GF-5 | *Staphylococcus aureus* |

**Table 3: Details of the experimental challenge infection of the bacteria isolated from disease goldfish**

| Bacterial strain | Experimentally infected fish | Mortality time (hours) |
|---|---|---|
| ***Micrococcus luteus*** | 1 | No death |
| | 2 | |
| | 3 | |
| | 4 | |
| | 5 | |
| ***Bacillus safensis*** | 1 | No death |
| | 2 | |
| | 3 | |
| | 4 | |
| | 5 | |
| ***Aeromonas caviae*** | 1 | No death |
| | 2 | |
| | 3 | |
| | 4 | |
| | 5 | |
| ***Staphylococcus aureus*** | 1 | No death |
| | 2 | |
| | 3 | |
| | 4 | |
| | 5 | |
| ***Edwardsiella tarda*** | 1 | 15 hours |
| | 2 | 19 hours |
| | 3 | 14 hours |
| | 4 | 13 hours |
| | 5 | 10 hours |

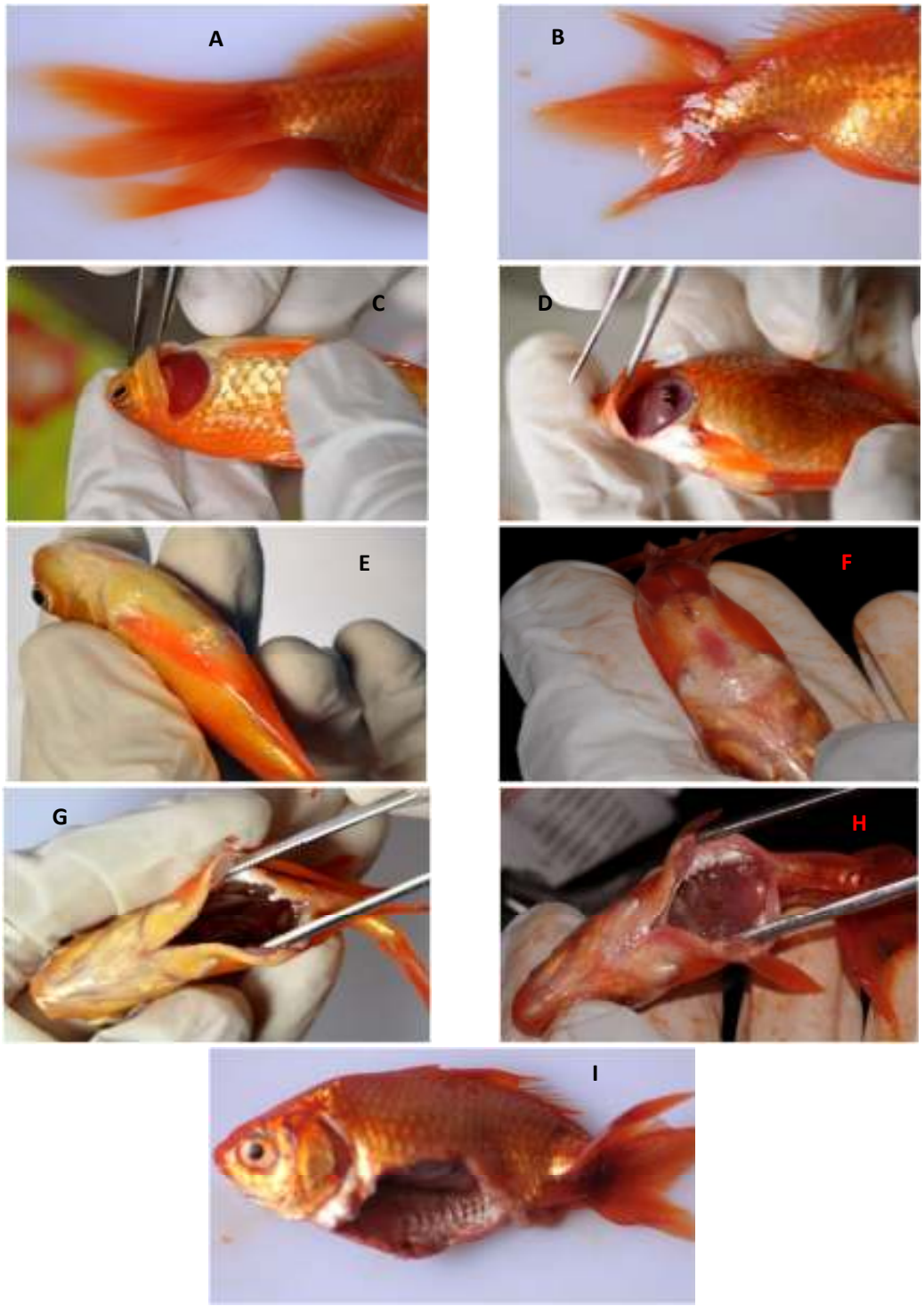


**Figure 1: Comparison of control injected with PBS and experimentally infected Gold fish with *Edeardsiella tarda.* [A) Control fish with no symptom on the caudal fin; B) Experimentally infected goldfish with internal haemorrhage at the caudal fin; C) Control fish with healthy gills; D) Experimentally infected fish with pale coloured gills; E) Healthy fish with no symptom at the base of the pelvic fin; F) Experimentally infected goldfish with haemorrhagic patches at the base of the pelvic fin; G) Control fish with no symptom in the internal organs; H) Experimentally**

**infected goldfish with congested internal organs; I) Black coloured spotted marks on the abdominal wall of experimentally infected goldfish.]**

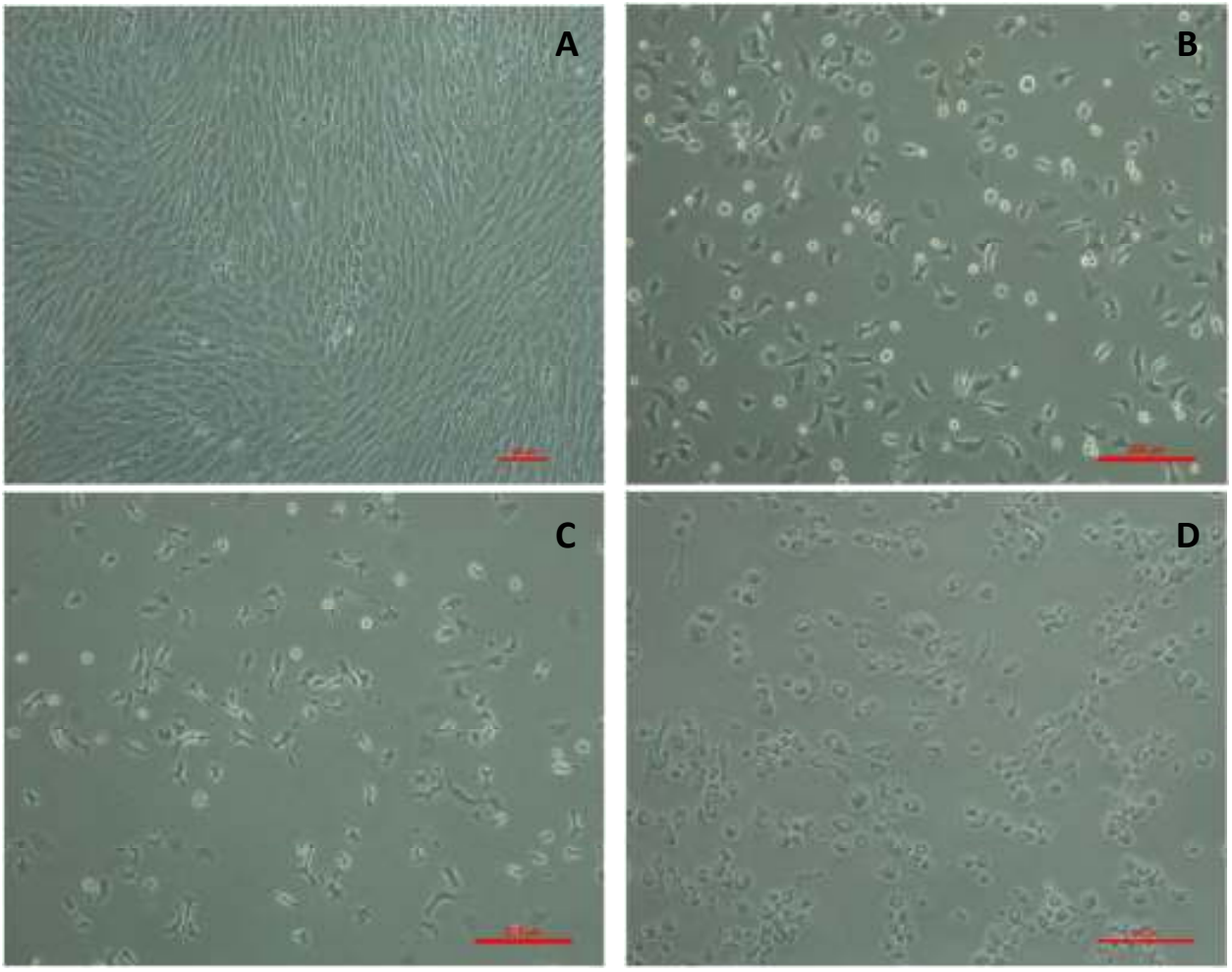


**Figure 2: Cytotoxicity assays of the ECP of *Edwardsiella tarda* on goldfishc ell line. [A) Normal cell line of Gold fish, B) Gold fish cells at 1 d post inoculation to ECP of *Edwardsiella tarda* ,C) 2nd d, D) 3rd d.]**

## DISCUSSION

Sample of diseased fan tail gold fish was collected from local ornamental fish farm in Ernakulam, Kerala shows hemorrhage by macroscopic examination. In this study, from each fish, liver, kidney, gills and spleen were separate and inoculated to nutrient broth. By spread plate technique, after serial dilution, five different isolates were obtained and are marked as GF1, GF2, GF3, GF4, and GF5. Of these, GF1 was appeared in large number. By biochemical test and genome sequencing, the isolates were identified as *Edwardsiella tarda, Micrococcus luteus, Bacillus safensis, Aeromonas caviae* and *Staphylococcus aureus* (Table no 1 and 2). Each of these isolates were already reported as normal flora of fish, but due environmental stresses it can be pathogenic to fishes. Among these isolates, *Edwardsiella tarda*belong to *Enterobacteraceae* family. The members of this family were considered as an indicator of sewage pollution and also these were opportunistic pathogen, usually cause diarrhea in fishes, while they exposed to environmental stress (Rajasekaran, 2008). And it is mainly found in feces of humans, animals and poultry (Wogu M.D. and Maduakol, 2010). According to the literature, *E. tarda* was sensitive to a wide variety of antimicrobials (Janda and Abbot, 1993) and, therefore, nearly any approved drug can be used to combat the infection.

In this study, *Edwardsiella tarda* was identified as pathogen of fishes. *E. tarda* was considered a serious pathogen of fishes. This bacterium was also important due its zoonotic aspects. Infected processed fish for human

consumption was a source of gastroenteritis and meningitis (Janda and Abbot, 1993; Inglis et al., 2001; Muratori et al. 2001). This bacterium was also found to be an intracellular pathogen to a broad range of hosts and causes diseases in fish, amphibians, reptiles, birds and mammals, including humans, throughout the world (Mohanty and Sahoo, 2007; Park et al., 2012) .Of the three recognized species of the genus *Edwardsiella*, only *E. tarda* had been demonstrated to be pathogenic for humans; *E. ictaluri*causes enteric septicemia of catfishes and infects only fish species, whereas *E. hoshinae*infects birds and reptiles. With the rapid developments in aquaculture, *E. tarda* had been recognized as one of the leading pathogens to freshwater and marine cultured fish worldwide. Infection by E. tarda often leads to the development of systematic diseases called Edwardsiellosis, characterized by symptoms of ascites, hernia, exophthalmia, brain and severe lesions of internal organs.

By challenge study, *Edwaedsiella tarda* injected fishes only died along with several symptoms(Table 3). The pathogenicity studies using live bacterial cells indicated that the mortality of fish is dose dependent pattern. $10^{-5}$cells/ml was observed as lethal dose for *Edwaedsiella tarda.*All the dead fish injected with *Edwardsiella tarda* displayed internal hemorrhages showed in Figure 1, comparing with control fish. The lethal dose value of the experimentally infected fish is about $220\times10^{5}$CFU/ml and the median lethal time for mortality was estimated to be 12 hours (Table not shown). After the death, they were examined for the morphological changes. One of the major symptoms was the internal hemorrhage in caudal fin and at the base of the pelvic fin. Additionally gills of the fishes were the primary target of contaminants, where it shows a pale colour(Fig1). Other bacteria such as, *Micrococcus luteus, Aeromonas caviae, Bacillu safensis* and *S .aureus* did not reproduce disease symptoms. The experimental infection assays proved that *Edwardsiella tarda* (GF-1) was virulent to gold fish and pathogenic. Bacterial challenge studies had been employed previously to determine the virulence of isolates viz., *Edwardsiella tarda* in steelhead trout (Amandi *et al.,* 1982), *Yersinia ruckeri* in salmonid fishes (Bullock G L*.,* 1984) and *C. freundii* in common carp *Cyprinus carpio*.Cell culture studies showed that virulent extracellular proteins were also produced by the organism.Extracellular enzymes were reported to be produced by pathogenic and nonpathogenic organisms and were lethal when they produced by pathogenic organism.The ECP from *Edwardsiella tarda* were cytotoxic for the gold fish cell line. Cytotoxic effects were started after 24h of inoculation of ECPs, then the gold fish cells became rounded, having serrated edges, degenerative changes were manifested by pyknotic nuclei, shrinking, dendritic elongations and detached from the surface leading to the destruction of the monolayer by 72 h (Fig.2).

In order to prevent this disease outbreak, treatment should be done as fast possible. *E. tarda* show high sensitive to various antibiotics such as, Oxytetracycline (O30), Cephalexin (CN30),Cefixime (CFM5),Amoxycillin (AMX25), Nitrofurantoin (NIT100), Ampicillin (AMP25), Kanamycin (K30), Gentamycin (GEN30), Furazolidone (FR100), Erythromycin (E15), Azithromycin (AZM30), Furazolidone (FR50), Bacitracin (B10) and Chloramphenicol (C25). According to the literature*, E. tarda* is sensitive to a wide variety of antimicrobials (Janda and Abbot, 1993) and, therefore, nearly any approved drug can be used to combat the infection. Antibacterial agents are mainly administered as supplementary feed for the treatment of diseased fish. However, due to loss of appetite in the severely affected fish, the antibiotics may not be consumed by the fish. So in this study, the adult goldfish were given drugs injection intra muscularly and we found the fish recovered after 5 days of treatment.

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## REFERENCES

Alexandrino A.C., Okumura M.P.M. and Baldassi L., 1999. Ocorrência de infecção por Edwardsiella tarda em truta arco-iris Oncorhynchus mykiss em cultivo intensivo. Bol. Inst. Pesca, **25**:121-123.

Aandi A., Hiu S.F., Rohovee J.S. and Fryer J.L., 1982. Isolation and charachterization of *Edwardsiella tarda* from Chinook salmon (*Onchorhynchus tshawytscha)*. Applied and Environmental Microbiology, **43**:1380-1384.

Bullock G.L., Conroy D.A. and Snieszko S.F., 1971. Bacterial diseases of fishes. Book 2A. Diseases of Fishes. Pp 139. (Eds) Siesziko S F and Axelrod H R. TFH Publications, Neptune, New Jersey.

Bullock G.L., 1984. Enteric Redmouth Disease of Salmonids. US Fish & Wildlife Publications. Paper 128.

Janda J.M. and Abbot S.L., 1993. Infections associated with the genus Edwardsiella: the role of Edwardsiella tarda in human disease. Clin. Infect. Dis., **17**:742-748.

Mohanty B. and Sahoo P., 2007. Edwardsiellosis in fish: a brief review. J. Biosci., **32**:1331–1344.

Miwa S. and Nobuhiro M., 2000. Infection with Edwardsiella tarda causes hypertrophy of liver cells in the Japanese flounder Paralichthys olivaceous. Dis. Aquat. Organ., **42**:227-231.

Novotny L., Dvonska L., Lorencova A., Beran V. and Pavlik L., 2004. Fish: A potential source of bacterial pathogens for human beings. Veterinarni Medicina-Czech, **49**(9):343-358.

Rajasekaran P., 2008. *Enterobacteriaceae* group of organisms in sewage-fed fishes. Advanced Biotech., **8**:12-14.

Subasinghe R.P. and Barg U., 1998. Challenges to health management in Asian aquaculture. Asian Fish. Sci., **11**: 177-193.

Wogu M.D and Maduakol., 2010. Evaluation of microbial spoilage of some aqua cultured fresh fish in Benin City Nigeria. Ethiopian Journal of Environmental Studies and Management, **3**(3): 18-22.