Simultaneous Detection of *Aeromonas hydrophila* and *Edwardsiella tarda* by Multiplex PCR in Diseased Catfish

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ABSTRACT

Diseases represent a serious setback in cultured fish by directly affecting production and causing serious economic losses. Early detection and precise identification of pathogens by polymerase chain reaction (PCR) can help in the selection of preventive and early treatment measures and reduce the losses. In this study, a multiplex PCR assay was demonstrated for simultaneous detection of *Edwardsiella tarda* and *Aeromonas hydrophila*. The primer pairs of the major fimbrial subunit (*etfA*) gene from type I fimbrial gene cluster of *E. tarda* (415 bp) and cytotoxic enterotoxin (*act*) gene of *A. hydrophila* (232 bp) were selected as targets. The multiplex PCR using the target genes simultaneously detected *E. tarda* and *A. hydrophila* in different organs (muscle, spleen, liver, and head kidney) of challenged *Pangasius pangasius*. This method was also capable of simultaneously detecting *E. tarda* and *A. hydrophila* in naturally infected catfish, thus indicating mixed bacterial infection in 37.50 % of the diseased population. The results suggested the usefulness of *etfA* and *act* genes for the rapid detection of mixed *A. hydrophila* and *E. tarda* infection in diseased catfish, irrespective of species. The multiplex PCR method can be useful in commercial aquaculture for performing routine disease diagnosis and detection of these bacterial pathogens.

Keywords: Aquaculture, Bacterial infection, Edwardsiellosis, Hemorrhagic septicemia, m-PCR

INTRODUCTION

Catfish farming has gained popularity with an impressive transformation from a highly traditional activity to a diversified commercial activity throughout the world. Bacterial diseases, often occurring as secondary infections following stress, are a serious problem in catfish aquaculture and cause massive mortalities and huge economic losses (Zhou *et al*., 2018; Rathinam *et al*., 2021). Among the bacterial diseases, the incidence of *Edwardsiella tarda* and *Aeromonas hydrophila*, the causative agents of edwardsiellosis and hemorrhagic septicemia, respectively were found to be notably high in a wide range of cultured finfish species, including catfish (Abraham *et al*., 2015; 2022; Paul *et al*., 2015; Hussein *et al*., 2018; Zhou *et al*., 2018; Rathinam *et al*., 2021). Bacterial disease diagnosis based on gross and clinical signs, and histopathological lesions, followed by elaborate laboratory procedures for the isolation and characterization of bacteria are not only time-consuming but also expensive (Sahoo *et al*., 2000). Detection of these pathogens using the traditional culture-based method is technically challenging and requires days to weeks. Considering the severity of these diseases, a more effective and accurate diagnosis is necessary to overcome the clinical consequences and high mortality rates.

Polymerase chain reaction (PCR)-based molecular techniques have been developed to rapidly diagnose diseases in aquaculture (Panangala *et al*., 2007; Lan *et al*., 2008; Chatterjee and Haldar, 2012).

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The detection of pathogens by PCR is highly specific and relatively less time-consuming than the conventional methods. Several PCR assays have been developed for the rapid and accurate diagnosis of diseases in infected fish (Sakai *et al*., 2007; Lan *et al*., 2008; Kingombe *et al*., 2010; Wang *et al*., 2012). However, using a PCR-based assay to detect a single species of pathogen would not be cost-effective, especially when used on a large number of clinical or experimental samples. A multiplex PCR (m-PCR) assay was also demonstrated consequently to simultaneously detect several pathogens at the same time, with different primer sets. Nonetheless, reports of this approach being used on a routine basis to detect fish pathogens by diagnostic laboratories are scanty (Chatterjee and Haldar, 2012; Castro *et al*., 2014). The selected target gene of the target pathogen used in the m-PCR diagnosis to detect fish disease should be stable, widely distributed, and species-specific (Chatterjee and Haldar, 2012). In commercial aquaculture, catfish are often infected with multiple bacterial pathogens (Abraham *et al*., 2022), and a suitable and rapid method is necessary for the correct diagnosis and early treatment. Therefore, the identification of multiple bacterial infections was attempted by developing an m-PCR assay in this study. Previously published species-specific primer pairs of the major fimbrial subunit (*etfA*) gene from type I fimbrial gene cluster of *E. tarda* (Sakai *et al*., 2007) and cytotoxic enterotoxin (*act*) gene of *A. hydrophila* (Kingombe *et al*., 2010) were used to develop and validate an m-PCR assay, as these primer sets have not been tested simultaneously in such assay. Additionally, culture-independent identification of mixed infections caused by *E. tarda* and *A. hydrophila* was also performed using DNA from the kidney tissues of naturally infected catfish.

MATERIALS AND METHODS

Bacterial strains, experimental infection, and culture-independent molecular characterization

Two bacterial pathogenic strains, namely *Edwardsiella tarda* CGH9 (NCBI accession number KX159725) and *Aeromonas hydrophila* $N_{10}P$ (accession number KC914628) from the collections of the Department of Aquatic Animal Health, Faculty of Fishery Sciences, Kolkata were used. The cell suspensions of both strains were prepared separately as described in Adikesavalu *et al*. (2016). For the experimental challenge, eight 500-L fiberglass reinforced plastic (FRP) tanks were first disinfected, cleaned, and dried for a week. The tanks were then filled with 200-L of clean bore-well water and labelled as group C (control) and experimental groups AE, A, and E, in duplicate. Six healthy catfish *Pangasius pangasius* from the acclimatized stock (57.33±6.11 g) were introduced into each of the treatment groups C, AE, A, and E. The tanks were covered with nylon netting for adequate protection. The group AE was intramuscularly administered with 0.1 mL of bacterial suspension adjacent to the dorsal fin. The bacterial suspension was prepared (Adikesavalu *et al*., 2016) by mixing the cell suspensions of *E. tarda* CGH9 ($\approx 2.17\times10^8$ cells·mL^{-1}) and *A. hydrophila* $N_{10}P$ ($\approx 2.30\times10^8$ cells·mL^{-1}) at a 1:1 ratio. Group A was administered with 0.1 mL of *A. hydrophila* $N_{10}P$ cell suspension ($\approx 2.30\times10^8$ cells·mL^{-1}). Similarly, group E was administered with 0.1 mL of *E. tarda* CGH9 cell suspension ($\approx 2.17\times10^8$ cells·mL^{-1}). Group C received 0.1 mL of sterile physiological saline. All fish groups were not fed for a day and maintained in their respective tanks (26-29 ˚C) with continuous aeration, and sampled 24-h post-injection.

Two catfish from each tank of the respective groups were randomly picked, euthanized using clove oil (100 µL·L^{-1} water), rinsed with sterile saline, and carefully dissected for harvesting different organs, viz., muscle, spleen, liver, and head kidney 24-h post-injection. The samples of the respective groups were pooled organ-wise and labelled appropriately. The DNA was extracted using the NucleoSpin tissue genomic DNA isolation kit (Macherey-Nagel, Germany) as per the manufacturer's protocol and the concentration of DNA was quantified for each sample. The m-PCR amplification of the major fimbrial subunit (*etfA*) gene of *E. tarda* (415 bp) and cytotoxic enterotoxin (*act*) gene (232 bp) of *A. hydrophila* was done with the DNA samples (30-50 ng·µL^{-1}) from different organs. The sequences of both primer sets and their amplification size are provided in Table 1. The PCR reaction mixture contained 25 µL 2×PCR TaqMixture (HiMedia,

Table 1. Primer sets of the genes used, their sequences, and amplification size.

Genes	Primer pairs	Product size in bp	Reference
Major fimbrial subunit (*etfA*)	F:5'-CGGTAAAGTTGAGTTTACGGGTG-3' R:5'-TGTAACCGTGTTGGCGTAAG-3'	415	Sakai *et al*. (2007)
Cytotoxic enterotoxin (*act*)	F: 5'-AGAAGGTGACCACCAAGAACA-3' R: 5'-ACTGACATCGGCCTTGAACTC-3'	232	Kingombe *et al*. (2010)

India), 2.0 µL each of forward (10 pMole·µL^{-1}) and reverse (10 pMole·µL^{-1}) primers of *etfA* and *act* genes, 4.0 µL DNA template, and 13 µL molecular biology grade water. Amplification was done by initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 25 s, annealing of primers at 55 °C for 30 s, and extension at 72 °C for 1 min. The final extension was at 72 °C for 5 min. The PCR-amplified *etfA* gene of *E. tarda* and *act* gene of *A. hydrophila* were viewed in the Gel Doc system (G-Box Syngene, UK) and analyzed by comparison with 100-bp molecular weight DNA ladder (Takara Biotech, Japan) on 1.5% agarose gel containing 0.5 µg·mL^{-1} ethidium bromide in 1× Tris-acetate-EDTA (TAE) buffer.

Collection of diseased catfish and bacterial analysis by culture-dependent and culture-independent methods

Diseased juvenile catfish, viz., *Clarias batrachus,* and *C. gariepinus* of size 22-28 g were collected from 20 semi-intensive farms between March and September 2016, and subadult *P. pangasius* of size 200-250 g from four farms between August 2021 and November 2021. The farms were located in North 24 Parganas and South 24 Parganas districts, West Bengal, India. At the farm site, about 60 catfish were examined for diseases on each sampling day as per Office International des Epizooties (OIE) guidelines (OIE, 2013). Morbid catfish with clear clinical signs of disease (n = 10) were brought to the laboratory in oxygen-filled polythene bags and sampled for bacteriology as per Heil (2009). The experimental catfish were euthanized with clove oil (100 µL·L^{-1} water), rinsed with sterile saline, and carefully dissected to expose the kidney. Inocula from the head kidneys were streaked onto the Rimler-Shotts agar (RSA; HiMedia, 2009) and *Edwardsiella ictalurid* agar (EIA; Shotts and Waltman II, 1990) plates and incubated at 30±2 °C for 24-48 h. Simultaneously, a part of the kidney tissues (10-20 mg) from each catfish of the respective lot was collected, pooled, and preserved in 70% alcohol. The extraction of DNA from the head kidney tissues and m-PCR amplification of the *etfA* and *act* genes were performed as described above.

Water samples from the diseased ponds were collected following standard protocol. The water temperature and pH were measured by mercury thermometer and digital pH meter (Waterproof Htestr 10, Oakton), respectively. The dissolved oxygen was determined by Winkler's method (Jhingran *et al*., 1969). The total hardness and alkalinity were determined by following APHA/AWWA/WEF (2012) methods. The levels of nitrite and ammonia were quantified spectrophotometrically as per standard methods (APHA/AWWA/WEF, 2012).

RESULTS AND DISCUSSION

In our preliminary study, we evaluated the effectiveness of nine *Edwardsiella tarda*-specific virulence genes, namely putative killing factor (*mukF*), citrate lyase ligase (*citC*), catalase precursor (*KatB*), type three secretion system (TTSS) regulatory protein (*esrB*; Wang *et al*., 2012), iron-containing superoxide dismutase (*sodB*; Yamada and Wakabayashi, 1999) and type I fimbrial gene cluster consisting of four genes, namely major fimbrial subunit (*etfA*), fimbrial chaperon protein (*etfB*), fimbrial usher protein (*etfC*) and fimbrial subunit (*etfD*) (Sakai *et al*., 2007) to assess and

identify the best virulence gene that can be used to detect *E. tarda* directly from the infected fish. The preliminary results demonstrated that only two of the nine tested *E. tarda* virulence genes, viz., citrate lyase ligase (*citC*), and major fimbrial subunit (*etfA*) were specifically present in all the tested *E. tarda* strains. The remaining tested virulence genes were either not amplified or resulted in non-specific amplification in all tested *E. tarda* strains (Data not shown). Earlier reports suggested that the *etfD* gene from the type I fimbrial gene cluster was specific to *E. tarda* when used in the culture-independent m-PCR assay for the simultaneous detection of *Tenacibaculum maritimum* and *E. tarda* in fish (Castro *et al*., 2014). In our study, the *etfD* gene was not amplified in one of the tested *E. tarda* strains, whereas the *etfA* gene was amplified in all *E. tarda* strains. Therefore, the *etfA* gene from the type I fimbrial gene cluster was selected for this m-PCR assay. The 232-bp region of the cytotoxic enterotoxin (*act*) gene described by Kingombe *et al*. (2010) was used to detect *Aeromonas* spp. The *act* gene plays a multifunctional role by encoding proteins with hemolytic, cytotoxic, and enterotoxin activities (Chopra *et al*., 1993). Detection of the *act* gene region of *Aeromonas* spp. in 70 % of water isolates from the United States and 67 % of clinical isolates from southern Taiwan was documented (Wu *et al*., 2007).

Over the years, several PCR methods based on the amplification of species-specific 16S rRNA genes have been developed for fast and sensitive diagnosis of pathogens in many aquatic animals (Darwish *et al*., 2004; Toranzo *et al*., 2005; Lan *et al*., 2008; Chang *et al*., 2009; Castro *et al*., 2014). In this study, we attempted the amplification of species-specific genes of *Edwardsiella tarda* (*etfA*) and *Aeromonas hydrophila* (*act*) in challenged *Pangasius pangasius*, as well as in diseased catfish for their simultaneous detection. As presented in Table 2, no amplification was observed in any organs of group C (Figure 1a). In group AE, both *etfA* and *act* genes were amplified in all the tested organs of catfish at 24-h post-inoculation (Figure 1b). In group A, the *act* gene of *A. hydrophila* alone was amplified, whereas the *etfA* gene of *E. tarda* was not amplified (Figure 1c). In group E, the *etfA* gene of *E. tarda* was alone amplified, while the *act* gene was not amplified (Figure 1d, Table 2). On EIA, typical *E. tarda* produced 0.5-1.0 mm green translucent colonies after 48-h as described by Shotts and Waltman II (1990). Colonies of *A. hydrophila* were 1.0-2.0 mm in diameter with a typical yellow colour on RSA (HiMedia, 2009). Colonies typical of *E. tarda* and *A. hydrophila* could be isolated on EIA and RSA in different organs of the challenged fish. The observations on bacterial growth were consistent with the results of the m-PCR assay.

Table 2. Presence of major fimbrial subunit (*etfA*) gene and/or cytotoxic enterotoxin (*act*) gene in different organs of *Pangasius pangasius* experimentally injected with *Aeromonas hydrophila* $N_{10}P$ and *Edwardsiella tarda* CGH9.

Organs (n = 2)	Amplification of *etfA* and *act* genes in experimental catfish			
	Group C	Group AE	Group A	Group E
Muscle	-/-	+/+	-/+	+/-
Spleen	-/-	+/+	-/+	+/-
Liver	-/-	+/+	-/+	+/-
Head kidney	-/-	+/+	-/+	+/-

Note: Experimental injections: Group C = sterile 0.85% saline (unchallenged control); Group AE = both *Aeromonas hydrophila* $N_{10}P$ and *Edwardsiella tarda* CGH9; Group A = *A. hydrophila* $N_{10}P$ alone; Group E = *E. tarda* CGH9 alone.

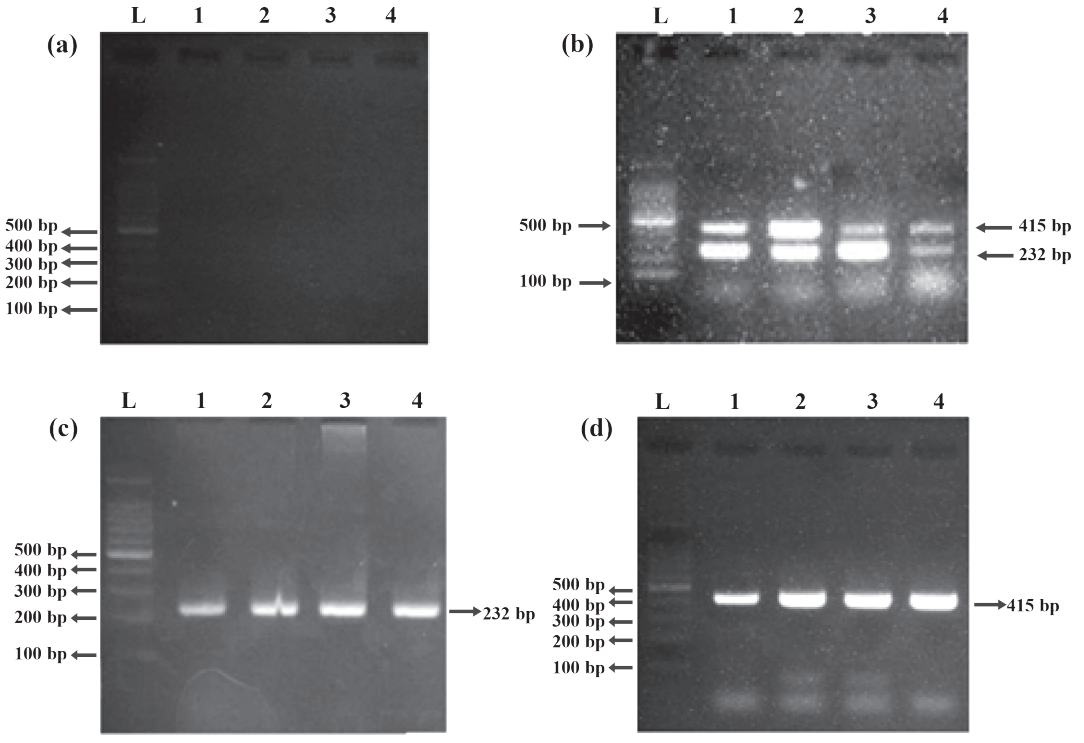

Figure 1. Images of the 1.5% agarose gel showing amplification of *etfA* gene of *Edwardsiella tarda* (415 bp) and *act* gene of *Aeromonas hydrophila* (232 bp) in different organs of *Pangasius pangasius*: (a) injected with sterile 0.85% saline (Group C), (b) injected with both *A. hydrophila* $N_{10}P$ and *E. tarda* CGH9 (Group AE), (c) injected with only *A. hydrophila* $N_{10}P$ (Group A) and (d) injected with only *E. tarda* CGH9 (Group E); L = molecular marker; 1 = muscle; 2 = spleen; 3 = liver; 4 = head kidney.

The naturally infected catfish were listless and exhibited abnormal swimming and spiral movement. The gross and clinical signs varied with the onset of the disease and these included excessive mucus secretion, loss of pigmentation, cutaneous lesions, abdominal swelling, swollen and haemorrhagic anus, and petechial haemorrhages on the fin and skin. The severity of abdominal swelling was more in *Clarias gariepinus*. Abscess development within muscles was noticed in a few cases, which progressed to form large cavities filled with gas and necrotic tissue fragments. Yellowish ascites in the intestine and abdominal cavity, foul smell, discoloured and congested liver, splenomegaly, and liquefied kidney were also observed. The water quality parameters that are regularly monitored in the aquaculture systems include temperature, dissolved oxygen, pH, alkalinity, hardness, ammonia, and nitrites (Boyd and Lichtkoppler, 1982). All these water quality parameters of the diseased ponds were observed to be within the desirable range (Table 3), except for hardness. The total hardness was particularly high in *C. gariepinus* and *P. pangasius* farms. A few *C. gariepinus* farms recorded pH above 9.2. The parameters including pH, alkalinity, and hardness can affect fish growth and survival, and can influence the toxicity of other compounds, such as ammonia (Boyd and Lichtkoppler, 1982; Cline, 2019). Possibly, these stressors might have triggered the onset of diseases in cultured catfish.

The results of the bacterial growth in the kidney samples of diseased catfish were also consistent with the molecular study. Of the 24 diseased catfish samples, 41.67 % and 20.83 % were positive for *act* and *etfA* genes, respectively,

indicating *A. hydrophila* and *E. tarda* infections. On the other hand, both *etfA* and *act* genes were amplified in 37.50 % of the diseased catfish, thus implying mixed infection by *A. hydrophila* and *E. tarda*. The diseased *C. batrachus* lots (n = 2) had only *A. hydrophila* infection, while in *C. gariepinus* and *P. pangasius*, *A. hydrophila* infection, *E. tarda* infection, and mixed infection were noticed (Table 4). The results corroborate our earlier study (Abraham *et al*., 2022). These results, thus, suggest the usefulness of *etfA* and *act* genes for the rapid detection of mixed *A. hydrophila* and *E. tarda* infection in diseased catfish, irrespective of species. Such precise identification of mixed bacterial pathogens by m-PCR assay would help take appropriate management measures in the prevention and early treatment of catfish diseases in aquaculture. Non-specific amplification which could interfere with the detection of the positive band was not observed in our study. Nevertheless, Chang *et al*. (2009) reported a noticeable loss of sensitivity of an m-PCR assay, while detecting *A. hydrophila, E. tarda, Photobacterium damselae* subsp. *piscicida* and *Streptococcus iniae*. Castro *et al*. (2014) described m-PCR to be less sensitive than single PCR because of competition for reaction reagents and the minimal quantity of DNA available, especially if one target organism is present in high numbers than the others. Therefore, the sensitivity of the culture-independent m-PCR assay of the present study should be carefully observed and modified if necessary.

Table 3. Water quality parameters of the diseased catfish farms.

Water quality parameters	***Clarias batrachus*** **(n = 2)**	***Clarias gariepinus*** **(n = 18)**	***Pangasius pangasius*** **(n = 4)**
pH	8.11±0.07	8.21±0.67	8.01±0.13
Temperature (ºC)	28.17±1.12	27.97±2.13	26.18±1.31
Dissolved oxygen ($mg \cdot L^{-1}$)	7.49±0.65	6.40±2.15	7.91±0.54
Ammonia (ppm)	0.11±0.05	0.29±0.15	0.19±0.04
Nitrite (ppm)	0.05±0.03	0.16±0.05	0.11±0.04
Alkalinity (ppm)	129.08±4.02	175.08±64.02	156.08±14.32
Hardness (ppm)	381.55±26.02	486.54±241.07	513.67±86.44

Table 4. Diagnosis and identification of mixed bacterial infection in the kidney of diseased *Clarias batrachus, Clarias gariepinus,* and *Pangasius pangasius*.

Bacterial infection (n = 24)	**Number positive/Number of kidney samples tested (Incidence, %)**			
	Clarias batrachus	***Clarias gariepinus***	***Pangasius pangasius***	**Total**
Aeromonas hydrophila	2/2	7/18	1/4	10/24
(AH) alone	(100.00)	(38.89)	(25.00)	(41.67)
Edwardsiella tarda	0/2	3/18	2/4	5/24
(ET) alone	(0)	(16.67)	(50.00)	(20.83)
Mixed AH and ET	0/2	8/18	1/4	9/24
infection	(0)	(44.44)	(25.00)	(37.50)

CONCLUSION

Our results indicated that the m-PCR method is suitable for the simultaneous detection of *Edwardsiella tarda* and *Aeromonas hydrophila*, and is capable of detecting mixed bacterial infection in naturally infected catfish, irrespective of species. The m-PCR method can be useful for performing routine fish disease diagnosis and early treatment in commercial aquaculture practices.

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