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Edwardsiella ictaluri: Pathogenicity and LD_{50} in *Pangasius nasutus*

S.Syafiq-Syauqi [1], M.Nur-Nazifah [1]*, M.Zudaidy Jaapar [2], M.Muhammad-Faizal [1]

ABSTRACT
This study focuses on pathogenicity and LD_{50} of *Pangasius nasutus* against *Edwardsiella ictaluri*. *Pangasius nasutus* or 'patin buah' is a native freshwater species of peninsular Malaysia and can be found in Pahang River. The market price for this species is high as it tastes better compared to other catfish. *Edwardsiella ictaluri* is a pathogenic bacteria and the causative agent that cause enteric septicemia of catfish (ESC) in the Pangasius species. However, the prevention measures against *Edwardsiella ictaluri* are still unknown for *Pangasius nasutus* due to the lack of research and study. Therefore, the objective of this study is to identify *Edwardsiella ictaluri* isolated from *Pangasius nasutus* cultured in Pahang River using polymerase chain reaction (PCR) and to determine the pathogenicity and LD_{50} of *Pangasius nasutus* against *Edwardsiella ictaluri* through injection method. There are four different concentrations of *Edwardsiella ictaluri* (1×10^{20}, 1×10^{9}, 1×10^{8}, 1×10^{7}) that were injected intraperitoneally including normal saline water as control to a total of 50 *Pangasius nasutus*. Observation of clinical signs and mortality were carried out for 30 days and LD_{50} was determined. The earliest clinical sign was observed at concentration of 1×10^{10} cfu/ml at 2 hours postinfection of *Edwardsiella ictaluri* where pale liver and congested kidney can be observed after dissection. Other clinical signs observed are inflammation on tail and fin, hemorrhagic fin, hemorrhagic upper mandible, discoloration, and inflammation on the lower part of body. The first mortality of *Pangasius nasutus* was at concentration 1×10^{10} cfu/ml at 2 hours of post infection. The highest cumulative mortality was recorded at concentration 1×10^{10} cfu/ml with 100% of mortality rate. From the result, the value of LD_{50} of *Edwardsiella ictaluri* calculated was 1×10^{6} cfu/ml. From this study, it can be concluded that *Edwardsiella ictaluri* does affect the survivability *Pangasius nasutus* in Malaysia.

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Introduction

Pangasius nasutus is a shark catfish family *Pangasiidae*, commonly found in freshwater habitats, particularly in South and Southeast Asia. It is known for its distinctive appearance and is often sought after by local communities for its delicious taste and nutritional value. In Malaysia, it is locally referred as 'Patin Buah' and is highly regarded as a delicacy, especially when cooked with fermented durian called 'tempoyak' [9]. This unique combination of flavors creates a culinary experience that is cherished by both locals and tourists, making it an important part of the cultural heritage of the region.

Pangasius nasutus is a highly sought-after food fish known for its exceptional culinary qualities. It boasts a delectable flavor profile, characterized by its very white, fine-grained, and sweet flesh. However, despite its undeniable appeal, this species remains relatively elusive in the market. The limited availability of *Pangasius nasutus* is primarily attributed to its dependence on wild catches, which, unfortunately, has resulted in a significant reduction in its natural resources [3]. This scarcity further contributes to the challenges of procuring this remarkable fish.

Furthermore, *Pangasius nasutus* is characterized by its slow growth rate, which significantly impacts its market price. The cost of acquiring this fish is nearly five times higher than *Pangasianodon hypophthalmus*. In light of these factors, the procurement and accessibility of *Pangasius nasutus* pose considerable challenges for both consumers and suppliers alike. There is typically limited information available about the fish. Therefore,

[1] Department of Marine Science, Kulliyah of Science, International Islamic University Malaysia, Jalan Istana, 25200 Kuantan, Malaysia
[2] Department of Fisheries, Fisheries Research Institute (FRI), Glami Lemi, Titi, 71650 Jelebu, Negeri Sembilan, Malaysia
*Corresponding Author: Nur Nazifah Mansor, e-mail: nurnazifah@iium.edu.my

additional research is required in areas such as nutrition, brood stock management, breeding technology, and genetics to promote the growth of the species in commercial aquaculture [1].

Edwardsiella ictaluri is a Gram-negative bacterium that is pathogenic to fish, particularly channel catfish (*Ictalurus punctatus*). It is the causative agent of enteric septicemia of catfish (ESC) which were a significant disease in the catfish aquaculture industry. *Edwardsiella ictaluri* was first described in the 1970s and has since been recognized as a major pathogen affecting catfish farms in the United States and other parts of the world [13]. *Edwardsiella ictaluri* is a type of bacteria that can cause severe infections in channel catfish. This bacterium is a highly contagious and deadly disease that affects the digestive system of catfish.

ESC caused by *Edwardsiella ictaluri* poses a significant threat to the catfish aquaculture industry. Infected fish experience symptoms such as bloating, lethargy, loss of appetite, and ultimately death [4]. The economic impact of this disease is substantial, leading to financial losses for catfish farmers. The discovery of *Edwardsiella ictaluri* dates back to the 1970s, and since then, it has gained recognition as a major pathogen affecting catfish farms worldwide. Owing to its ability to rapidly spread and cause devastating outbreaks, strict biosecurity measures are necessary to control the transmission of this bacterium within aquaculture facilities.

However, there has been relatively little research conducted on the best identification method used to identify Edwardsiella ictaluri, the bacterium that can infect Pangasius nasutus in Malaysia. Furthermore, there has been a complete absence of studies exploring the LD50 (lethal dose 50%) of Edwardsiella ictaluri on Pangasius nasutus in Malaysia. This knowledge gap has raised significant concerns among researchers, prompting the urgent need for investigation in this area. Hence, the objective of this study is to identify isolate of Edwardsiella ictaluri using molecular biology approach (PCR) and to determine the pathogenicity and LD50 of Edwardsiella ictaluri in Pangasius nasutus through injection method.

Material and Methods

Selection of Pangasius nasutus for bacterial isolation

Samples of Pangasius nasutus were taken from a farm located at Kampung Lebak Seberang, Temerloh, Pahang. Random sampling was done where 10 pieces of fish with severe clinical signs such as fish gasping at water surface were taken and dissected. All procedures when handling and dissecting fish has obeyed the Animal Welfare act which was carried out solely for research purposes. Organs such as brain, kidney, liver and spleen in every fish sample were streaked onto TSA agar (Oxoid, England) to acquire bacterial isolates. All bacterial isolates were incubated at 28°C for 48 hours in the incubator.

Identification of bacteria *Edwardsiella ictaluri*

Isolation of *Edwardsiella ictaluri*

The isolate of *Edwardsiella ictaluri* was isolated from *Pangasius nasutus* cultured in a cage system. Then, sample of bacteria was cultured again on TSA agar (Oxoid, England) in order to obtain single and pure colony. The optimum condition for *Edwardsiella ictaluri* to grow was 28°C at 48 hours of incubation period.

DNA Extraction (G-Spin™ Total DNA Extraction Kit, Protocol F)

DNA extraction was carried out by using G-Spin™ Total DNA Extraction Kit according to protocol F for bacteria. Single colony from TSA agar were pick up and inoculated into 5 ml of Brain Heart Infusion (BHI) broth (Oxoid, England). Then, the BHI broth was incubated for 48 hours at 28°C, 100 rpm. After that, bacteria were pelleted by centrifugation for 1 min at 13,000 rpm, and supernatant was discarded. The cell pellet was resuspended completely with remnant supernatant by vigorously vortex.

To prepare the sample, 200 µl of Buffer CL, 20 µl of Proteinase K, and 5 µl of RNase A Solution was added to the sample tube. The mixture was vortexed vigorously to ensure thorough mixing. The lysate was incubated at 56°C using a preheated heat block for 6 minutes. During the incubation, the sample tube was inverted gently every 2 minutes to aid in sample lysis. Once the lysis is complete, 200 µl of Buffer BL was added to the upper sample tube and mixed it thoroughly. Then, the mixture was incubated at 70°C for 2 minutes.

The sample tube was centrifuged at 13,000 rpm for 5 min to remove un-lysed tissue particles. Then, 350 - 400 µl of the supernatant was transferred into a new 1.5 ml tube. 200 µl of absolute ethanol was added into the lysate, and mixed well by pulse vortex. After mixing, the 1.5 ml tube was briefly centrifuged to remove drops from inside of the lid.

After that, the mixture was carefully applied to the Spin Column. Then without wetting the rim, close the cap, and centrifuged at 13,000 rpm for 1 min. The filtrate was discarded and the Spin Column was placed in a new 2 ml Collection Tube.

Next, 700 µl of Buffer WA was added to the Spin Column without wetting the rim, and centrifuged for 1 min at 13,000 rpm. The flow-through was discarded and the Collection Tube was reused. Then, 700 µl of Buffer WB was added to the Spin Column without wetting the rim, and centrifuged for 1 min at 13,000 rpm. The

flow-through was discarded and placed the Column into a new 2.0 ml Collection Tube. Then, centrifuged again for additional 1 min to dry the membrane. The flow-through and Collection Tube were discarded altogether. The Spin Column was placed into a new 1.5 ml tube. Then, 50 µl of Buffer CE was added directly onto the membrane. After that, the tube was incubated for 1 min at room temperature and then centrifuged for 1 min at 13,000 rpm to elute and DNA was obtained until further used.

Polymerase Chain Reaction (PCR) and Gel Electrophoresis

The conventional Polymerase Chain Reaction (PCR) method was carried out on DNA of *E. ictaluri* extracted earlier. The species-specific primers of *Edwardsiella ictaluri* (Table 1) were employed for sequence amplification [7] and to distinguish *Edwardsiella ictaluri* from *Edwardsiella tarda*. The composition of master mix for PCR by using (Thermo Scientific™, America) was shown in (Table 2) and the final volume PCR was 50 µL.

Table 1 Specific primers used for sequence amplification

Primer	Genetic Code
IVS	5'-TTA AAG TCG AGT TGG CTT AGG G-3'
IRS	5'-TAC GCT TTC CTC AGT GAG TGT C-3'

Table 2 The composition of PCR master mix

PCR Component	Volume
Pure distilled water	43 µL
Buffer	2 µL
Mg2+	2 µL
IVS	0.5 µL
IRS	0.5 µL
Deoxynucleotide triphosphates (dNTPs)	0.5 µL
DNA polymerase	0.5 µL
Template DNA	1 µL

Edwardsiella ictaluri ATCC was used as positive control while distilled water was used as negative control. The total time taken to process PCR products was 2 hours 29 minutes. PCR condition for both of primers pairs (IVS and IRS) was shown in Table 3.

Table 3 PCR Amplification Parameter

Process	Duration	Temperature
initialization	4 minutes	94°C
denaturation	1 minute	94°C
annealing	1 minute	50°C
extension	1 minute	72°C
final elongation	10 minutes	72°C

After that, 2 µL of PCR product and 2 ul of 6× loading dye (First Base, Singapore) were mixed and electrophoresed through a 1% of agarose (First Base, Singapore) gel containing Gel Red (First Base, Singapore) and observed under Gel Doc XR+ Gel Documentation System (BioRad, California). The band sizes which can be visible for *Edwardsiella ictaluri* were 2000-bp and 1kb DNA Ladder (Thermo Scientific™ GeneRuler, America) was used.

Pathogenicity and LD_{50}

Bacteria Revirulent

The single colony of *Edwardsiella ictaluri* were cultured in 250 ml of Brain Heart Infusion (BHI) broth (Oxoid., England) and incubated at 28°C for 48 hours with 100 rpm shaking movement. Then, 1 ml of the inoculum was injected to the experimental fish via intraperitoneal and left for a period of 12 hours. After 12 hours of post-injection, the symptomatic injected fish was dissected. Bacterial samples from organs (spleen, liver, kidney, brain) were isolated and streaked onto TSA (Oxoid., England) agar plates. All agar plates were sealed with parafilm and incubated at temperature of 28°C for 48 hours in the incubator. After 48 hours, the re-virulent bacteria were obtained.

Serial Dilution and Colony Forming Unit (CFU)

A total of 10 colonies of virulent bacteria were inoculated into 250 ml of Brain Heart Infusion (BHI) broth (Oxoid., England) and then incubated at 28ºC for 48 hours with 100 rpm in the incubator shaker. Next, tenfold serial dilution was done where the ratio must be 1:10 according to Robert Koch method [17]. Next, spread plate method was done to determine the concentration of inoculum. The number of colonies in each plate was counted after 48 hours of incubation at 28ºC. The concentrations of bacteria suspension were determined by using the colony-forming unit (CFU) formula (Table 4) [18]. There were 4 different concentrations of bacteria used in this experiment which were (1×10^{10}, 1×10^{9}, 1×10^{8}, 1×10^{7}) (cfu/ml) and one control treatment.

Table 4 CFU Formula

Formula
CFU/ml = (Number of colonies × dilution factor) / volume of culture plate

Experimental Designs

In this experiment, a total of 100 fish of *Pangasius nasutus* were used with the average weight of 40 g ± 5g. These fish were acclimatized in 1-ton tank for a week to enable them to be accustomed in new environmental conditions. A total of 4 different concentrations of *Edwardsiella ictaluri* which consist of (1×10^{10} cfu/ml, 1×10^{9} cfu/ml, 1×10^{8} cfu/ml, 1×10^{7} cfu/ml), and one control treatment (saline water) was used in this experiment. Tank for each concentration of *Edwardsiella ictaluri* and control treatment was duplicated. The size of the tank used is 50 L and were set up with aeration system. The stocking density was 10 fish per tank.

All fish were injected via intraperitoneal according to concentration of *Edwardsiella ictaluri* and saline water (control). All activities when conducting this experiment has obeyed the Animal Welfare act including handling, feeding, and dissecting that was carried out solely for research purposes. Observations of fish in every tank was carried out for a period of 30 days and any clinical signs and mortality was observed and recorded daily. The experimental design conducted was shown as below (Fig 1).

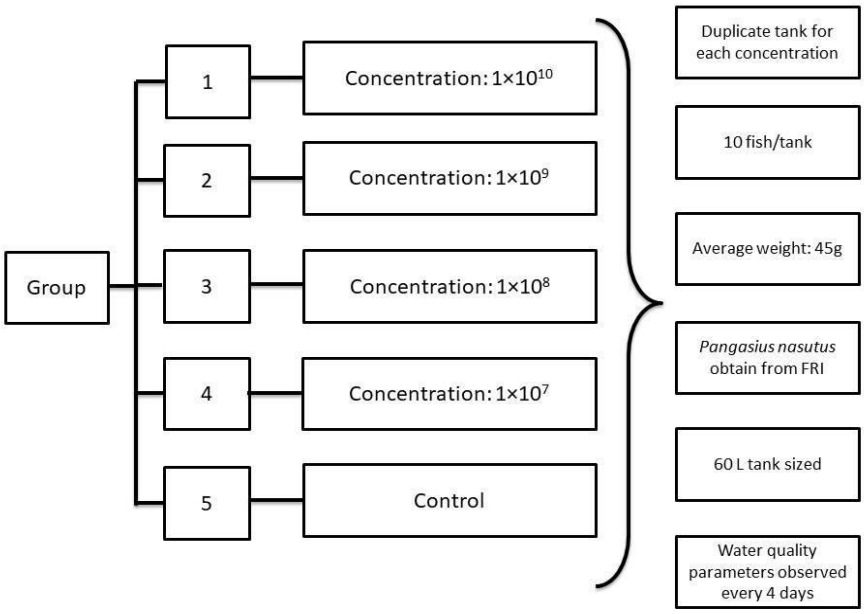

Fig 1 Experimental design

Data Analysis

Data obtained from the experiment was analyzed through statistical analysis using Paired Samples T-Test. This type of statistical analysis was used to differentiate the mean of mortality percentage and concentration of the *Edwardsiella ictaluri*. The data were analyzed by using IBM SPSS Statistic Version 20 with 95 % confidence interval. Meanwhile, LD_{50} for this experiment was measured using Curve Expert Professional Software (Version 2.7.4).

Results

Identification of *Edwardsiella ictaluri* using PCR

In this study, the species-specific primers IVS and IRS was used to identify *Edwardsiella ictaluri*. *Edwardsiella ictaluri* ATCC was used as positive control and distilled water was used as negative control. From PCR product, it showed the bacterial samples is positive *Edwardsiella ictaluri*. Positive PCR *Edwardsiella ictaluri* amplified at 2000bp. The image of PCR product on gel electrophoresis was shown in (Fig 2).

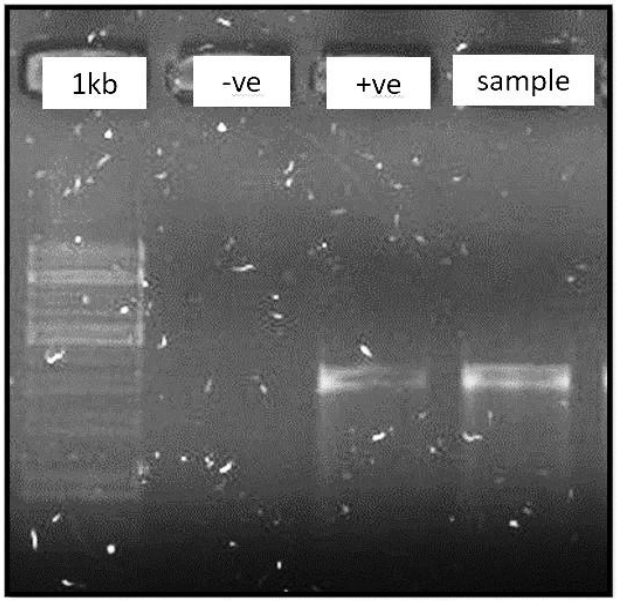

Fig 2 The image of PCR products on gel electrophoresis

Pathogenicity and Clinical Sign

The data analysis of cumulative and percentage mortality rates of *Pangasius nasutus* in 4 different concentrations of *Edwardsiella ictaluri* were obtained and calculated after 30 days of observation with results tabulated in (Table 5). The highest mortality percentage was recorded in tank with concentration of 1×10^{10} cfu/ml where mortality rate was 100%. Meanwhile, the second highest percentage of mortality was recorded in tank with concentration of 1×10^{8} cfu/ml with 80% mortality rate. The third highest mortality percentage was recorded in tank with concentration of 1×10^{7} and 1×10^{9} cfu/ml where the mortality percentage was 60%. The lowest percentage of mortality was recorded in control tank with with 0% mortality rate.

Table 5 The cumulative and percentage of mortality of *Pangasius nasutus* after been infected with *Edwardsiella ictaluri* in 4 different concentrations

Concentration	Number of experimental fish	Cumulative mortality	Percentage of mortality (%)
1×10^{10}	10	10	100
1×10^{9}	10	6	60
1×10^{8}	10	8	80
1×10^{7}	10	6	60
Control	10	0	0

The first mortality of *Pangasius nasutus* was observed in the tank with concentration of 1×10^{10} cfu/ml where fish died 2 hours post infection of *Edwardsiella ictaluri* (Fig 3). Half mortality of *Pangasius nasutus* from the whole population has been observed in the tank with concentration of 1×10^{10} cfu/ml on 19th days after post-injection. The last mortality of *Pangasius nasutus* recorded in this experiment was on 26th day after post-injection in tank with concentration of 1×10^{9} cfu/ml.

Fig 3 First mortality of *Pangasius nasutus* 2 hours post-injection

For tank infected with *Edwardsiella ictaluri* concentration of 1×10^{10} cfu/ml, the percentage of mortality was 100% where 10 fish died out of 10. Meanwhile, for tank infected with *Edwardsiella ictaluri* concentration of 1×10^{7} and 1×10^{9} cfu/ml, the percentage mortality was 60% where 6 fish died out of 10 fish. For tank infected with *Edwardsiella ictaluri* concentration of 1×10^{8} cfu/ml, the percentage mortality was 80% where 8 fish died out of 10 fish. For control tank with no infection of *Edwardsiella ictaluri*, the percentage mortality was 0% as there was no mortality recorded.

After 30 days of experiment, there were several clinical signs which has been observed including fish behaviour, external and internal organs. For fish behaviour, it has been observed that infected fish in tank infected with *Edwardsiella ictaluri* concentration of 1×10^{8} cfu/ml demonstrates erratic swimming and tend to swim vertically before dying (Fig 4).

As for external organ, it has been observed that infected fish in tanks infected with *Edwardsiella ictaluri* concentration of 1×10^{10}, 1×10^{9}, 1×10^{8}, and 1×10^{7} cfu/ml have inflammation on its tail and fin (Fig 5), hemorrhagic fin, hemorrhagic upper mandible, discoloration, and inflammation on the lower part of the body.

Fig 4 Vertical swimming of fish

Fig 5 Inflammation of tail and fin

As for internal organs, it has been observed that fish infected with all 4 concentrations of *Edwardsiella ictaluri* shown pale liver (Fig 6), congested kidney (Fig 7), and patchy liver (Fig 8). Meanwhile, infected fish in tank infected with *Edwardsiella ictaluri* concentration of 1×10^{7} cfu/ml shown white nodular on liver (Fig 9). On the other hand, dark liver (Fig 10) has been spotted in infected fish infected with *Edwardsiella ictaluri* concentration of 1×10^{8} cfu/ml. However, there were no clinical signs observed on fish in the control tank.

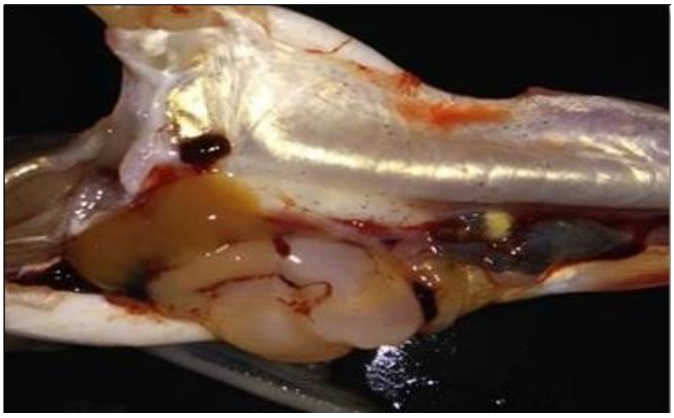

Fig 6 Pale liver

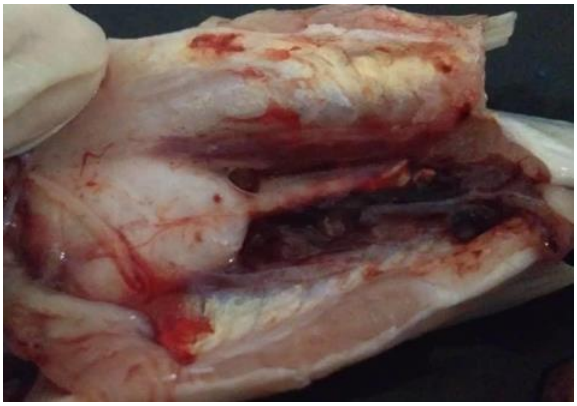

Fig 7 Congested kidney

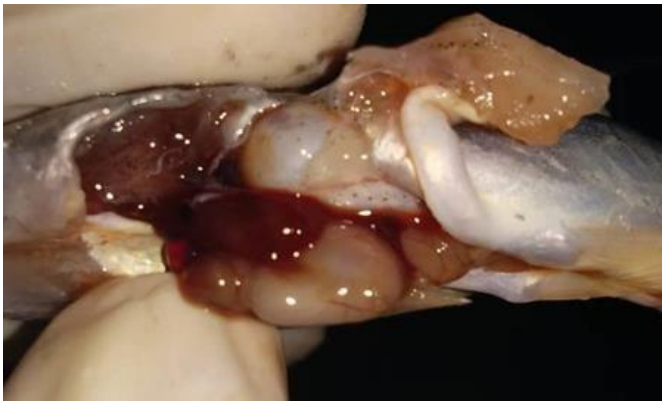

Fig 8 Patchy liver

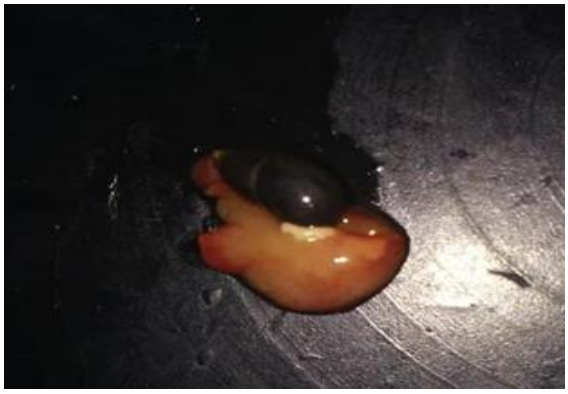

Fig 9 White nodules on liver

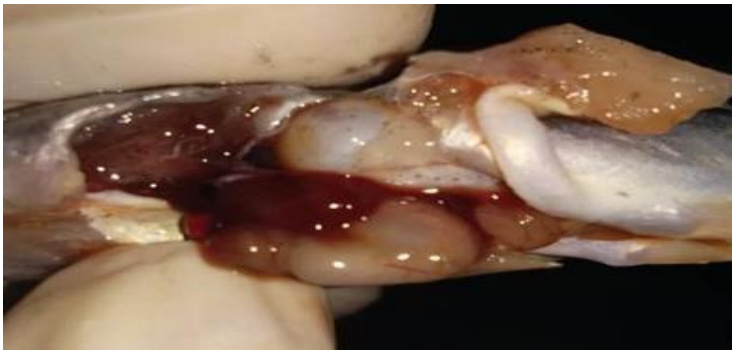

Fig 10 Dark liver

Lethal Dose 50 of *Pangasius nasutus*

The lethal dose 50 (LD_{50}) of *Edwardsiella ictaluri* was determined by plotting the graph of mortality percentage of *Pangasius nasutus* against different concentrations of *Edwardsiella ictaluri* as variables by using the Curve Expert software. Based on (Fig 11), the graph has been plotted and the linear line could be drawn which was satisfyingly validated to represent a trend line and almost pass all the plotted point in the graph. The calculation of LD_{50} has been computed by using the graph analysis and the value of LD 50 calculated in the graph was 1×10^6 cfu/ml (Fig 12).

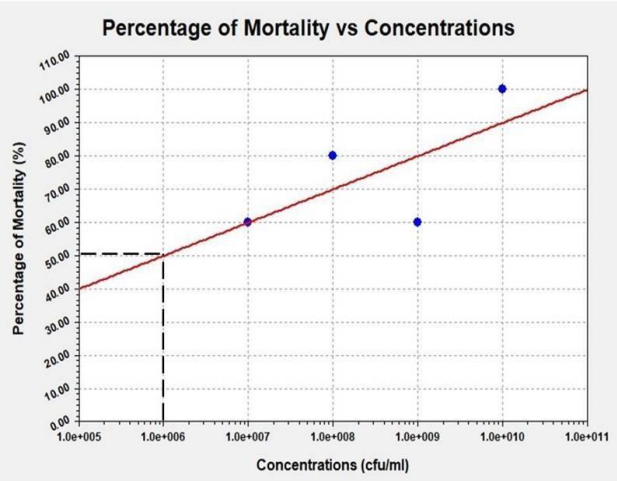

Fig 11 Graph of mortality percentage of *Pangasius nasutus* against concentration of *Edwardsiella ictaluri*

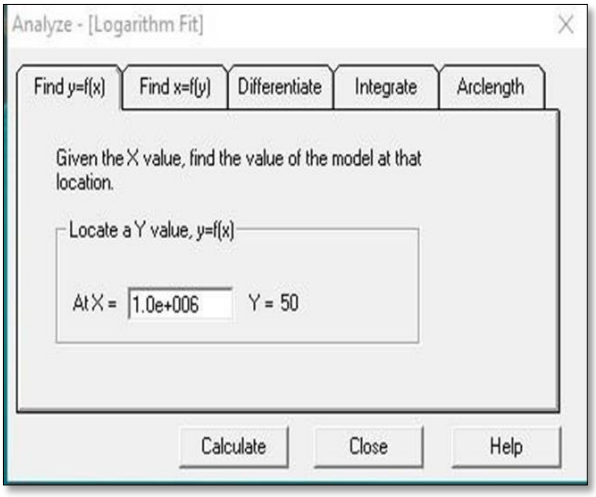

Fig 12 LD_{50} of *Edwardsiella ictaluri* on *Pangasius nasutus*

Statistical Analysis

It has been analysed that there was no significant difference between percentage mortality of *Pangasius nasutus* and concentration of *Edwardsiella ictaluri* as P or significant value is more than 0.05 ($p > 0.05$). Therefore, the null hypothesis is not rejected.

Discussion Lethal Dose 50 (LD_{50})

Table 6 Size of experimental fish and LD_{50} based on published literature

Studies on LD_{50} and Pathogenicity of *Edwardsiella ictaluri* in Catfish	**Size of fish**	**LD_{50}**
Susanti, Indrawati & Pasaribu, 2017	6-10 g	2.8×10^4 cfu/ml
Dong et al. 2015	26.7 g ±8.7 g	2.6×10^6 cfu/ml
Koswara, 2009	5-6 g	1.3×10^4 cfu/ml
Phuoc, Richard & Crumlish, 2020	15-20 g	1.1×10^7 cfu/ml
Current study	40 g ± 5g	1×10^6 cfu/ml

Based on the results of this comprehensive study, it has been determined that the LD_{50} (lethal dose for 50% of the population) of *Edwardsiella ictaluri* on *Pangasius nasutus* is 1×10^6 cfu/ml. This finding is supported by a study conducted by [12], which found that the LD_{50} of *Edwardsiella ictaluri* causing 50% mortality in *Pangasianodon hypophthalmus* with the size of 6-10 g is 2.8×10^4 cfu/ml. Moreover, [2] reported in their study that an infection of *Edwardsiella ictaluri* with a concentration of 2.6×10^6 cfu/ml can result in 50% mortality in *Pangasianodon hypophthalmus*. Another noteworthy research study conducted by [6] reported that the LD_{50} of *Edwardsiella ictaluri* on *Clarius sp* is 1.3 x 10^4 cfu/ml. Finally, according to [10], LD_{50} of *Edwardsiella ictaluri* causing 50% mortality in *Pangasianodon hypophthalmus* is 1.1×10^7 cfu/ml.

Based on findings of multiple comprehensive literatures conducted in this study, it can be inferred that the LD_{50} result obtained from current study was relatively higher by using local isolates in approximately 40 g fish. One of the most significant factors identified is the notable difference in the size of the catfish used between those literatures. Theoretically, it is wellestablished that the LD_{50} value tends to be lower in smaller-sized fish when compared to their larger counterparts. This is primarily due to the relatively underdeveloped immune system of smaller fish, rendering them more susceptible to bacterial infections [15]. The less mature immune system of smaller fish creates a vulnerability that allows bacteria to easily infect the host [16]. On the

other hand, larger fish typically possess a more evolved and robust immune system, necessitating a higher concentration of bacteria to successfully infect the host [15].

Pathogenicity and Clinical Signs

Based on the results of this experiment, it has been observed that the earliest clinical sign recorded is infected fish with a concentration of 1×10^{10} cfu/ml. After a period of 2 hours postinjection, the infected fish exhibit a pale liver and congested kidney after the dissection procedure. Furthermore, one of the notable clinical signs that can be observed in a tank with a concentration of 1×10^{8} cfu/ml is the catfish displaying a tendency to swim vertically, with its head positioned on the water surface shortly before succumbing to the infection. According to [12], their study found that *Pangasianodon hypophthalmus* infected by *Edwardsiella ictaluri* with concentrations ranging from 1×10^{4} - 1×10^{10} cfu/ml results in fish swimming vertically with their head on the water surface. Additionally, [5] also noted in their research that the infection of *Edwardsiella ictaluri* in fingerling yellow catfish can lead to the fish swimming with their head hanging on the water surface just prior to death.

The main possible reason for the observed clinical sign in fish is hypoxia, a condition characterized by low oxygen levels in the body. This can occur when the defence mechanism, particularly the immune system, is compromised. In the case of fish infected with *Edwardsiella ictaluri,* it is believed that fat degeneration occurs due to the presence of a toxin that disrupts the normal pathways of fat metabolism. This disruption ultimately leads to hypoxia, resulting in reduced blood flow and oxygen supply to the fish's body. As a consequence of hypoxia, fish may exhibit abnormal swimming behaviour, such as erratic and vertical movements. It appears as if the fish are hanging their heads on the water surface, struggling to maintain their equilibrium. This behaviour can be attributed to the fish's attempt to obtain more air supply, as the reduced blood flow limits the amount of dissolved oxygen reaching their tissues [6]. Other clinical signs that can be observed in this experiment include inflammation on the lower part of the body, tail, and fin. The main cause of inflammation on the lower abdomen of catfish is the effect of *Edwardsiella ictaluri* Lipopolysaccharide (LPS). Lipopolysaccharides, which are the main component of the exterior membrane of Gram-negative bacteria, act as endotoxins. They play a crucial role in the pathogenesis of infections caused by these bacteria. The Opolysaccharide chains of LPS are involved in resistance to complement-mediated killing. Complement is a part of the immune system that helps in clearing microbes and damaged cells from the body. By interacting with antibodies and phagocytic cells, LPS enhances the ability of the immune system to identify and eliminate pathogens. However, this immune response also leads to the promotion of inflammation and the attack on the pathogen's cell membrane.

A study by [8] highlighted the role of LPS in promoting inflammation and its impact on the pathogen's cell membrane. It was found that LPS has the ability to induce an inflammatory response and cause tissue damage in catfish. According to [11], *Edwardsiella ictaluri* LPS specifically affects internal inflammation and tissue damage in catfish. In summary, the presence of *Edwardsiella ictaluri* LPS in catfish leads to inflammation in various parts of the body, including the lower abdomen, tail, and fin. This inflammation is a result of the interaction between LPS and the immune system, which aims to clear the infection. However, this immune response can also cause tissue damage.

Next, based on the findings of this study, the clinical signs that can be observed in infected catfish include the presence of patchy, lesions, and white nodules on the liver. According to the research conducted by [14], it has been described that a majority of *Edwardsiella ictaluri* strains exhibit haemolytic activity across a range of temperatures. Haemolysin, in essence, can be defined as an extracellular toxic protein that plays a crucial role in the penetration of mucosal layers. By facilitating intracellular survival, haemolysin enables the bacteria to spread more effectively within the host organism. This phenomenon elucidates the ability of *Edwardsiella ictaluri* to colonize the layer of peritoneal muscle and internal organs of catfish, as the bacteria are capable of penetrating the mucosal layer, which serves as the fish's primary defense mechanism in its immune system.

Furthermore, based on the findings of this study, the clinical signs that can be observed in infected catfish include the presence of patchy, lesions, and white nodules on the liver. The significance of these findings is highlighted by the research conducted by [14], which indicates that a majority of *Edwardsiella ictaluri* strains exhibit haemolytic activity across a range of temperatures. Haemolysin, an extracellular toxic protein, plays a crucial role in the pathogenicity of *Edwardsiella ictaluri*. It facilitates the penetration of mucosal layers, allowing the bacteria to establish an intracellular presence. This ability to survive and replicate within host cells enhances the spread of the bacteria throughout the host organism. Consequently, *Edwardsiella ictaluri* is able to colonize the peritoneal muscle layer and internal organs of catfish. This colonization is made possible by the bacteria's ability to penetrate the mucosal layer, which serves as the first line of defense in the fish immune system.

Furthermore, clinical signs that can be observed on the catfish are hemorrhagic in the upper mandible and fin. The main cause of hemorrhagic symptoms on the fish's body is due to damaged capillary endothelium, where infectious agents are circulating in the blood vessels [12]. Therefore, this indicates that *Edwardsiella ictaluri* is a destructive septicemia blood vessel, as bleeding occurs and forms an accumulation of blood fluid in the peritoneal cavity.
This is the main reason for the congested kidney that occurs internally in the catfish's body.

Conclusion

The LD_{50} of local bacterial isolate of *Edwardsiella ictaluri* on *Pangasius nasutus* was 1×10^6 cfu/ml in approximately 40 g fish. The tank infected with *Edwardsiella ictaluri* concentration of 1×10^{10} cfu/ml recorded the highest cumulative mortality rate, with 100% mortality. The earliest clinical sign was observed in the tank with concentration of 1×10^{10} cfu/ml 2 hours post infection where pale liver and congested kidney observed after dissection procedure. The finding of this research could lead to a better treatment and prevention of *Edwardsiella ictaluri* in *Pangasius nasutus.*

Abbreviations
Brain Heart Infusion (BHI); CFU: Colony Forming Unit; E. ictaluri: Edwardsiella ictaluri; PCR: Polymerase Chain Reaction: TSA; Trypticase soy agar; ESC: Enteric Septicemia of Catfish

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