The arrival, establishment and spread of a highly virulent Edwardsiella ictaluri strain in farmed tilapia, Oreochromis spp.

Doan Thi Nhinh[1], Nguyen Thi Huong Giang[2], Kim Van Van[1], Lua Thi Dang[3], Ha Thanh Dong[4], and Truong Dinh Hoai[1]

[1]Vietnam National University of Agriculture
[2]VNU University of Science
[3]Research Institute for Aquaculture No 1 Bac Ninh 16352 Vietnam
[4]Asian Institute of Technology

April 16, 2024

Abstract

Edwardsiella ictaluri is an emerging bacterial pathogen affecting farmed tilapia (*Oreochromis* spp.). This study reports the arrival, establishment and widespread of *E. ictaluri* in farmed tilapia in Vietnam. Among 26 disease outbreaks from 9 provinces in Northern Vietnam during 2019-2021, 19 outbreaks originated from imported stocked seeds while outbreaks in 7 farms were from domestic sources. Clinically sick fish showed appearance of numerous white spots in visceral organs, and accumulative mortalities reached 30-65%. Twenty-six representative bacterial isolates recovered from 26 disease outbreaks were then identified as *E. ictaluri* based on a combination of phenotypic tests, genus- and species-specific PCR assays, *16S rRNA* and *gyrB* sequencing and phylogenetic analysis. All isolates harbored the same virulence gene profile *esrC* $^{+}$, *evpC* $^{+}$, *ureA-C* $^{+}$, *eseI*$^{-}$, *escD*$^{-}$ and *virD4*$^{-}$. Antimicrobial susceptibility tests revealed 80.8-100% isolates were multidrug-resistant, with resistance to 4-8 antimicrobials in the groups of penicillin, macrolide, sulfonamide, amphenicols, and glycopeptide. Experimental challenge successfully induced disease that mimicked natural infection. The median lethal dose (LD_{50}) of the tested isolates (n = 4) were 42 to 61 CFU/fish, indicating their extremely high virulence. This emerging pathogen has already been established and spread to various geographical locations and causing serious impact to farmed tilapia in Northern Vietnam. It is likely that this pathogen will continue to occur through possibly contaminated stocks (both imported and domestic sources) and persist spreading. Thus, increased awareness combined with biosecurity measures and emergent vaccination programs are essential to mitigate the negative impact of this emerging disease on the tilapia farming industry.

Original article

The arrival, establishment and spread of a highly virulent *Edwardsiella ictaluri* strain in farmed tilapia, *Oreochromis* spp.

Doan Thi Nhinh[1,3], Nguyen Thi Huong Giang[2], Kim Van Van[1], Lua Thi Dang[3], Ha Thanh Dong[4], Truong Dinh Hoai[1]*

[1]*Faculty of Fisheries, Vietnam National University of Agriculture, Hanoi 131004, Vietnam*

[2]*Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Hanoi 131004, Vietnam*

[3]*Research Institute for Aquaculture No 1, Bac Ninh 16352, Vietnam*

[4]*Department of Food, Agriculture and Bioresources, School of Environment, Resources & Development (SERD), Asian Institute of Technology (AIT), Klong Luang, Pathumthani, Thailand*

Corresponding author:

Truong Dinh Hoai, Faculty of Fisheries, Vietnam National University of Agriculture, Hanoi, Vietnam

Email: tdhoai@vnua.edu.vn

ORCID: https://orcid.org/0000-0002-2271-849X

SUMMARY

Edwardsiella ictaluri is an emerging bacterial pathogen affecting farmed tilapia (*Oreochromis* spp.). This study reports the arrival, establishment and widespread of *E. ictaluri* in farmed tilapia in Vietnam. Among 26 disease outbreaks from 9 provinces in Northern Vietnam during 2019-2021, 19 outbreaks originated from imported stocked seeds while outbreaks in 7 farms were from domestic sources. Clinically sick fish showed appearance of numerous white spots in visceral organs, and accumulative mortalities reached 30-65%. Twenty-six representative bacterial isolates recovered from 26 disease outbreaks were then identified as *E. ictaluri* based on a combination of phenotypic tests, genus- and species-specific PCR assays, *16S rRNA* and *gyrB* sequencing and phylogenetic analysis. All isolates harbored the same virulence gene profile *esrC* $^{+}$, *evpC* $^{+}$,*ureA-C* $^{+}$, *eseI*$^{-}$,*escD*$^{-}$ and *virD4*$^{-}$. Antimicrobial susceptibility tests revealed 80.8-100% isolates were multidrug-resistant, with resistance to 4-8 antimicrobials in the groups of penicillin, macrolide, sulfonamide, amphenicols, and glycopeptide. Experimental challenge successfully induced disease that mimicked natural infection. The median lethal dose (LD_{50}) of the tested isolates (n = 4) were 42 to 61 CFU/fish, indicating their extremely high virulence. This emerging pathogen has already been established and spread to various geographical locations and causing serious impact to farmed tilapia in Northern Vietnam. It is likely that this pathogen will continue to occur through possibly contaminated stocks (both imported and domestic sources) and persist spreading. Thus, increased awareness combined with biosecurity measures and emergent vaccination programs are essential to mitigate the negative impact of this emerging disease on the tilapia farming industry.

Keywords : *Edwardsiella ictaluri* , outbreaks, tilapia, virulence genes, antimicrobial resistance

INTRODUCTION

The interests in tilapia are increasing in aquaculture because the species is affordable to produce, is an inexpensive source of protein and nutrients and has high tolerance to stress induced by handling, high density condition and a wide range of environmental conditions (Prabu et al., 2019). To date, tilapia is the second most important farmed fish worldwide after the carps, which has been farmed in over 135 countries (FAO, 2018; Prabu et al., 2019). In 2015, global tilapia production was 6.4 million tons with an estimated value of $9.8 billion and a trade value of $1.8 billion (FAO, 2017). Vietnam is among the top 7 tilapia producers in the world (FAO, 2017). The yield of tilapia in Vietnam reached 255,000 tons in 2018 and the production aims to reach 400,000 tons by 2030 (MARD, 2019). Intensive production and growing numbers of tilapia farms have led to the emergence and rapid widespread of infectious diseases which is likely a significant impact to overall tilapia production (Li et al., 2015; Romero et al., 2012).

Edwardsiella ictaluri , a Gram-negative rod-shaped bacterium belonging to the family Enterobacteriaceae, is the causative pathogen of enteric septicemia in channel catfish (ESC) in USA (Hawke et al., 1981). The bacterium is a facultative intracellular pathogen that can survive inside channel catfish phagocytes such as macrophages and neutrophils (Baldwin & Newton, 1993; Waterstrat et al., 1991). So far, the pathogen has been reported to infect other catfish species including walking catfish and hybrid catfish in Thailand (Kasornchandra et al., 1987; Suanyuk et al., 2014), striped catfish in Vietnam, Indonesia and Thailand (Dong et al., 2015; Rogge et al., 2013; Yuasa et al., 2003), yellow catfish in China (Liu et al., 2010) and non-catfish species including zebrafish in USA (Hawke et al., 2013), tilapia in USA (Soto et al., 2012), and wild ayu in Japan (Hassan et al., 2012; Sakai et al., 2008). Naturally disease outbreaks caused 40-90% mortality (Dong et al., 2019; Dung et al., 2004; Iwanowicz et al., 2006) while experimental infection resulted in up to 100% mortality (Dong et al., 2019; Ngoc Phuoc et al., 2020; Plumb & Sanchez, 1983; Sakai et al., 2008), indicating that *E. ictaluri* is a deadly pathogen of multiple freshwater fish species.

Vietnamese catfish industry has suffered from Edwardsiellosis caused by *E. ictaluri* for almost two decades

since the early 2000s (Ferguson et al. (2001). Emergence of the natural cases of *E. ictaluri* in red tilapia in open floating cages in Northern Vietnam in 2016 raised an alarm of widespread of this emerging pathogen in this significantly important industry (Dong et al., 2019). A recent comparative genomic analysis of *E. ictaluri* from different fish hosts revealed four distinct host-specific genotypes, indicating that the *E. ictaluri* strain from tilapia is an emerging, unique genotype (Machimbirike et al., 2021). However, little is known regarding the arrival, establishment and spread of this *E. ictaluri* strain in tilapia aquaculture industry in Vietnam. The present study provides comprehensive information on the establishment and widespread of a highly pathogenic *E. ictaluri* strain in Northern Vietnam and highlights the importance of biosecurity measures, especially for imported stocks to prevent wider spread of this emerging pathogen.

2. MATERIALS AND METHODS

2.1. Disease outbreaks, sampling and bacterial isolation

After the first report of *E. ictaluri* infection in red tilapia (*Oreochromis* sp.) in one Northern province of Vietnam (Dong et al., 2019), similar disease outbreaks with massive mortalities continually occurred in various tilapia farms in 9 provinces in Northern Vietnam during the period from February 2019 to March 2021. Epidemiological investigations and fish sampling were conducted at 26 affected farms (Table 1), including 5 earth-pond farms and 21 cage-culture farms located in 9 provinces in Northern Vietnam (Figure 1).

During the study, the information on the source of fish stocking (imported or domestic), the estimation of fish mortality caused by*E. ictaluri* was obtained by interviewing the farmers. In each affected farm, the water temperature was measured at the time of fish sampling using a water quality meter (YSI Professional Plus, YSI Incorporated, USA). Ten to fifteen diseased fish were collected, placed in sterile sealed plastic bags, and transferred to the laboratory of the Department of Aquatic Environment and Fish Pathology – Faculty of Fisheries, Vietnam National University of Agriculture (VNUA) in a cold box (below 4^{o}C) for microbial analysis. Clinical signs and gross features of all diseased fish were observed and recorded.

Bacteria were isolated from the head kidney, spleen and liver of the affected fish using brain heart infusion agar (BHIA). The plates were then incubated at 28°C for 48 h. Twenty-six representative isolates corresponding to 26 disease outbreaks were selected and preserved in brain heart infusion broth (BHIB) containing 20% glycerol and kept at –80°C for further examinations.

2.2. Biochemical tests

Twenty-six putative *E. ictaluri* isolates (whitish, pinpoint colonies) were cultured on BHIA at 28°C for 48 h and then subjected to biochemical characteristics examination. Gram staining was conducted and bacterial morphology was examined under a light microscope. Oxidase and catalase tests were carried out on all 26 isolates as described by Crumlish et al. (2002). Other biochemical tests were performed using API 20E kit (bioMérieux, France) following the manufacturer's instructions.

2.3. DNA extraction and PCR confirmation of E. ictaluri

Genomic DNA of all bacterial isolates (n = 26) were extracted using InstaGene Matrix kit (Bio-Rad, USA). PCR tests were performed using genus- and species-specific primers targeting Fimbrial gene of *E. ictaluri* (generating 848 and 470 bp amplicon, respectively) as previously described (Sakai et al., 2009) (Table 2). Genomic DNA of*E. ictaluri* LMG7860 from striped catfish (Purchased from BCCM/LMG Bacteria collection) was used as a positive control and nuclease-free water was used as a negative control. PCR reaction mixtures (total 20 µl) included 10 µl of Gotaq Green Master Mix (Promega), 1.5 µl (10 µM) of specific primer (each of forward and reverse), 3 µl DNA template and 4 µl of DNA-free distilled water. The mixtures were then placed in a thermal cycler for amplification under the following conditions: an initial denaturation of 4 min at 94°C; 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 60 s; and a final extension for 7 min at 72°C. The amplified products were then analyzed by electrophoresis on a 1% agarose gel containing Redsafe nucleic acid staining solution (Intron, Korea). The images were captured digitally using a Gel image system (Bio-rad, USA).

2.4. *16S rRNA* and *gyrB* amplification, sequencing and phylogenetic analysis

Nine representative isolates (Ed.HB-02, Ed.TQ-06, Ed.HD-09, Ed.TB-07, Ed.YB-08, Ed.BN-04, Ed.HY-06, Ed.SL-07, and Ed.HNa-02) originated from nine different provinces were chosen for further genetic analysis. The*16S rRNA* and *gyrB* genes were amplified using the universal bacterial primer (27F/1525R, ˜1500 bp) (Weisburg et al., 1991) and *gyrB* primer (1245F/1949R, 1860 bp) (Griffin et al., 2014); and the purified PCR products were submitted for sequencing (Macrogen, South Korea). Bacterial species identification was performed using Blast N search on the GenBank database. The nucleotide sequences of *16S rRNA* and *gyrB* genes of representative *E. ictaluri* isolates in this study and closely related sequences retrieved from GenBank were aligned using ClustalW (Thompson et al., 1994). Phylogenetic trees were then constructed using the neighbor-joining method with bootstrap of 1000 replicates (Saitou & Nei, 1987) by MEGA 10 software (Kumar et al., 2018).

2.5. Detection of putative virulence genes

Amplifications of the 6 virulence genes of *E. ictaluri* including type III secretion system (T3SS), *ersC* , putative T3SS effector*eseI* and its chaperone, *escD* , type IV secretion system (T4SS), *virD4* , type VI secretion system (T6SS), *evpC* and ureA-C genes of the urease operon were performed on all the 26 isolates using specific primers and protocols outlined by Rogge et al. (2013) (Table 2). Nuclease-free water was used as no template control. Thermal conditions were used for each respective primer set as described previously (Rogge et al., 2013) with 1 cycle of 98°C for 30 s, 35 cycles of 98°C for 10 s, 56°C for 30 s and 72°C for 2 min, followed by a final extension of 72°C for 5 min. PCR products were stained and visualized as described above.

2.6. Antimicrobial susceptibility test

The susceptibility of *E. ictaluri* isolates to antibiotics was examined using the disk diffusion method according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2018). Sixteen antibiotics (Oxoid, England) of 11 antibiotic classes/subclasses were tested including two penicillin: oxacillin (Ox, 1 µg) and amoxicillin (Ax, 10 µg); one β-Lactam/β-Lactamase inhibitor combination(BL/BLIs): amoxicillin-clavulanic acid (Ac, 20/10 µg); three cephalosporins: cefotaxime (Ct, 30 µg), cefuroxime (Cu, 30 µg), and ceftriaxone (Cx, 30 µg); one macrolide: erythromycin (Er, 15 µg); one quinolone: nalidixic acid (Na, 30 µg); one sulfonamide: sulfamethoxazole/trimethoprim (ST, 23.75/1.25 µg); one aminoglycosides: neomycin (Ne, 30 µg); one glycopeptide: vancomycin (Va, 30 µg); two fluoroquinolones: ofloxacin (Of, 5 µg) and norfloxacin (No, 10 µg); two tetracyclines: doxycycline (Dx, 30 µg) and oxytetracycline (OTC, 30 µg); and one amphenicol: florfenicol (Fl, 30 µg).

Edwardsiella ictaluri isolates were grown in Mueller Hinton (MH) broth and were adjusted to reach a McFarland turbidity of 0.5. The suspension was then spread onto MH agar by a sterilized cotton swab. Antibiotic discs were placed on the inoculated plates and incubated at 28°C for 48 h. The inhibition zone diameters were recorded and classified as susceptible, intermediate, and resistant according to the standard CLSI (2020) valid for Enterobacterales. In the case of ammoxicillin and neomycin for which CLSI (2020) assessment does not exist, the clinical breakpoints according to the EUCAST standard (EUCAST, 2021) were applied. The multiple antibiotic resistance (MAR) index of the isolates was calculated as described by Krumperman (1983), in which MAR = a/b, where 'a' represents the number of antibiotics to which the isolate was resistant and 'b' represents the total number of antibiotics to which the isolates were exposed for susceptibility testing.

2.7. Challenge experiment

Apparently healthy Nile tilapia juveniles, *O. niloticus*(˜28 g) were obtained from a commercial tilapia hatchery in Northern Vietnam for the challenge experiments. The fish were acclimatized to the experimental conditions for one week before conducting experiments. Ten fish were randomly checked for *E. ictaluri* free status by Gram staining and inspection of spleen and head kidney for the presence of bacteria and by streaking these tissue samples on BHIA plate prior to commencing the experiments. Four representative bacterial

isolates (Ed.HB-02, Ed.HD-09, Ed.YB-08, Ed.TB-07) were chosen for experimental infection. Each isolate was grown in BHIB at 28°C for 36 h. The viable bacterial density of the stock suspension was determined by plate count method. The bacterial density of the stock suspension was then adjusted to approximately 1×10^8 CFU/ml by adding an equivalent volume of PBS buffer. Ten-fold serial dilutions of bacteria from $10^2$to 10^8 CFU/ml were prepared for the virulent test. With each bacterial isolate, fish were divided into eight 100 L groups (15 fish/tank, two replicates). Fish from the 7 groups were intraperitoneally (i.p.) injected with 0.1 ml of the serial bacterial suspensions to reach the respective bacterial concentrations of 10^1 to 10^7 CFU/fish. In control group, the fish were injected with normal saline solution without bacteria. Mortality was observed daily for 2 weeks. Representative moribund and freshly dead fish (n = 3) from each challenge group and apparently healthy fish from control groups (at the end of the experiment, n = 3) were subjected to bacterial re-isolation and histopathological analysis.

2.8. Histopathological examination

Representative moribund tilapia (n = 3) from each challenge group and apparently healthy tilapia (n = 3) from control groups were examined for the histopathological changes. Tissues of affected fish (spleen, kidney, liver and brain) were collected and preserved in buffer formalin 10%. After being fixed for 24 h, sampled tissues were dehydrated in a series of ethanol, embedded in paraffin, and sectioned at 5 µm thickness. The sections were then stained with hematoxylin and eosin (H&E) following the standard histology protocol. The histopathological changes were examined under light microscope equipped with a digital camera.

3. RESULTS

3.1. Disease history and epidemiological factors

During the period from February 2019 to March 2021, 26 tilapia disease outbreaks, located in 9 provinces in Northern Vietnam, were investigated. In tilapia farming systems using earth-pond and floating cage on rivers in Delta region (Hai Duong, Thai Binh, Hung Yen, Bac Ninh and Ha Nam provinces), the disease outbreaks often occurred from July to October, while for the cage culture in reservoirs in mountainous provinces such as Hoa Binh, Yen Bai, and Tuyen Quang and Son La, the outbreaks were from December to March. Water temperature during the disease outbreaks ranged from 23.3 to 29.1ºC. The 26 affected farms included 5 earth pond and 21 floating cage farms, culturing red tilapia (8 farms) and Nile tilapia (18 farms). The tilapia seed were imported (19 farms) and domestic (7 farms). The mortality estimated by farmers during disease outbreaks were ranged within 30-65% (Table 1). In all disease outbreaks, clinically sick fish showed gross signs of pale gills due to anemia; no clearly external symptoms were observed except for darkened body in Nile tilapia and pale color in hybrid red tilapia (at a low frequency). Internally, numerous white spots appeared on the spleen and head kidney, and were occasionally observed in the liver. Hemorrhage or congestion on liver was also recorded at a high frequency.

3.2. Bacterial isolation and identification

Totally, 341 infected fish collected from 26 tilapia farms (10-15 fish/farm) were subjected to bacterial isolation. A typical type of whitish, pinpoint colonies was dominantly recovered from the spleen, kidney and liver of the majority of diseased fish from each farm (Table 1). Twenty-six representative isolates (one per farm) were chosen for further identification. All the isolates were Gram negative, rod-shaped bacteria, oxidase-negative and catalase-positive. Other biochemical features were homogeneous among the 26 isolates and identical to the reference isolates of *E. ictaluri* from red tilapia (Dong et al., 2019) and from striped catfish (Crumlish et al., 2002), except for Voges-Proskauer which was variable amongst isolates (Table 3). The specific PCR results showed that all 26 isolates were positive to both*Edwardsiella* genus and *E. ictaluri* species, evidenced by presence of 848 bp and 470 bp amplicon, respectively (Figure S1).

Partial *16S rRNA* and *gyrB* sequences (1500 bp and 1860 bp, respectively) were successfully amplified and sequenced from all nine representative isolates. Nucleotide sequences of these isolates were deposited in the GenBank database under the accession number of MZ382896 - MZ382904 for *16S rRNA* , and MZ576507 - MZ576515 for *gyrB* . Nucleotide BLAST results revealed that all isolates showed 99.93-100% and 99.41-100%

nucleotide identity to the respective *16S rRNA*and *gyrB* sequences of the reference strains, *E. ictaluri*ATCC 33202 (NR024769), *E. ictaluri* 2234 (MH540086.1) and less than 95% identity to other species in the *Edwardsiella* genus. Phylogenetic analysis for both *16S rRNA* and *gyrB* gene sequences also demonstrated that nine isolates in this study were grouped in the same cluster with other *E. ictaluri* isolates and were separated from other species in the same genus (Figure 2).

Taken together, the results of biochemical tests, genus- and species-specific PCR, sequencing of *16S rRNA* and *gyrB*confirmed that 26 bacterial isolates recovered from disease outbreaks in this study were *E. ictaluri* .

3.3. Detection of virulence genes of E. ictaluri

PCR amplification results for 6 virulence genes revealed that all 26 isolates were *esrC* $^{+}$,*evpC* $^{+}$, and *ureA-C* $^{+}$(Figure 3, Table 4). However, all of these isolates were PCR negative for the three remaining genes (*eseI* $^{-}$,*escD* $^{-}$, and *virD4*$^{-}$) (Table 4).

3.4. Antimicrobial susceptibility

Overall, the *E. ictaluri* isolates were susceptible to the antimicrobials belonging to the classes/subclasses of β-Lactam/β-Lactamase inhibitor combination, Cephems, Tetracyclines and Fluoroquinolones, while resistant to antimicrobials belonging to the groups of Penicillin, Macrolide, Sulfonamide, Amphenicol, and Glycopeptide (Tables 4 and 5). Specifically, more than 80% of the isolates were susceptible to cefotaxime, ceftriaxone, cefuroxime, doxycycline, oxytetracycline, ofloxacin, and norfloxacin; 73.1% of the isolates were also susceptible to the combination of amoxicillin and clavulanate (Tables 4 and 5). However, 80.8 to 100% isolates were resistant to amoxicillin, oxacillin, erythromycin, sulfamethoxazole/trimethoprim, florfenicol and vancomycin. The resistant frequencies of *E. ictaluri* isolates to nalidixic acid and neomycin were 27.0 and 19.2%, respectively (Table 5). The multi-antibiotic resistance values (MAR) of *E. ictaluri* isolates ranged from 0.25 to 0.5 corresponding to 4 to 8 antibiotics or 12 resistant phenotypes (Table S1). The highest frequency of the isolates (34.6%) was observed to resist to 7 tested antimicrobial agents, followed 5 and 6 agents (23% both). The frequencies of the isolates resistant to 2 and 8 drugs were 11.5 and 7.7%, respectively (Table S1).

3.5. Virulence test and histopathology

The experimental challenge using four multi-drugs resistant *E. ictaluri* isolates, Ed.HB-02, Ed.HD-09, Ed.YB-08, Ed.TB-07 (Table 1), resulted in LD_{50} values of 42, 54, 46, and 61 CFU/fish, respectively (Figure 4). Overall, mortality rates were dose-dependent. The fish that received high doses (10^6-10^7 CFU/fish) showed 77-97% mortality within 3 days and reached almost 100% at 5-6 days post infection (dpi). The fish that died at day 3 or earlier showed visceral decay, fluid accumulation in fish abdomen and unclear visceral white spots. All dead fish after 3 dpi exhibited clear white spots in the viscera similar to those of the naturally infected fish collected from ponds/cages (Figure 5). In the groups injected with lower doses (10^1-10^2 CFU/fish), white spots appeared clearly in the spleen, head kidney and posterior kidney after 4-5 dpi and in the liver after 10 dpi. Noticeably, apart from a small number of affected fish with darker color, most infected fish in challenge test showed no obvious external clinical signs. Bacterial isolation from internal organs of the infected fish resulted in dominant pinpoint colonies which were identical to the colony morphology of*E. ictaluri* and tested positive by species-specific assay (figure not shown). No bacteria were recovered from clinically healthy fish of the control groups.

Histopathological manifestation of *E. ictaluri* infected fish was consensus at similar challenged dose amongst four bacterial isolates used in experiments. The lesion exhibited accurately reflected the gross features in affected organs. Severe multifocal necrosis and pyogranulomas were observed in the spleen and kidney (Figure 6 A, C). At high magnification, splenic focal necrosis surrounded by collagenous fibers, infiltration of inflammatory cells, the presence of basophilic rod-shaped bacterial clumps, and pyknosis and karyorrhexis were clearly observed (Figure 6B). Similarly, the kidney of infected fish exhibited pyogranulomas, multifocal necrosis and hyaline droplet accumulation in the kidney tubular epithelium (Figure 6D). The affected livers

showed severe congestion, hepatic lipidosis and tissue degeneration with occasional presence of multifocal necrosis areas (Figure 6E-F). The brain of diseased fish also exhibited severe congestion and inflammations in the primitive meninges and periventricular grey zone of optic tectums (Figure 6 G-H).

4. DISCUSSION

Edwardsiellosis caused by *E. ictaluri* in Nile tilapia (*O. niloticus*) was reported for the first time in Western Hemisphere (Soto *et al.* , 2012) and later in farmed hybrid red tilapia (*Oreochromis* sp.) in Vietnam, a country in Southeast Asia, in 2016 (Dong et al., 2019). Comparative genomic analysis of *E. ictaluri* from different fish hosts revealed that*E. ictaluri* isolates from tilapia is a novel genotype, which differ from currently circulated catfish genotypes (Machimbirike et al., 2021; Reichley et al., 2017). This comprehensive follow-up investigation and findings suggest that this emerging pathogen has been well-established and spread in tilapia farms in Northern Vietnam. Although the introduction (sources of infection) remains inconclusive, the notation of majority of affected farms that used imported stocks for aquaculture with improper diagnostic screening suggests possible foreign introduction of this pathogen. On the other hand, there were a proportion of disease outbreak farms that used domestic stock sources. This implies that this pathogenic *E. ictaluri* may have already circulated domestically from previously unknown introduction to Vietnam (Dong et al., 2019) and continued to spread thereafter through contaminated seeds and/or contaminated water bodies during disease outbreaks. The detection of *E. ictaluri* associated with disease outbreaks from two different continents (America and Asia) highlights the risk of transboundary spread and potential impact to tilapia industry. The countries that rely on imported tilapia stocks for aquaculture like Vietnam may have the same theoretical risk on introduction of this emerging pathogen. Therefore, active surveillance, early diagnostic screening and biosecurity measures are highly recommended for these tilapia farming countries.

The present study also identified some potential risk factors associated with disease outbreaks caused by *E. ictaluri* in tilapia including the influence of temperature, fish size and belated detection due to unclear clinical symptoms. The occurrence of the disease was in cool seasons including autumn (ponds and cage culture on the rivers) and winter time (cage culture on deep lake/ hydropower reservoir) when temperature range within around 23-29°C. This temperature condition is probably suitable for this pathogen in propagating and causing the disease outbreak. The incidence of *E. ictaluri* infections associated with cool seasons in other host species including catfish and non-catfish species were demonstrated in previous reports (Hawke et al., 1998; Pham et al., 2021; Takeuchi et al., 2016). In addition, our survey results revealed that tilapia at the small size ($<$ 350 g) were more susceptible to *E. ictaluri* infection which tends to cause acute death with higher mortality than at adult stages. Farming observation revealed that in cage line raising tilapia culture on the same rivers/lakes, cages raising fish less than 350 g were found to be susceptible and have higher mortality rate than that of other cages raising marketable size fish. The fish stages influenced the susceptibility of infection similar to catfish *E. ictaluri*infection, in which the disease occurs in all ages of catfish, but fingerlings and juvenile were demonstrated to be more susceptible than adult fish (Dung et al., 2008; Hawke et al., 1998). Further works on epidemiology and experimental studies is needed to determine the optimal temperature and influence of ages on the circulating and outbreaks cause of this pathogen in tilapia. Moreover, *E. ictaluri* affected fish did not exhibit recognizable external symptoms, causing misleading presumptive disease diagnostic and untimely treatment efforts.

Virulence factors of pathogenic bacteria may be encoded by particular regions of the prokaryotic genome, termed pathogenicity islands (Hacker et al., 1990). Pathogenicity islands are present in the genomes of pathogenic strains but absent in the genomes of nonpathogenic members of the same or related species (Hacker & Kaper, 2000). Detection of the identical virulence gene profile among 26 isolates from 9 provinces implies the circulation of a homologous strain. The presence of*esrC* , *evpC* , *ureA-C* genes, which are well-known for enabling the bacteria to survive and replicate intracellularly (Booth et al., 2006; Chen et al., 2017; Hu et al., 2014; Moore et al., 2002; Rogge & Thune, 2011), in all identified isolates in the present study suggests their potential virulence and difficulty in management using antimicrobials. While *virD* , *eseI* , and its chaperone*escD* genes were present in the US channel catfish (*Ictalurus punctatus*) isolates, they were absent from all 26 isolates identified in this study as well as from isolates collected from striped catfish

(*Pangasianodon hypophthalmus*) in Vietnam (Rogge et al., 2013) and from tilapia in Western Hemisphere (Griffin et al., 2016). Our findings support previous studies that the variability in virulence genes of *E. ictaluri* is in relation to host fish species and geographic origins (Griffin et al., 2016).

Although disease caused by *E. ictaluri* was first detected and affected farmed tilapia in Vietnam only few years ago, their high levels of antibiotic resistance pose potential risks and thus emergent action is needed to mitigate the disease outbreak and spread of this pathogen. High resistance frequencies of this bacterium to various antimicrobials belonging to several classes/subclasses might be the consequence of inappropriate usage of these drugs for disease control in tilapia farms, leading to 12 multi-drugs resistance phenotypes. Of note, these AMR isolates may have also been introduced to Vietnam elsewhere and continue to spread and acquire more resistance. Some resistances were likely intrinsic resistance. Specifically, almost 85% of the *E. ictaluri* isolates from tilapia were resistant to erythromycin which is consistent with the previous report on the intrinsic resistance of*Edwardsiella* species to macrolide (Stock & Wiedemann, 2001). The intrinsic resistance of Gram negative bacteria and specifically of*Edwardsiella* species to glycopeptide is also widely revealed (Breijyeh et al., 2020; Stock & Wiedemann, 2001) and was reaffirmed by the present result of extremely high frequency of *E. ictaluri*resistant to vancomycin. However, some resistances were likely resulted from misuse of antimicrobials. For example, florfenicol was highly effective in the control of *E. ictaluri* infection in catfish (Gaunt et al., 2015), but all *E. ictaluri* isolates from tilapia in present study were resistant to this drug. Similarly, high resistant frequencies were also recorded with two other approved antibiotics used in aquaculture in Vietnam, namely, amoxicillin (80.8%) and sulfamethoxazole/trimethoprim (100%). Nevertheless, alternatives to antibiotics such as vaccines, probiotics, and bioactive metabolites should be further explored to tackle this emerging, pathogenic, multi-drug resistant bacterium.

The multidrug resistant *E. ictaluri* isolates in this study were extremely highly virulent. In comparison with previous studies where the LD_{50} was 3.2×10^4 CFU/ml in yellow catfish (Kim & Park, 2015) and 5.1×10^4 CFU/ml in Nile tilapia (Soto et al., 2013), *E. ictaluri* isolates in this study required only 42 to 61 CFU/fish to kill 50% tilapia population. At higher dose, the results were similar to that reported by Dong et al. (2019) when fish challenged with 10^5-10^7 CFU/fish resulted in 95-100% mortality within 9 days post infection. Gross signs and histopathological manifestation were similar to that of previous studies that showed severe tissue destructions, especially in the spleen and head kidney, two major lymphoid organs playing important roles in defense against infection. The failure of these organs may explain the high mortality in experimental challenged fish (Dong et al., 2019; Soto et al., 2013). Since our investigation suggested homologous strains of the collected *E. ictaluri* isolates, an autogenous vaccine might be the best option to combat this emerging disease in present time before a better vaccine candidate for a wider region is discovered.

In conclusion, this follow-up investigation from our previous case report highlights the establishment and widespread of extremely high virulent, multidrug resistant *E. ictaluri* isolates as an emerging threat for tilapia farming industry in Vietnam. The arrivals of this pathogen were likely involving both imported and domestic stocks. Therefore, increased awareness, early diagnostic testing and biosecurity measures at both national and international levels are needed to prevent its transboundary spread and negative impact to the tilapia industry at large.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Saengchan Senapin (National Center for Genetic Engineering and Biotechnology – BIOTEC, Pathum Thani, Thailand) for her valuable comments on the manuscript. This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106.05-2020.18

CONFLICT OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. Ethic approval for the challenged experiments was obtained from Faculty of Fisheries, Vietnam National University of Agriculture Animal Care and Use committee FFVNUA-ACUC, approval number: 15620-1-KHCN-FFVNUA.

REFERENCES

Baldwin, T. J., & Newton, J. C. (1993). Pathogenesis of enteric septicemia of channel catfish, caused by*Edwardsiella ictaluri* : bacteriologic and light and electron microscopic findings. *Journal of Aquatic Animal Health, 5* (3), 189-198. doi:10.1577/15488667(1993)005<0189:POESOC>2.3.CO;2

Booth, N. J., Elkamel, A., & Thune, R. L. (2006). Intracellular replication of *Edwardsiella ictaluri*in channel catfish macrophages. *Journal of Aquatic Animal Health, 18*(2), 101-108. doi:*10.1577/H05-025.1*

Breijyeh, Z., Jubeh, B., & Karaman, R. (2020). Resistance of Gram-negative bacteria to current antibacterial agents and approaches to resolve it. *Molecules, 25* (6), 1340. doi:10.3390/molecules25061340

Chen, H., Yang, D., Han, F., Tan, J., Zhang, L., Xiao, J., Zhang, Y., & Liu, Q. (2017). The bacterial T6SS effector EvpP prevents NLRP3 inflammasome activation by inhibiting the Ca2+-dependent MAPK-Jnk pathway. *Cell Host & Microbe, 21* (1), 47-58. doi:10.1016/j.chom.2016.12.004

CLSI (2018*). Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals* . 3rd Edition. CLSI document M31-A3. Wayne, PA: Clinical Laboratory Standards Institute

CLSI. (2020). *Performance Standards for Antimicrobial Susceptibility Testing* . 30th Edition. CLSI supplement M100. Wayne, PA: Clinical Laboratory Standards Institute.

Crumlish, M., Dung, T., Turnbull, J., Ngoc, N., & Ferguson, H. (2002). Identification of *Edwardsiella ictaluri* from diseased freshwater catfish, *Pangasius hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam.*Journal of Fish Diseases, 25* (12), 733-736.

Dong, H., Senapin, S., Jeamkunakorn, C., Nguyen, V., Nguyen, N., Rodkhum, C., Khunrae, P., & Rattanarojpong, T. (2019). Natural occurrence of edwardsiellosis caused by*Edwardsiella ictaluri* in farmed hybrid red tilapia*(Oreochromi* s sp.) in Southeast Asia. *Aquaculture, 499* , 17-23. doi:10.1016/j.aquaculture.2018.09.007

Dong, H. T., Nguyen, V. V., Phiwsaiya, K., Gangnonngiw, W., Withyachumnarnkul, B., Rodkhum, C., & Senapin, S. (2015). Concurrent infections of *Flavobacterium columnare* and*Edwardsiella ictaluri* in striped catfish, *Pangasianodon hypophthalmus* in Thailand. *Aquaculture, 448* , 142-150. doi:10.1016/j.aquaculture.2015.05.046

Dung, T., Crumlish, M., Ngoc, N., Thinh, N., & Thy, D. (2004). Investigate the disease caused by the genus *Edwardsiella* from Tra catfish (*Pangasianodon hypophthalmus*). *Journal of Science, Can Tho University, 1* , 23-31.

Dung, T. T., Haesebrouck, F., Tuan, N. A., Sorgeloos, P., Baele, M., & Decostere, A. (2008). Antimicrobial susceptibility pattern of *Edwardsiella ictaluri* isolates from natural outbreaks of bacillary necrosis of *Pangasianodon hypophthalmus* in Vietnam. *Microbial Drug Resistance, 14* (4), 311-316. doi:10.1089/mdr.2008.0848

EUCAST. (2021). *The European Committee on Antimicrobial Susceptibility Testing* . Breakpoint tables for interpretation of MICs and zone diameters. Version 11.0, 2021. Retrieved from https://eucast.org/

FAO. (2017). Outbreaks of Tilapia lake virus (TiLV) threaten the livelihoods and food security of millions of people dependent on tilapia farming. Rome: Italy. Retrieved from http://www.fao.org/documents/card/en/c/3ce1da5b-1529-4e7c-8b88-7adfef8d138c/

FAO. (2018). The state of world fisheries and aquaculture 2018.*Meeting the Sustainable Development Goals.* Rome. Licence: CC BY-NC-SA 3.0 IGO.

Ferguson, H., Turnbull, J., Shinn, A., Thompson, K., Dung, T. T., & Crumlish, M. (2001). Bacillary necrosis in farmed *Pangasius hypophthalmus* (Sauvage) from the Mekong Delta, Vietnam. *Journal of Fish Diseases, 24* (9), 509-513. doi:10.1046/j.13652761.2001.00308.x

Gaunt, P. S., Chatakondi, N., Gao, D., & Endris, R. (2015). Efficacy of florfenicol for control of mortality associated with *Edwardsiella ictaluri* in three species of catfish. *Journal of Aquatic Animal Health, 27* (1), 45-49. doi:10.1080/08997659.2014.976672

Griffin, M., Reichley, S., Greenway, T., Quiniou, S., Ware, C., Gao, D., Gaunt, P., Yanong, R., Pouder, D., & Hawke, J. (2016). Comparison of *Edwardsiella ictaluri* isolates from different hosts and geographic origins. *Journal of Fish Diseases, 39* (8), 947-969. doi:10.1111/jfd.12431

Griffin, M. J., Ware, C., Quiniou, S. M., Steadman, J. M., Gaunt, P. S., Khoo, L. H., & Soto, E. (2014).*Edwardsiella piscicida* identified in the southeastern USA by gyrB sequence, species-specific and repetitive sequence-mediated PCR.*Diseases of Aquatic Organisms, 108* (1), 23-35. doi:*10.3354/dao02687*

Hacker, J., Bender, L., Ott, M., Wingender, J., Lund, B., Marre, R., & Goebel, W. (1990). Deletions of chromosomal regions coding for fimbriae and hemolysins occur in vitro and in vivo in various extra intestinal *Escherichia coli*isolates. *Microbial Pathogenesis, 8* (3), 213-225. doi:10.1016/0882-4010(90)90048-U

Hacker, J., & Kaper, J. B. (2000). Pathogenicity islands and the evolution of microbes. *Annual Reviews in Microbiology, 54* (1), 641-679. doi:10.1146/annurev.micro.54.1.641

Hassan, E. S., Mahmoud, M. M., Kawato, Y., Nagai, T., Kawaguchi, O., Iida, Y., Yuasa, K., & Nakai, T. (2012). Subclinical *Edwardsiella ictaluri* infection of wild ayu*Plecoglossus altivelis* . *Fish Pathology, 47* (2), 64-73. doi:10.3147/jsfp.47.64

Hawke, J. P., Durborow, R., Thune, R., & Camus, A. (1998). Enteric septicemia of catfish. *SRAC Publication* (477).

Hawke, J. P., Kent, M., Rogge, M., Baumgartner, W., Wiles, J., Shelley, J., Savolainen, L. C., Wagner, R., Murray, K., & Peterson, T. S. (2013). Edwardsiellosis caused by*Edwardsiella ictaluri* in laboratory populations of zebrafish*Danio rerio* . *Journal of Aquatic Animal Health, 25* (3), 171-183. doi:10.1080/08997659.2013.782226

Hawke, J. P., McWhorter, A. C., Steigerwalt, A. G., & Brenner, D. J. (1981). *Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicemia of catfish. *International Journal of Systematic and Evolutionary Microbiology, 31* (4), 396-400. doi:10.1099/00207713-31-4-396

Hu, W., Anand, G., Sivaraman, J., Leung, K. Y., & Mok, Y.-K. (2014). A disordered region in the EvpP protein from the type VI secretion system of *Edwardsiella tarda*is essential for EvpC binding. *PloS One, 9* (11), e110810. doi:10.1371/journal.pone.0110810

Iwanowicz, L. R., Griffin, A. R., Cartwright, D. D., & Blazer, V. S. (2006). Mortality and pathology in brown bullheads *Amieurus nebulosus* associated with a spontaneous*Edwardsiella ictaluri* outbreak under tank culture conditions.*Diseases of Aquatic Organisms, 70* (3), 219-225. doi:10.3354/dao070219

Kasornchandra, J., Rogers, W., & Plumb, J. (1987). *Edwardsiella ictaluri* from walking catfish,*Clarias batrachus* L., in Thailand. *Journal of Fish Diseases, 10* (2), 137-138. doi:10.1111/j.1365-2761.1987.tb00729.x

Kim, J. D., & Park, S. W. (2015).*Edwardsiella ictaluri* infection in cultured yellow catfish*Pelteobagrus fulvidraco* fingerlings in Korea. *Korean Journal of Fisheries and Aquatic Sciences, 48* (5), 725-730. doi:*10.5657/KFAS.2015.0725*

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution, 35* (6), 1547-1549.

doi:10.1093/molbev/msw054

Li, L., Wang, R., Liang, W., Huang, T., Huang, Y., Luo, F., Lei, A., Chen, M., & Gan, X. (2015). Development of live attenuated *Streptococcus agalactiae* vaccine for tilapia via continuous passage in vitro. *Fish & Shellfish Immunology, 45* (2), 955-963. doi:10.1016/j.fsi.2015.06.014

Liu, J. Y., Li, A. H., Zhou, D. R., Wen, Z. R., & Ye, X. P. (2010). Isolation and characterization of*Edwardsiella ictaluri* strains as pathogens from diseased yellow catfish *Pelteobagrus fulvidraco* (Richardson) cultured in China.*Aquaculture Research, 41* (12), 1835-1844. doi:10.1111/j.1365-2109.2010.02571.x

Machimbirike, V. I., Uthaipaisanwong, P., Khunrae, P., Dong, H. T., Senapin, S., Rattanarojpong, T., & Sutheeworapong, S. (2021). Comparative genomics of *Edwardsiella ictaluri* revealed four distinct host-specific genotypes and thirteen potential vaccine candidates. *Genomics, 113* (4), 1976-1987. doi:10.1016/j.ygeno.2021.04.016

MARD. (2019). *Decision to approve the plan of tilapia farming development by 2020, driven by 2030* . Issued on May, 6th 2016 by the Ministry of Agriculture and Rural Development, Vietnam (MARD).

Moore, M. M., Fernandez, D. L., & Thune, R. L. (2002). Cloning and characterization of *Edwardsiella ictaluri* proteins expressed and recognized by the channel catfish*Ictalurus punctatus* immune response during infection.*Diseases of Aquatic Organisms, 52* (2), 93-107. doi:*10.3354/dao052093*

Ngoc Phuoc, N., Richards, R., & Crumlish, M. (2020). Establishing bacterial infectivity models in striped Catfish *Pangasianodon hypophthalmus* (Sauvage) with*Edwardsiella ictaluri* . *Journal of Fish Diseases, 43* (3), 371-378. doi:10.1111/jfd.13135

Pham, K. D., Nguyen, S. V., Ødegård, J., Gjøen, H. M., & Klemetsdal, G. (2021). Case study development of a challenge test against *Edwardsiella ictaluri* in Mekong striped catfish (*Pangasianodon hypophthalmus)* , for use in breeding: Estimates of the genetic correlation between susceptibilities in replicated tanks. *Journal of Fish Diseases, 44* (5), 553-561. doi:*10.1111/jfd.13292*

Plumb, J., & Sanchez, D. (1983). Susceptibility of five species of fish to *Edwardsiella ictaluri* .*Journal of Fish Diseases, 6* (3), 261-266. doi:10.1111/j.1365-2761.1983.tb00075.x

Prabu, E., Rajagopalsamy, C., Ahilan, B., Jeevagan, I. J. M. A., & Renuhadevi, M. (2019). Tilapia - an excellent candidate species for world aquaculture: a review.*Annual Research & Review in Biology* , 1-14. doi:10.9734/arrb/2019/v31i330052

Reichley, S. R., Waldbieser, G. C., Soto, E., Lawrence, M. L., & Griffin, M. J. (2017). Complete genome sequence of *Edwardsiella ictaluri* isolate RUSVM-1 recovered from Nile tilapia (*Oreochromis niloticus*) in the Western Hemisphere.*Genome Announcements, 5* (24), e00390-00317. doi:*10.1128/genomeA.00390-17*

Rogge, M. L., Dubytska, L., Jung, T. S., Wiles, J., Elkamel, A. A., Rennhoff, A., Oanh, D. T. H., & Thune, R. L. (2013). Comparison of Vietnamese and US isolates of*Edwardsiella ictaluri* . *Diseases of Aquatic Organisms, 106* (1), 17-29. doi:*10.3354/dao02620*

Rogge, M. L., & Thune, R. L. (2011). Regulation of the *Edwardsiella ictaluri* type III secretion system by pH and phosphate concentration through EsrA, EsrB, and EsrC.*Applied and Environmental Microbiology, 77* (13), 4293-4302. doi:*10.1128/AEM.00195-11*

Romero, J., Feijoó, C. G., & Navarrete, P. (2012). *Antibiotics in aquaculture–use, abuse and alternatives* (Vol. 159). Croatia: InTech.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4* (4), 406-425. doi:10.1093/oxfordjournals.molbev.a040454

Sakai, T., Kamaishi, T., Sano, M., Tensha, K., Arima, T., Iida, Y., Nagai, T., Nakai, T., & Iida, T. (2008). Outbreaks of *Edwardsiella ictaluri* infection in ayu*Plecoglossus altivelis* in Japanese rivers. *Fish Pathology, 43* (4), 152-157. doi:10.3147/jsfp.43.152

Sakai, T., Yuasa, K., Sano, M., & Iida, T. (2009). Identification of *Edwardsiella ictaluri* and*E. tarda* by species-specific polymerase chain reaction targeted to the upstream region of the fimbrial gene. *Journal of Aquatic Animal Health, 21* (2), 124-132. doi:10.1577/H08-061.1

Soto, E., Griffin, M., Arauz, M., Riofrio, A., Martinez, A., & Cabrejos, M. E. (2012). *Edwardsiella ictaluri* as the causative agent of mortality in cultured Nile tilapia.*Journal of Aquatic Animal Health, 24* (2), 81-90. doi:10.1080/08997659.2012.675931

Soto, E., Illanes, O., Revan, F., Griffin, M., & Riofrio, A. (2013). Bacterial distribution and tissue targets following experimental *Edwardsiella ictaluri* infection in Nile tilapia *Oreochromis niloticus* . *Diseases of Aquatic Organisms, 104* (2), 105-112. doi:*10.3354/dao02593*

Stock, I., & Wiedemann, B. (2001). Natural antibiotic susceptibilities of *Edwardsiella tarda, E. ictaluri,* and *E. hoshinae* . *Antimicrobial Agents and Chemotherapy, 45* (8), 2245-2255. doi:*10.1128/AAC.45.8.2245-2255.2001*

Suanyuk, N., Rogge, M., Thune, R., Watthanaphiromsakul, M., Champhat, N., & Wiangkum, W. (2014). Mortality and pathology of hybrid catfish, *Clarias macrocephalus* (Günther)×*Clarias gariepinus* (Burchell), associated with *Edwardsiella ictaluri* infection in southern Thailand. *Journal of Fish Diseases, 37* (4), 385-395. doi:10.1111/jfd.12127

Takeuchi, H., Hiratsuka, M., Oinuma, H., Umino, Y., Nakano, D., Iwadare, M., Tomono, R., Hori, K., Imai, T., & Ishikawa, T. (2016). Infection status of ayu and other wild fish with*Flavobacterium psychrophilum* and *Edwardsiella ictaluri* in the Tama River, Japan. *Fish Pathology, 51* (4), 184-193. doi:10.3147/jsfp.51.184

Waterstrat, P., Ainsworth, A., & Capley, G. (1991). In vitro responses of channel catfish,*Ictalurus punctatus* , neutrophils to *Edwardsiella ictaluri* .*Developmental & Comparative Immunology, 15* (1-2), 53-63. doi:*10.1016/0145-305X(91)90047-3*

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology, 173* (2), 697-703. doi:*10.1128/jb.173.2.697-703.1991*

Yuasa, K., Kholidin, E. B., Panigoro, N., & Hatai, K. (2003). First isolation of *Edwardsiella ictaluri* from cultured striped catfish*Pangasius hypophthalmus* in Indonesia. *Fish Pathology, 38* (4), 181-183. doi:10.3147/jsfp.38.181

Hosted file

`3. Figure edited final.docx` available at https://authorea.com/users/444851/articles/712022-the-arrival-establishment-and-spread-of-a-highly-virulent-edwardsiella-ictaluri-strain-in-farmed-tilapia-oreochromis-spp

Hosted file

`4. Table Fixed final.docx` available at https://authorea.com/users/444851/articles/712022-the-arrival-establishment-and-spread-of-a-highly-virulent-edwardsiella-ictaluri-strain-in-farmed-tilapia-oreochromis-spp