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Identification and characterization of *Edwardsiella ictaluri* from diseased *Pangasius pangasius*, cultured in Cirata Lake, Indonesia

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Abstract. *Mawardi M, Jaelani, Zainun Z, Mundayana Y, Chilora BS, Hardi EH. 2018. Identification and characterization of* Edwardsiella ictaluri *from diseased* Pangasius pangasius*, cultured in Cirata Lake, Indonesia. Biodiversitas 19: 766-772.* In January 2016, there were reported extensive mortalities of *Pangasius pangasius* cultured on cage, in Cirata Lake. *Edwardsiella ictaluri* is primarily recognized as a disease-causing pathogen in catfish species, causing an economically important bacterial infection hence affecting productivity of aquaculture enterprises in many regions across the *E. ictaluri*. The samples were 17 fishes collected from organs containing spleen, kidney, and liver. The total samples were 51 organs of fishes. The high bacteria infection prevalence level was found in kidney (29.41%). The gross pathological signs of sample of organs were abnormal sizes and abnormal color including white nodules. Bacteria were identified by conventional biochemical test, polymerase chain reaction (PCR) analysis and nucleotide sequencing. The results showed that *P. pangasius* cultured in cages in Cirata Lake is positively infected by *E. ictaluri* strain (accession number KF9071291). Other infection cases by *Aeromonas* sp. and *Plesiomonas shigelloides* as co-infection bacteria in *P. pangasius* were also found.

Keywords: Aquaculture, characterization, *Edwardsiella ictaluri*, identification, *Pangasius pangasius*

INTRODUCTION

Edwardsiella ictaluri is a gram-negative bacterium which causes enteric septicemia in majority of catfish species and Japanese eels (*Anguilla japonica*) in Japan and Taiwan and infecting fish only (Hawke et al. 2014). In 1976 there were observed natural outbreaks of *E. ictaluri* in Georgia, Alabama, USA, Japan, South Africa and also in Asia. *E. ictaluri* is primarily recognized as a disease of catfish species and is known to cause an economically important bacterial disease of farmed catfish in some countries (Hawke and Khoo 2004; Suanyuk et al. 2014). Species *E. ictaluri* is the most widespread family member of the genus Edwardsiella, which had been detected in over 20 species of freshwater and marine fish from 25 countries in the America, Europe, Asia, Australia, Africa and the Middle East (Hawke and Khoo 2004). *E. ictaluri* can survive in both freshwater and marine ecosystems and its hosts can range from fingerling to table sized fish (Ainsworth et al. 1986). The optimum temperature for growth of *E. ictaluri* is around 35°C, but can also thrive within the temperature range of 10°C to 45°C. During cold seasons, edwardsiellosis can thrive at temperature range of 10°C and 18°C (Lui and Tsai 1980; Castro et al. 2006). *E. ictaluri is* known to be causative agents of edwardsiellosis disease and it can also cause infection in yellow catfish, white catfish, blue catfish and walking catfish (Neema et al. 2011; Matt et al. 2014).

The pathogenic effects of *E. ictaluri* can cause mortalities of up to 50% as it has been recorded (Williams et al. 2008). In one comparative study, an injection of 1.5 x 10^3 cells of the pathogen was sufficient to cause 100% mortality among a group of channel catfish (Plumb and Sanchez 1983 in Austin and Austin. 2007). Little is known about the pathogenicity mechanisms of *E. ictaluri*, however extracellular products have been associated with its virulence (Williams et al. 2008). Survival of natural outbreaks has led to high humoral antibody levels and protection from fresh onslaught with *E. ictaluri* (Plumb 1993). Certainly, it has been firmly established that channel catfish are highly susceptible to the organism, with an injected dose of 1.5 x 10^3 cells capable of killing the host within 10 days at a water temperature of 26°C (Plumb and Sanchez 1983 in Austin and Austin. 2007). *E. ictaluri* infects fish by several routes and one of which is the water-borne bacteria which can invade the olfactory organ via the nasal opening and migrate into the olfactory nerve, then into the brain meninges and finally to the skull and skin (Miyazaki and Plumb 1985; Shotts et al. 1986; Morrison and Plumb 1994). It enters through the blood and intestinal wall and is engulfed by bacteriophages, resulting in septicemia (Shotts et al. 1986; Newton et al. 1989).

In February 2002 high mortalities of cultured *Pangasius hypophthalmus* associated with *E. ictaluri* was reported for the first time in freshwater ponds in central Sumatra, Indonesia. It is reported in this case that mortality of *Pangasius hypophthalmus* fish is 50-100% in 2 to 3 weeks (Yuasa et al. 2003). The aim of this research was to characterized and identified *E. ictaluri* isolated from *Pangasius pangasius* with natural infection reared in cage in Cirata Lake, West Java, Indonesia.

MATERIALS AND METHODS

Location

Cirata Lake, West Java, Indonesia is located on 223 m dpl, at $107^0$14'15"-$107^0$22'03"S and $06^0$41'30"-$06^0$48'07"E. The main fish species cultured in the lake includes common carp species, *Oreochromis niloticus*, and catfish. The fish breeding method in Cirata Lake is floating net cages (KJA) for the fish growth phase. The breeding systems used by the breeders are different, such as monoculture or polyculture. The main function of Cirata Lake is a hydroelectric dam which is the biggest in Southeast Asia.

Procedures

Animals

Fish which used in this study was *P. pangasius*, with sizes ranging from 200 to 250 grams (N=17) cultured in cages from a farm, in Cirata Lake. The fishes were not physically examined for external lesions before necropsy and collection of tissue samples (spleen, kidney, and liver) and observation of the internal organ of fish like organ color and size. Sampling is taken when death fish case found. Fish sampling is taken in critical situation (moribound) and in healthy situation shown by normal swimming. Fishes were taken to Main Center for Freshwater Aquaculture Fisheries Laboratory, Sukabumi, West Java, Indonesia with closed transportation system, using plastic which has filled with gas.

Medium for culturing bacteria and biochemical characterization

Conventional microbiology analysis was used for laboratory examination of the fish samples and comprised the following equipment and chemicals; Trypticase Soy Agar (TSA) Himedia, Brain Heart Infusion Agar (BHIA) Oxoid, *E. ictaluri* Medium (EIM), the gram's stain kit (Merck), immersion oil (Merck), SIM (Merck), OF (Merck), TSIA (Oxoid), Tryptone Soy Broth (TSB) Himedia, Oxidase test (Merck), catalase H_2O_2 3%, Kovac reagent (Merck), malonate (Merck), L-arabinose (Applichem), D-mannitol (Applichem), sucrose (Merck), trehalose (Applichem) and API 20E (Biomerieux) kit. Each medium was prepared according to protocol described by manufacturer.

Bacteria isolation from fish specimens

Fish specimens were swabbed with cotton wool dabbed in 70% ethanol to prevented the internal (visceral) organs from external contamination. The visibly infected fish was dissected, and all the necropsied visceral organs (kidney, spleen, and liver) were removed. A part of each organ was preserved without squashing them in 70% ethanol for microbiology analysis and preserved by squashing them in 70% ethanol then stored at 4°C for PCR.

Figure 1. Location of this study in Cirata Lake (●), West Java, Indonesia

To isolated the bacteria, each organ was placed on sterilized Petri-dishes, torn apart by a sterilized scalpel to make a homogeneous mixture, and the resulted suspensions were streaked on to TSA and BHIA media, which inoculated under aseptic condition. Each plate was appropriately labeled, and the samples were incubated in an incubator at 28ºC for 24-48 hours under aerobic conditions to allow growth of colonies. Morphological characteristics of each bacteria colony were scrutinized. A single colony was isolated on to medium TSA and BHIA, and incubated at 28°C for 24-48 hours under aerobic condition to allow growth of colony to get pure culture of bacteria. The colonies of bacteria were analyzed using the conventional (Biochemical test), API kit 20E, PCR, and nucleotide sequencing.

Biochemical test

The isolated colonies of bacteria pass through some biochemical tests such as gram staining test, oxidase, mortality, oxidative-fermentative, indol, H_2S, malonate, L-arabinose, D-mannitol, sucrose and trehalose test. It is weakly motile at 25-30°C. It is also lactose and indol negative, catalase-positive, cytochrome oxidase-negative and glucose fermentative and reduces nitrate to nitrite (Plumb 1999).

Maintenance of bacteria

Each one of the isolate cultures was maintained at 4°C in TSA or BHIA media for 15 days and later sub-cultured at 15 days intervals. The result cultures were stored for 4 weeks in sterilized paraffin oil on the medium agar. Preparation of the samples was carried out in 10 mL TSB and incubated under a temperature of 28°C for 24 hours, and then 3 mL 85% glycerin sterile, 1 mL aliquot of vortex was added to 2 mL microtubes and keep at -80°C for a period for one year.

Polymerase chain reaction (PCR)

PCR was carried out on DNA extracted from each of the visceral organs and the pure culture of bacteria. DNAzol kit (Invitrogen) was used to perform the extraction of DNA following the recommended protocol. The species-specific primers of *E. ictaluri* IVS 5'-TTA AAG TCG AGT TGG CTT AGG G-3' and IRS 5'-TAC GCT TTC CTC AGT GAG TGT C-3' were employed for sequence amplification (Williams and Lawrence 2010). The sequence of universal primer pairs for bacteria was 16R-5'-CGG TTA CCT TGT TAC GAC TT-3' and 16F-5'-ACA GTT TGA TCC TGG CTC AG-3'. The initial stage was to conduct a PCR premix by hot star tag polymerase (Qiagen). The composition of master mix for PCR was 25 µL HotStarTaq master mix 2x; 5 µL of each primer (0,2 µM of each primer) and 13 µL $2dH_2O$ and 2 µL DNA sample (<1 µg/reaction) or added 15 µL $2dH_2O$ for 1 colony of bacteria *E. ictaluri*, and the final volume PCR was 50 µL. PCR condition for both of primers pairs was pre-denaturation step at 95°C for 5 minutes 1 cycle; denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 2 minutes 30 cycles and elongation at 72°C for 7 minutes per cycle. The PCR was kept running for 2 hours. Analysis was conducted using the PCR machine from Applied Biosystems (AB).

The samples of 9 µL PCR product and 1 ul 5x loading dye (Invitrogen) were mixed and were electrophoresed (Owl) through a 1.5% (m/v) agarose (Vivantis) gel containing ethidium bromide (Merck) (1 µg/mL) and observed under ultraviolet light (AB). Observable band sizes for *E. ictaluri* were 2000-bp and 450-bp for universal primers. The PCR products were sent to a company (Genetika Science) for nucleotide sequencing.

RESULTS AND DISCUSSION

Results

Cirata Lake is multifunctional lake which one of it is as a place for developing freshwater fisheries sector using cages system. Generally, fish breeding system which is applied to the fish growth phase, where fingerlings come from different area in West Java. The fast-growing breeding makes the quality water decreasing. This is indicated by many Water Hyacinth found on the surface of the water, causing narrower lake surface. Natural case like up-welling always happens every year. Despite this situation, fish breeding in Cirata Lake is still running.

In January 2016, 17 *P. pangasius* fish samples were collected due to fish death report in cages at a farm in Cirata. This is reported as the first case found in cages, so complete information about this disease is needed. The mortality recorded in this case is up to 95% acute, and numerous fish died within 2 weeks. The sample fish condition which was taken varies from the dying fish (moribound) to healthy-look fish shown by swimming activeness. Although deformities on body, fin, anus, and mouth are not physically seen, the inside organ like kidney, liver, and lymph have color and size changing as shown in Figure 2.

A characteristic feature of an *E. ictaluri* colony is small size about 1-2 mm, white in color and oval in shape. The growth of colony cultured on media is slow and requires a period of about 36-48 hours to form punctate colonies on BHIA at 28-30°C. It exhibits poor growth if incubated at 37°C. TSA and BHIA media provide a conducive environment for the growth of *E. ictaluri,* which is gram negative, short, pleomorphic rod, and about 0.75 µm x1.5-2.5 µm. The colony of *E. ictaluri* on Edwardsiella Ictaluri Medium (EIM) forms small, translucent and greenish colonies. They display slow growth on EIM for a 36-48 hours period, at the temperature of 35°C. The colony has smooth circular edges which are measuring about 2 mm diameter, slightly convex, entire, non-pigmented colonies develop (Hawke et al. 2014).

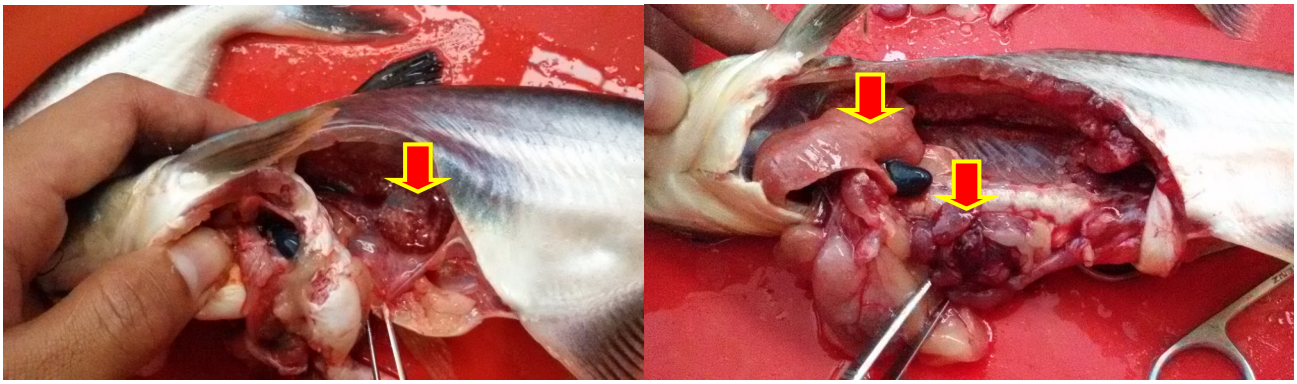


Figure 2. The clinical appearance of organs, kidney (left), spleen and liver (right) of *Pangasius pangasius* observed in this study. The kidney of fish abnormal size, with white nodules and colorless. The liver was yellow and less color, abnormal size with white nodule. The spleen of fish was colorless, abnormal size with white nodules. Lymph, kidney, and liver are stiff when pushed. Bile's color is thick green and stiff

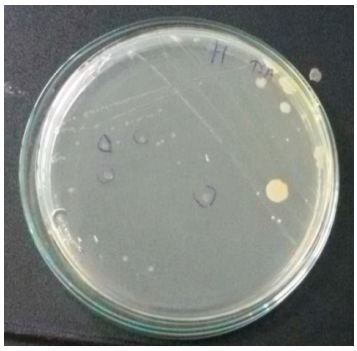
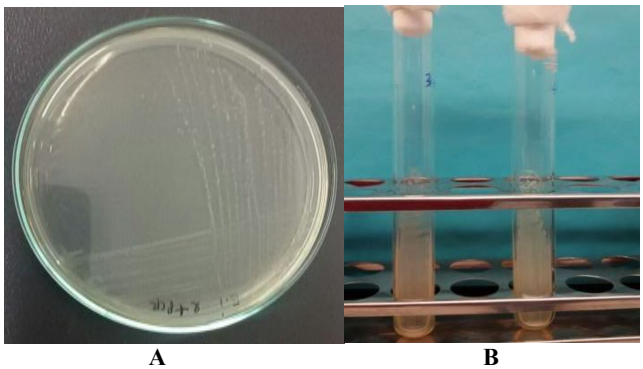


Figure 3. Bacteria *Edwardsiella ictaluri* colonies isolated from *Pangasius pangasius* on TSA medium after 48 hours incubation. The bacteria collected from internal organs were streaked on TSA medium and afterward incubated at 28°C for 48 hours. The colonies of bacteria are good growth. The morphology of colonies of bacteria dominant was smooth, white, uniform. The average diameter of the colonies is 0.15 mm. That bacteria colony is more dominantly grow compared to other bacteria colony. In addition to that, other types of bacteria colony are also grown. Yellow colony in bigger size and white colony in medium size. The next identification process is by bacteria purification based on some different morphologies of colony. It shows that there are other bacteria types found in the fish samples.



A **B**

Figure 4. That results of isolation bacteria (pure culture). (A) The colonies of bacteria as pure culture on TSA medium plate and (B) on TSA medium tube. They were incubated at 28°C for 48 hours. The colonies were thin growing on TSA medium tube (B). The bacteria colonies isolated from A and B have the same sizes and morphologies, and are ready for further identification test.

The characterization of *E. ictaluri* was performed by conventional biochemical testing. The results were shown in Table 1. The results of biochemical analysis using API 20E kit were unacceptable. Other bacteria identification result found on *P. pangasius* fish organ using API 20E kit is good identification with percentage of 99.0% in genus *Aeromonas* sp. and Excellent Identification with percentage of 99.9% in *Plesiomonas shigelloides. Aeromonas* sp. and *Plesiomonas shigelloides* colonies are not dominantly grown on TSA medium, which could be assumed that this kind of bacteria is a co-infection and are not the main cause of *P. pangasius* mortality.

The primer of IVS-IRS was positive in 2000 bp for *Edwardsiella ictaluri* and the primer of 16S for universal bacteria positive in 450 bp (Figure 6). The result of electrophoresis from PCR result, negative control (line 10) and positive control for IVS-IRS primer pairs (line 11). The positive result of *E. ictaluri* from kidney (line 1), spleen (line 2) and liver (line 3) of *P. pangasius.* The positive result of *E. ictaluri* from isolate 1 (line 4); isolate 2 (line 7) and isolate 3 (line 9). The positive universal bacteria isolate 1 (line 5); isolate 2 (line 6); isolate 3 (line 8). The electrophoresis was running on 1.5% agarose (Vivantis) and M: marka of DNA ladder 100 bp plus (Vivantis). From the PCR result can be seen that sample which is taken from *P. pangasius* fish organ tissue can be detected positively infected by *E. ictaluri* bacteria. It demonstrated that the *E. ictaluri* infection level is high in the fish organ. The sequence of 16S ribosomal RNA gene isolated from pure culture bacteria is close to Sequence of *E. ictaluri* with accession number ID: KF907129.1 and number of identification match 1.

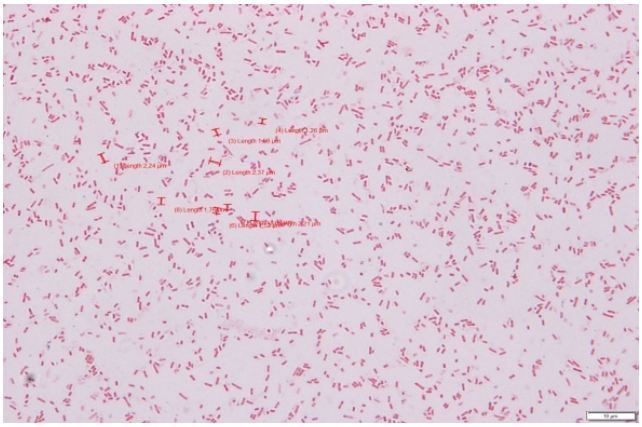


Figure 5. Micrograph of Gram staining of *Edwardsiella ictaluri* isolated from kidney of fish. The color of cell bacteria was red, gram-negative, straight rod, the measure of cell bacteria about 1.84 µm (microscope Olympus BX53, 10 µm).

Table 1. Results of conventional biochemical test of *Edwardsiella ictaluri*

Substrate	Result
Oxidase	Negative
Gram stain	Negative, rod
Motility 37°C	Negative
O/F test	F
Indol	Negative
H2S	Negative
Malonate (utilization)	Negative
L-arabinose	Negative
D-mannitol	Negative
Sucrose	Negative
Trehalose	Negative

Table 2. Distribution of *Edwardsiella ictaluri* among the organs of *Pangasius pangasius*

Organ	Result		Total
	Positive	Negative	
Spleen	13	4	17
Kidney	15	2	17
Liver	7	10	17

The results of distribution *E. ictaluri* on the organs of fish base on clinical sign on the organs and laboratory analysis results. The prevalence distribution of bacteria on kidney was the higher infection than spleen and liver of *P. pangasius* (Table 2). Prevalence of *E. ictaluri* on the organs was kidney 29.41%; spleen 25.49% and liver 13.73%, respectively. It means that the kidney is more sensitive to infection than another organ of *P. pangasius.*

Discussion

However, because the standard bacterial detection procedure prescribes the testing of multiple fish samples or pieces (Thoesen 1994), the occasional isolation failure among clinically infected fish is not problematic. The use of EIM agar enhances *E. ictaluri* isolation when there is an existence of multiple infections. *Aeromonas hydrophila* was found to co-infect fish suffering from ESC (Hanson and Grizzle 1985; Antonio and Hedrick 1994) and tends to overgrow and mask *E. ictaluri* on a non-selective agar (Earlix et al. 1996). Acute mortalities in *Pangasius hypophthalmus* facilities are more likely to be associated with minimal or un-compensated physiologic stress, and area frequently manifested as an acute stress response, arising perturbations from water quality conditions (Hawke 2015). Opportunistic pathogens may have a negative impact on bacterial infections such as *Aeromonas* sp., *Pseudomonas* sp. and *Plesiomonas* sp. (Hawke 2015). Lusiastuti et al. (2012) stated that there is a tendency of the emergence of cases of superinfection *A. hydrophila* and *E. ictaluri* was found in case of death of larval catfish on size ¾ to 2 inches in some subdistricts in Subang, West Java, Indonesia that need to be observed in anticipation of the spread and control in the event of similar case. Outbreaks in several subdistricts in Subang of acute mortality showed a symptom both external and internal. Clinical symptoms such as corkscrew spiral swimming and then die immediately. Some fish showed enlarged abdominal, exopthalmus, and small foci measuring 1-3 mm in the liver. These results indicate that the fish can rid their self from *E. ictaluri* in blood circulation, but super-infection with *A. hydrophila* aggravated latent clinical symptoms of edwardsiellosis and obtained only *A. hydrophila* that dominated almost all organs. Vaccination using *A. hydrophila* vaccine can prevent super infection but need more in-depth review of whether the vaccine could provide cross-protection against the infection of edwardsiellosis.

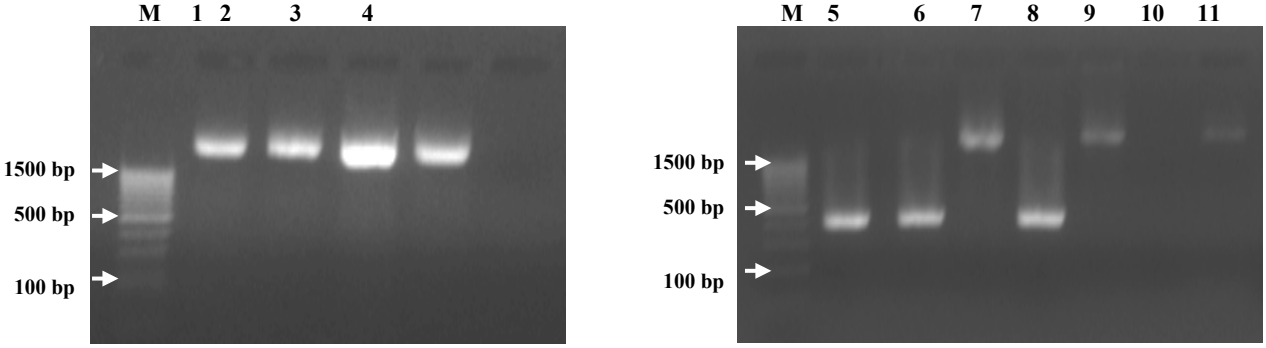


Figure 6. Electrophoretogram of PCR products amplified with IVS-IRS primer pairs and 16-S primer pairs for the DNA templates from pure culture bacteria *Edwardsiella ictaluri* and organs of *Pangasius pangasius*

The morphology of *E. ictaluri* colonies found in this study was smooth, white, uniform. The averaged diameter of colonies is 0.15 mm on the TSA medium after incubated at 28°C for 48 h. Crumlish et al. (2002) demonstrated that the size of *E. ictaluri* colony on TSA medium which is incubated at 28°C for 48 h is small from 0.03 to 0.14 mm. The colony appeared to be off-white and translucent and displaying an irregular surface and edge. *E. ictaluri* have an analogous structure and adjoining regions in comparison with other members of the family Enterobacteriaceae. In this case, the size of the cells of *E. ictaluri* had an average of 1.84 µm (Figure 5), straight rod, gram-negative. While *E. ictaluri* morphology on Pangasius, while *E. ictaluri* morphology on *Pangasius hypophthalmus* found in Sumatra is gram-negative rod-shaped organisms of variable size (1.2-15 µm) (Yuasa et al. 2003). But, this research doesn't test the characterization using sequencing method.

Typically, *E. ictaluri* is described as a small straight rod, which measures on average, 1 µm x 2-3 µm (Plumb 1993). However, the investigation conducted in Vietnam isolates showed a much greater variation in length and width, often with very large rods clearly visible. This variation in size was constantly observed in all 12 isolates that were recovered from clinically diseased *Pangasius hypophthalmus* (Crumlish et al. 2002).

In this study, the sequencing result showed that the isolated type of bacteria from *P. pangasius* was *E. ictaluri* with sequence ID number KF9071291. The Confirmatory result using PCR was positive *E. ictaluri* from organs of *P. pangasius* and from isolated bacteria (Figure 6), while the result of analysis using API kit 20E was unacceptable because the kit doesn't provide for *E. ictaluri* identification. Characterization of *E. ictaluri* by biochemical testing. The result showed that the motile at 37 °C temperature, fermentative and oxidative test, indole test, H_2S, malonate, L arabinose, D mannitol, sucrose dan trehalose were negative.

The distribution of *E. ictaluri* was found high prevalence infection in the kidney of *P. pangasius* (Table 2). It was also found in other types of bacteria, *Aeromonas* sp., and *Plesiomonas shigelloides*, as co-infection bacteria on *P. pangasius*. These kinds of bacteria could be assumed are not the main cause of *P. pangasius* mortality, because it can be seen in the picture Figure 3. The morphology of colony distribution is not much compared to *E. ictaluri* which was dominantly grown on the medium.

Practically, multiple bacterial pathogens exist in aquaculture production systems and are possibly responsible for disease outbreaks and significantly outweigh single infection. Therefore, it was imperative to investigate whether single or concurrent bacterial pathogens were involved in the naturally diseased striped catfish and subsequently also investigate the pathogenicity of single- and dual-infection through experimental challenges/investigations. The investigations revealed co-infections of *Flexibacter columnare* and *E. ictaluri* that were found in naturally diseased Thai stripedcat fish which exhibited columnaris and edwardsiellosis diseases (Dong et al. 2015).

Enteric septicemia disease of catfish which is caused by *E. ictaluri* has been a long-standing problem in aquaculture. Some methods could be used for *E. ictaluri* bacteria identification, among all are using Polymerase Chain Reaction Assay method, conventional Method, and Sequencing Method. Polymerase chain reaction assay may be employed for the early detection of *E. ictaluri* on visceral organs of fish.

REFERENCES

Ainsworth AJ, Capley G, Waterstreet P, Munson D. 1986. Use of monoclonal antibodies in the indirect fluorescent antibody technique (IFA) for the diagnosis of *Edwardsiella ictaluri*. J Fish Dis 9: 439-444.

Antonio DB, Hedrick RP. 1994. Effects of the corticosteroid Kenalog on the carrier state of juvenile channel catfish exposed to *Edwardsiella ictaluri*. Aquat Anim Health 6: 44.

Austin B, Austin D. 2007. Bacterial Fish Pathogens Diseases of Farmed and Wild Fish. Springer, Chichester, UK.

Castro N, Toranzo AE, Barja JL, Nunez S, Magarinos B. 2006. Characterization of *Edwardsiella tarda* strain isolated from turbo, *Psetta maxima*. J Fish Dis 29: 541-547.

Crumlish M, Dung TT, Turnbull JF, Ngoc TNT, Ferguson WH. 2002. Identification of *Edwardsiella ictaluri* diseased freshwater catfish, *Pangasius hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam. J Fish Dis 25 (12): 733-736.

Dong HT, Nguyen VV, Phiwsaiyab K, Gangnonngiw W, Withyachumnarnkulb B, Rodkhuma C, Senapin S. 2015. Concurrent infections of *Flavobacterium columnare* and *Edwardsiella ictaluri* in striped catfish, *Pangasianodon hypophthalmus* in Thailand. Aquaculture 448: 142-150.

Earlix D, Plumb JA, Rogers AW. 1996. Isolation of *Edwardsiella ictaluri* from channel catfish by tissue homogenization, filtration and enzyme-linked immunosorbent assay. Dis Aquat Org 27: 19-24.

Hanson LA, Grizzle JM. 1985. Nitrite-induced predisposition of channel catfish to bacterial diseases. Prog Fish Cult 47: 98-101.

Hawke JP, Kent M, Rogge M, Baumgartner M, Wiles J, Shelley JL, Savolainen C, Wagner R, Murray K, Peterson TS. 2014. Edwardsiellosis caused by *Edwardsiella ictaluri* in laboratory populations of Zebrafish *Danio rerio*. J Aquat Anim Health 25 (3): 171-183.

Hawke JP, Khoo LH. 2004. Infectious diseases. In: Tucker CS, Hargreaves JA (eds.). Biology and Culture of Channel Cat-Fish. Elsevier, San Diego, CA.

Hawke JP. 2015. Enteric Septicemia of catfish. Southern Regional Aquaculture Center 477: 1-6

Lui CI, Tsai SS. 1980. Edwardsiellosis in pond cultured eel in Taiwan. Council of Agriculture. CAO Fisheries series, Taipei, Taiwan.

Lusiastuti AM, Sumiati T, Hadie W, Sularto, Taukhid. 2012. Case study super infection of aeromoniasis against edwardsiellosis on catfish fingerling in Subang district, West Java, Proceeding Forum Inovasi Teknologi Akuakultur 2012. [Indonesian]

Matt JG, Ware C, Quiniou SM, Steadman JM, Gaunt PS, Khoo LH, Soto E. 2014. *Edwardsiella piscicida* identified in the southeastern USA by *gyrB* sequence, species-specific and repetitive sequence-mediated PCR. Dis Aquat Org 108: 23-35.

Miyazaki T, Plumb JA. 1985. Histopathology of *Edwardsiella ictaluri* in channel catfish, *Ictalurus punctatus* (Rafinesque). J Fish Dis 8: 389-392.

Morrison EE, Plumb JA. 1994. Olfactory organ of channel cat- fish as a site of experimental infection. J Aquat Anim Health 6 (2): 101-109.

Neema M, Karunasagar I,, Karunasagar I. 2011. Structural and functional characterization of outer membrane protein N in *Edwardsiella ictaluri*: A bioinformatic approach. Intl J Pharma Sci Res 2 (1): 13-26.

Newton JC, Wolfe LG, Grizzle JM and Plumb JA. 1989. Pathology of experimental enteric septicemia in channel catfish *Ictalurus punctatus* Rafinesque following immersion exposure to *Edwardsiella ictaluri*. J Fish Dis 12: 335-348.

Plumb JA. 1993. *Edwardsiella septicaemia*. In: Inglis V, Roberts RJ, Bromage NR (eds.). Bacterial Diseases of Fish. Blackwell, Oxford, UK.

Plumb JA. 1999. *Edwardsiella septicemias*. In: Woo PTK, Bruno DW (eds.). Fish Diseases and Disorders: Viral, Bacterial and Fungal Infections. CABI Publishing, Wallingford, UK.

Shotts EB, Blazer VS, Waltman WD. 1986. Pathogenesis of experimental *Edwardsiella ictaluri* infections in channel catfish (*Ictalurus punctatus*). Canadian J Fish Aquat Sci 43: 36-42.

Suanyuk N, Rogge M, Thune R, Watthanaphiromsakul M, Champhat N, Wiangkum W. 2014. Mortality and pathology of hybrid catfish, *Clarias macrocephalus* (Gunther), *Clarias gariepinus* (Burchell), associated with *Edwardsiella ictaluri* infection in southern Thailand. J Fish Dis 4: 385-395

Thoesen JC. 1994. Suggested procedures for the detection and identification of certain fin-fish and shellfish pathogens. Fish Health Section. American Fisheries Society, Bethesda, MD, USA.

Williams K, Lawrence ML. 2010. Verification of an *Edwardsiella ictaluri*-specific diagnostic PCR. Lett Appl Microbiol 50: 153-157.

Williams ML, Waldbieser GC, Dyer DW, Gillaspy AF, Lawrence ML. 2008. Characterization of the operons in the channel catfish pathogen *Edwardsiella ictaluri*. J Appl Microbiol 104: 1790-1796.

Yuasa K, Kholidin EB, Panigoro N, Hatai K. 2003. First isolation of *Edwardsiella ictaluri* from cultured striped catfish *Pangasius hypophthalmus* in Indonesia. Fish Pathol 38 (4): 181-183.