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Outbreak of *Edwardsiella tarda* in Cultured *Pangasius hypophthalmus* (Sauvage, 1878)

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Abstract

Mass mortality and morbidity were reported from cultured *Pangasius hypophthalmus* (Sauvage, 1878) in an aquaculture farm in Kerala, India. Pure bacterial cultures were isolated and identified as *Edwardsiella tarda* from body ulcers and internal organs of moribund *P. hypophthalmus* and from sediments of farms without an outbreak. LD_{50} in *P. hypophthalmus* models of the isolates from diseased fish (1.25×10^5 CFU per fish) and isolates from sediment samples (1.18×10^5 to 3.8×10^5 CFU per fish) were similar. PCR targeting *esaV* gene was unable to discriminate the pathogenic and non-pathogenic *E. tarda.* However, ERIC-PCR followed by dendrogram analysis differentiated isolates based on the presence of *esaV* gene. The isolates that did not carry the *esa*V gene also were found to be virulent in *P. hypophthalmus* fingerlings. Hence it can be assumed that the type III secretory system along with other virulence determinants contributes to virulence by *E. tarda* in fishes.

Keywords: Edwardsiellosis, *Edwardsiella tarda*, *Pangasius hypophthalmus*, pathogenicity, antibiotic susceptibility, ERIC-PCR

Introduction

Pangas catfish, *Pangasius hypophthalmus* (Sauvage, 1878) was first introduced in India from Bangladesh in 1997. It is estimated that the total production of *P. hypophthalmus* in India is 820,000 to 1500,000 tons. Intensification of culture practices for mass *P. hypophthalmus* production has resulted not only increase in the overall production of freshwater fishes in India but also its susceptibility to the infection by various pathogens such as *Edwardsiella tarda* causing enteric septicemia, *Edwardsiella ictaluri* causing bacillary necrosis of pangasius (BNP) and *Aeromonas hydrophila* causing (Red spot diseases) and other parasitic diseases too (Singh & Lakra, 2012). Edwardsiellosis caused by *E. tarda* is a generalized septicemia in fishes, often associated with poor water quality and stress (Plumb, 1999). The organism can cause disease in humans, with symptoms of gastroenteritis and extra-intestinal infection (Janda & Abbott, 1993; Mohanty & Sahoo, 2007). *Edwardsiella* infection has been reported in many cultured fish such as Mullet (Kusuda et al., 1976), Tilapia (Galal et al., 2005), Bloch (Sahoo et al., 2000), Asian catfish (Sahoo et al., 1998), Spotted snakehead (Kumar et al., 2007), Zebra Fish (Pressley et al., 2005), Striped catfish (Shetty et al., 2014). In addition, *E. tarda* can infect reptiles, birds, amphibians and aquatic mammals (Plumb, 1999). Fish affected with *E. tarda* shows abdominal dropsy, enteritis, liver congestion and vent protrusion (Yu et al., 2009).

In this study, *E. tarda* was isolated from diseased *P. hypophthalmus* from an aquaculture farm in Thrissur district, Kerala, India. The pathogenic potential of the bacterial isolates was confirmed by challenge experiment in *P. hypophthalmus* fingerlings. The histopathological changes in the organs of diseased fish were also examined. The distribution of T3SS gene *esa*V in the pathogenic and non-pathogenic isolates of *E. tarda* was investigated by PCR assay. The ERIC-PCR analysis was used to determine the genetic variability of the isolates from diseased fish and sediment samples.

Materials and Methods

A disease outbreak was reported from Pangas catfish, *P. hypophthalmus* in a multi-species farm located in Thrissur District, Kerala, India. Morbid *P. hypophthalmus* with symptoms were collected and

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brought to the laboratory within two hours in sterile sample bags for detailed analysis. Direct swabs from body ulcers and internal organs of the infected fish were taken and the samples were inoculated onto Tryptic soy agar (Becton-Dickinson, USA) and Salmonella-Shigella agar (Becton-Dickinson, USA). After incubation at 30ºC for 18-24 h, transparent small colonies from TSA and small black centred colonies from SS agar were picked and restreaked onto Tryptic soy agar to obtain a pure culture. The isolates were identified using biochemical tests as described by Bergey's manual of systematic bacteriology.

The crude DNA of the isolates was prepared by the method of Cabrera-Garcia et al. (2004). The 16S rRNA sequence of the isolated bacterial strains was amplified by PCR using 27 F and 1544 R primers (Lane, 1991). The PCR was performed in 25 µL reaction; the mixture contained 2.5 µL of 10X PCR buffer (Finnzymes, USA), 200 µM of dNTP mix (Fermentas, USA), 0.4 µM of each primer, 2 µL of the cell lysate and 2U Taq DNA Polymerase (Finnzymes, USA). The PCR conditions were as follows: 94ºC for 3 min followed by 35 cycles of 94°C for 45s, 55°C for 45s, and 70°C for 1.5 min, and a final extension of 72°C for 10 min. The PCR product was analyzed using agarose gel electrophoresis. The PCR product was purified using GenElute gel extraction kit (Sigma, USA). The purified product was sequenced on an ABI PRISM 377 genetic analyzer (Applied Biosystems Inc., Faster city, CA, USA). The sequence was aligned and compared to other bacterial strains using BLAST in NCBI for species identification.

A Duplex PCR method targeting *rpoS* and *esaV* genes were employed as described by Li et al. (2013) with modifications to confirm the species of *Edwardsiella* as well as its pathogenic potential. Along with *E. tarda* isolate from diseased fish, twelve strains previously isolated from sediment samples of various aquaculture farms in Kerala were also used for comparison. PCR was performed in 25 µL reaction volumes containing 2.5 µL of 10X PCR buffer (Finnzymes, USA), 200 µM of dNTP mix (Fermentas, USA), 1U Taq DNA polymerase, 0.2 µM each primer and 2 µL of cell lysate. The cycling conditions were as follows: 94ºC for 3 min followed by 35 cycles of 94ºC for 45s, 60.8ºC for 45s and 68ºC for 1.5 min and a final extension at 72°C for 10 min.The PCR product was analyzed using agarose gel electrophoresis.

Experimental infection was performed as described by Li et al. (2011). Healthy *P. hypophthalmus* fingerlings (weight=10±1.1 g) were maintained in aquarium tanks. The study was conducted in duplicates with test and control groups comprising 20 individual *P. hypophthalmus* fingerlings. The tanks were aerated and water temperature maintained at 24ºC. To determine the virulence, an isolate of *E. tarda* from diseased fish and three isolates from sediment samples were injected 100 µL intramuscularly in fish with concentrations ranging from 10^4 to 10^6 CFU per fish. Duplicates were maintained for each dilution and a control group was injected with normal saline. Fishes were observed daily for signs of disease and mortality for five days. The mean lethal dose (LD_{50}) values were calculated as described by Reed & Muench (1938).

Diseased fishes were sacrificed and samples of gills, liver, kidney and skin lesions from sacrificed were fixed in 10% phosphate-buffered formalin for 24 h, and dehydrated in ethanol. The organs were embedded in paraffin wax and sections were stained with haematoxylin-eosin stain.

Antimicrobial susceptibility testing (AST) was performed for *E. tarda* isolates by disc diffusion method (Bauer et al., 1966) in Mueller Hinton medium (Oxoid, UK). Antimicrobial used in the experiment were Imipenem, Ciprofloxacin, Tobramycin, Moxifloxacin, Ofloxacin, Ceftazidime, Levofloxacin, Norfloxacin, Co-trimoxazole, Colistin, Nalidixic acid, Cefoxitin, Gatifloxacin, Gentamicin, Amikacin, Aztreonam, Ceftriaxone, Cefpodoxime, Nitrofurantoin, Cephalothin, Clindamycin, Erythromycin, Penicillin, Vancomycin, Ampicillin, Chloramphenicol, Oxacillin, Linezolid, Azithromycin, Clarithromycin, Teicoplanin, Methicillin, Amoxyclav, Novobiocin, and Tetracycline. The plates were incubated at 30ºC for 24 h and zones of growth inhibition were compared with the guidelines of CLSI, 2006.

The ERIC-PCR was performed as per the protocol described by Puente-Redondo et al., 2000. The reaction was set in a volume of 25 µl using 1× PCR buffer, 2 mM $MgCl_2$, 125 µM each dNTP, 1.0 µM each primer, 2.5U Taq DNA polymerase (Thermo Scientific). The PCR program consisted of an initial denaturation of 95ºC for 5 min, followed by another 35 amplification cycles of 92ºC for 45 s, 52ºC for 1 min, 70ºC for 10 min and a final extension at 70ºC for 20 min. The amplicons generated by ERIC-PCR were electrophoresed on a 1.5% agarose gel at 90 V

for 4 h. A 100 bp Plus DNA marker (Thermo Scientific, USA) was used as the molecular size standard.

Results and Discussion

The affected fish showed clinical signs such as spiralling movement near the surface of the water, excess mucous secretions over the body surface, ulceration on the ventral surface of mouth and tail, haemorrhages on the body, distended abdomen and perianal oedema (Fig. 1). The affected fish had enteritis with enlargement of liver and kidney and abdominal cavity was filled with serosanguineous fluid. The bacterial isolates were identified as *E. tarda* using the biochemical tests as described by Bergey's manual of systematic bacteriology. The isolates were also confirmed by 16S rRNA analysis. The 16S rRNA sequence of one of the isolates was deposited in GenBank under accession number KP240955. Antibiotic susceptibility testing revealed that the isolates were sensitive to all the antibiotics except nalidixic acid.

Fig. 1. Lateral view image of farmed moribund striped Catfish, *P. hypophthalamus* infected with *E. tarda*. Haemorrhages are seen near the mouth and on the pectoral fin. Sloughing off of skin on the dorsal part of the body is also visible.

The primer set targeting *rpos* gene produced a 223 bp amplicon in all the isolates, which is specific to both *E. tarda* and *E. ictaluri*. A 955bp amplicon targeting *esaV* gene was amplified in the isolate from diseased fish (outbreak) as well as three isolates from sediment sample (1soil93, K1W34, K1W15) (Fig. 2).

The isolates were checked for its pathogenic potential in *P. hypophthalmus* by determination of the LD_{50} values. The *E. tarda* isolate from diseased fish as well as the isolates from sediment samples were

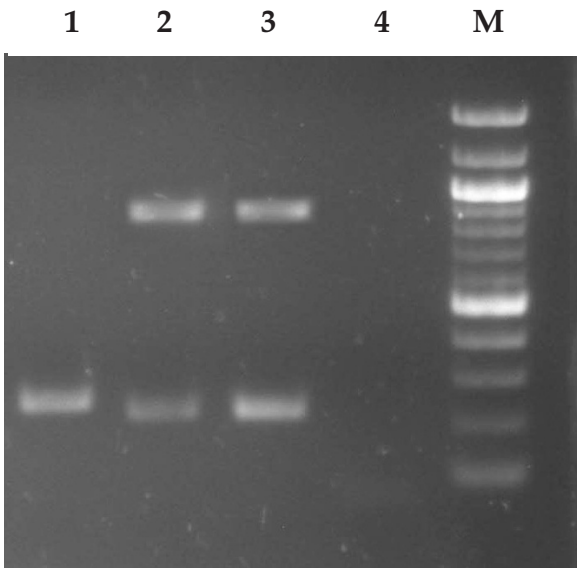

Fig. 2. Duplex PCR for *rpoS and esaV* genes; M is the molecular weight 100bp Plus DNA marker

found to be pathogenic to *P. hypophthalmus*. The LD_{50} of the isolate from diseased fish was found to be 1.25 X 10^5 CFU per fish where as the isolates from sediment samples showed an LD_{50} value ranged from 1.18 X 10^5 to 3.8 X 10^5 CFU per fish. Gross pathological signs of a typical edwardsiellosis were observed in the infected fish which included distended abdomen with sero-sanguineous fluid accumulation in the abdominal cavity, spiralling movement and haemorrhages. *E. tarda* could be re-isolated from the dead and moribund fish.

Histopathological examination of gills revealed diffused haemorrhages on lamellae and necrosis of the epithelial cells (Fig. 3a). Congestion and vacuolar changes in the epithelial cells of the kidney were observed indicating nephritis. Renal tubules showed necrosis with degeneration of tubular epithelial cells (Fig. 3b). Hepatic cells showed diffused macular and vesicular fatty changes. Necrotic cells were observed in the glomeruli (Fig. 3c).

A distinctive ERIC profile was obtained for each isolate of *E. tarda*. ERIC-PCR profiles showed 8–11 bands. The molecular size of the bands ranged from approximately 50 to 2000 bp (Fig. 4a). *E. tarda* ATCC 23685 was included in the ERIC-PCR as a control strain. Genetic fingerprints generated by ERIC-PCR were analyzed using the GelCompar II software (Applied Maths, Kortrijk, Belgium) and a dendrogram was generated via Unweighted Pair Group

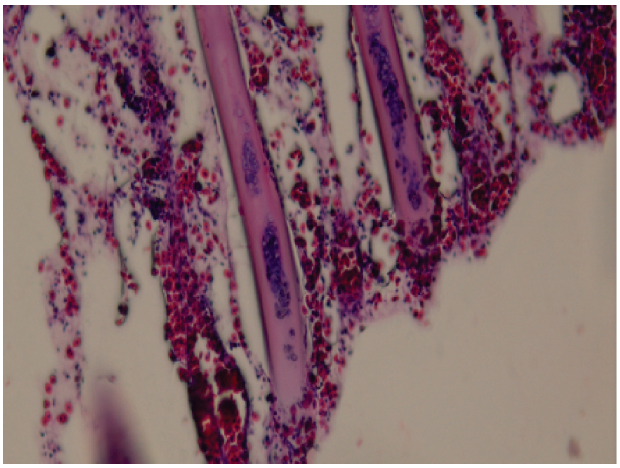

Fig. 3a.Gill section of the infected catfish showing lamellae with diffused hemorrhages

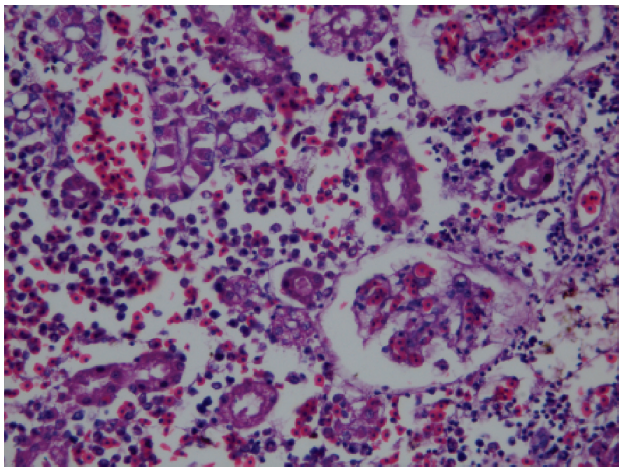

Fig. 3b. Kidney section of infected catfish with diffused congestion and vacuolation of tubular epithelium

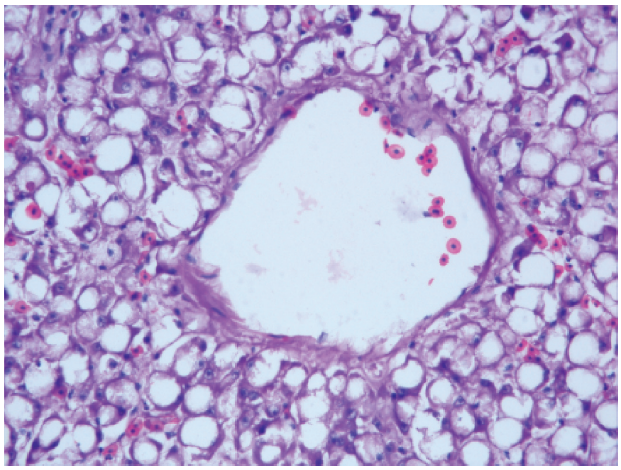

Fig. 3c. Liver section of infected catfish showing diffused macular and vesicular fatty changes

Mathematical Averaging (UPGMA) and Pearson Coefficient. Three major genetic groups for *E. tarda* isolates were obtained with similarity ranging from 75-95% (Fig. 4b). All the isolates carrying virulence gene, *esaV* (Outbreak, 1Soil93, K1W34 and K1W15) formed a single cluster together with *E. tarda* ATCC23685, whereas all the other isolates formed two separate clusters.

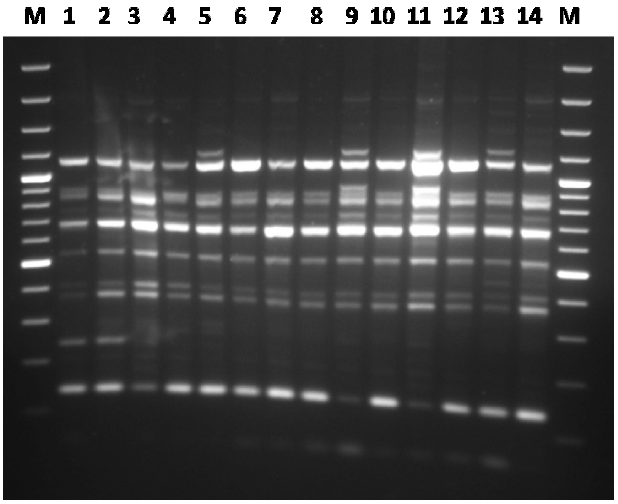

Fig. 4a. BOX PCR Fingerprint of *E. tarda* isolates

M: 100bp Plus DNA marker, Lane1-14: Outbreak strain, ATCC 15947, K1W34, K1W15, 1Soil93, 1Soil4, K2S36, K2W22, 3S454, K3W21, 3S_E.tarda, TF129, TF132, 2TS104

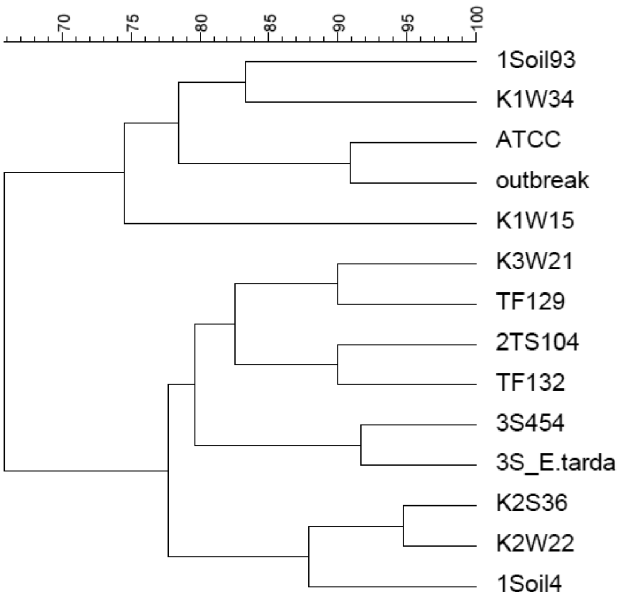

Fig. 4b. Dendrogram of *E. tarda* isolates by GelCompar II software using UPGMA and Pearson Coefficient on the basis of the ERIC-PCR profiles.

E. tarda is a fish pathogen that causes Edwardsiellosis and is characterized by septicemia (Thune et al., 1993). Investigation on the infection in a catfish farm in Thrissur District, Kerala (India) revealed that the causative agent is *E. tarda*. It is the only species in its genus that can cause disease in humans, with signs of gastroenteritis and extra-intestinal infection (Janda & Abbott, 1993). Conventional biochemical test and molecular identification by 16S rRNA sequencing were used to identify the causative bacteria. Pathogenicity of *E. tarda* in their host is a multifactorial process, which involves adhesion, penetration and replication in the host cell and also secretion of several virulent proteins such as hemolysin, collagenase and catalase to survive and adapt in the host (Park et al., 2012). Type III secretion system (T3SS) and type VI secretion system (T6SS) play important roles in the secretion of virulent proteins into the host tissue (Tan et al., 2005; Lan et al., 2007; Wang et al., 2009). The presence of *esa*V gene, a component of T3 SS, in *E. tarda* was effectively used as a marker to assess the pathogenic potential (Li et al., 2011). However in our study, it was observed that *esa*V was present in some pathogenic *E. tarda* isolates, but not all pathogenic isolates possess *esa*V gene. Thus even in the absence of T3SS, the *E. tarda* isolates could be pathogenic to fish. Hence *esa*V gene alone cannot be used as a virulence marker in *E. tarda*. The experimental infectivity study showed that the isolates from diseased fish as well as from sediment samples had almost similar LD_{50} values.

Environmental stresses such as high temperature, high organic content, poor water quality and high stocking density contribute to the onset and severity of *E. tarda* infection in fish (Plumb, 1999; Noga, 2011). Shedding of *E. tarda* by the affected fish into the water before and after death play an important role in spreading of Edwardsiellosis (Matsuoka, 2004). It is difficult to determine and control the underlying stress factors that might have contributed to the onset of infection. Eventhough the farm was also stocked with Catla and Rohu, both these species were unaffected by the disease. Catla and rohu were reported to the susceptible to infection by *E. tarda* (Swain & Nayak, 2003; Devi et al., 2016). The exact mechanism by which Catla and Rohu were resistant to *E. tarda* infection could not be ascertained.

Fish affected by Edwardsiellosis showed gaped mouth and spiralling movement which are due to anaemia of fish leading to oxygen insufficiency (Mohanty & Sahoo, 2007). Edwardsiellosis cause significant production losses in freshwater fishes (Meyer & Bullock, 1973; Castro et al., 2006; Mohanty & Sahoo, 2007). The gross pathology of fish in this study are similar to those reported that include excessive mucus secretion, ulcers on the lateral sides of mouth and on tail, distension of the abdomen, hemorrhagic lesions on the skin, swelling of the anal region (Meyer & Bullock, 1973; Padroset al., 2006; Yu et al., 2009)

In the present study LD_{50} values of *E. tarda* in Pangas catfish ranged from 1.18×10^5 to 3.8×10^5 CUF mL^{-1}. Xiao et al. (2009) reported an LD_{50} that ranged between 3.8×10^3 to 3.2×10^5 CFU per fish for seven isolates of diseased turbots. Immersion challenge of Zebrafish with *E. tarda* had LD_{50} values between 10^4 and 10^5 CFU per fish (Pressley et al., 2005).

Maiti et al. (2009) have reported ERIC-PCR to be most discriminatory in typing of *E. tarda* compared to plasmid profiling and phenotypic analysis. In accordance with the virulence gene-based grouping profile of the isolates, the three environmental isolates with virulence gene clustered in one group along with the isolate from diseased fish and *E. tarda* ATCC 23685. This entire group could be easily distinguished from the environmental isolates without virulence gene.

All Edwardsiella are naturally sensitive to tetracyclines, aminoglycosides, most â-lactam antibiotics, quinolones, antifolates, chloramphenicol, nitrofurantoin and fosfomycin. Studies on the antibiotic resistance in bacteria from shrimp ponds (Tendencia & de la Pena, 2001; Nascimento & Araújo, 2014; Albuquerque et al., 2015) demonstrated a correlation between multiple bacterial antibiotic resistance levels and use of particular drugs. The use of antibiotics will lead to selection pressure for the emergence of multiresistant strains. The absence of antibiotic resistance of the isolates was unexpected since the hatcheries and farmers have a history of using antibiotics.

The present study confirmed that the causative agent for mortality of fishes in the farm in Kerala as *E. tarda* and the isolate was sensitive to most of the antibiotics tested except nalidixic acid. ERIC PCR was able to differentiate the isolates with high discriminatory power. Overall results showed that the isolates from diseased fish and environment

were almost similar in terms of virulence potential. We were not able to ascertain as to why the other species in the pond with infection were unaffected. Since the environment plays a major role in the occurrence of disease, better aquaculture practices will help in controlling the occurrence of the disease.

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Conflicts of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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