International Journal of Life Sciences and Biotechnology
Syahidah N.A., et al., Prevalence of *Edwardsiella ictaluri* in Cage Cultured *Pangasius spp* in Pahang River and Their Risk Factors. International Journal of Life Sciences and Biotechnology, 2024 7(2): p. 37-45.
https://doi.org/10.38001/ijlsb.1414371
Araştırma Makale/ Research article

Prevalence of *Edwardsiella ictaluri* in Cage Cultured *Pangasius spp* in Pahang River and Their Risk Factors

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ABSTRACT
Striped catfish, *Pangasianodon hypophthalmus* is a native species to most Asian countries, including Malaysia which faced series of bacterial disease such as edwardsiellosis caused by *E.ictaluri*. *Edwardsiella ictaluri* is a Gram-negative intracellular bacterial pathogen which had caused mortalities in farmed or wild fish in lots of countries and accountable for large economic losses. This study was conducted to determine disease status of Pangasius spp due to *Edwardsiella ictaluri* and to determine the risk factors of *E.ictaluri* in striped catfish in Pahang River, Malaysia. Four sampling sites were chosen, two sites in Pekan (Kampung Belimbing and Kampung Tanjung Pulai) and two sites in Temerloh (Kampung Teluk Ira and Kampung Bintang). Thirty samples were collected from each farm for 6 months consecutively and four organ tissues were taken which were liver, spleen, kidney, and brain. External and internal clinical signs were recorded accordingly. DNA was extracted from all tissues and pursued for PCR to detect the presence of *Edwardsiella ictaluri*. Results show that the infected fish has gross internal clinical signs such as patchy liver, white nodular spleen, and congested kidney. The prevalence rate of *E.ictaluri* was highest at Kampung Tanjung Pulai in June with 16.67% compared to all farms. No infection of *E.ictaluri* shown at Kampung Belimbing during the whole sampling session. Spearman's rho correlation showed that the prevalence of *E.ictaluri* has strong correlation with temperature in Kampung Tanjung Pulai ($p > 0.05$). Meanwhile those bacteria prevalence have strong relationship with ammonia, sulfide and total suspended solid (TSS) with $p< 0.05$. The susceptible size for this bacterium in striped catfish is between 1 to 50g. To our knowledge, this paper is the first report for *Edwardsiella ictaluri*in cage cultured *P.hypopthalmus* in Pahang River, Malaysia.

ARTICLE HISTORY
Received
05 January 2024
Accepted
01 March 2024

KEYWORDS
Prevalence,
Edwardsiella ictaluri,
Pangasius hypophthalmus,
risk factor, PCR

Introduction

Pangasius hypophthalmus or striped catfish is classified into Pangasidae family and is one of the most popular species raised through aquaculture. Primarily, striped catfish are native to the Mekong Delta in Vietnam and recent years are extending most to Asian continent [1]. In Malaysia, the production of Pangasius spp showed tenfold increase from 10,891.51 tons in 2011 to 20, 861.9 tons in 2022 [2]. The culture of Pangasius spp in Malaysia become one of the primary activities for locals as their source of income, especially in Pahang River which located at Peninsular of Malaysia.

Despite the positive contribution of aquaculture, it is still an intensive agricultural practice and health-related problems that hinder both economic and socio-economic expansion of the sector. The primary constraint to many aquaculture species is the emergence of infectious diseases caused by pathogens such as bacteria, viruses, fungi, and infestations caused by parasites [3]. The majority of disease-related deaths in the catfish industry emanate from bacterial diseases, with the most common bacteria are caused by Edwardsiella ictaluri, Flavobacterium columnare, and Aeromonas hydrophila in channel catfish [4,5].

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Edwardsiella ictaluri is a Gram-negative and rod-shaped bacterium, emerging from the family of Enterobacteriaceae. It had become one of the most threatening bacteria which had caused massive loss to the catfish industry. This bacterium is the aetiological agent of Enteric Septicaemia of Catfish (ESC) in channel catfish (Ictalurus punctatus) and Bacillary Necrosis of Pangasius in striped catfish (Pangasianodon hypophthalmus) [1,6]. The external part of infected fish possessed swollen abdomen and petechial haemorrhages on the tail, fins, and occasionally over the rest of the body while internally presenting pinpoint white spot at the liver, kidney and spleen [7].

According to [8], a five years of survey study conducted in the Tama River, Japan, results indicate that mortality of ayu due to Edwardsiella ictaluri was driven by large water temperature fluctuations and low streamflow during unusual hot summer. Clinically sick fish were emaciated, and several had internally, white 1±3 mm diameter miliary lesions were observed under the capsular surface and throughout the parenchyma in the liver, kidney and spleen of diseased fish. Meanwhile, another in vitro challenge study showed that a pH value of 6.5 and NaCl concentration of 0.5% was optimal for the growth of bacteria E.ictaluri on striped catfish [9]. Given the importance of environmental conditions on the host-pathogen interaction, the aim of this study is to determine the disease status of E.ictaluri and to determine their risk factors in cage cultured striped catfish in Pahang River, Malaysia.

Material and Methods

Sampling sites

This study was carried out at Pahang River, Malaysia. Two different districts were chosen for this study which were at Pekan and Temerloh. Temerloh located upstream than Pekan which located more towards downstream. In Temerloh, two sampling sites were chosen which were Kampung Teluk Ira and Kampung Bintang, meanwhile in Pekan were at Kampung Belimbing and Kampung Tanjung Pulai. (Figure 1). These two districts are well known with their aquaculture activities especially for *Oreochromis* and *Pangasius* industry among local.

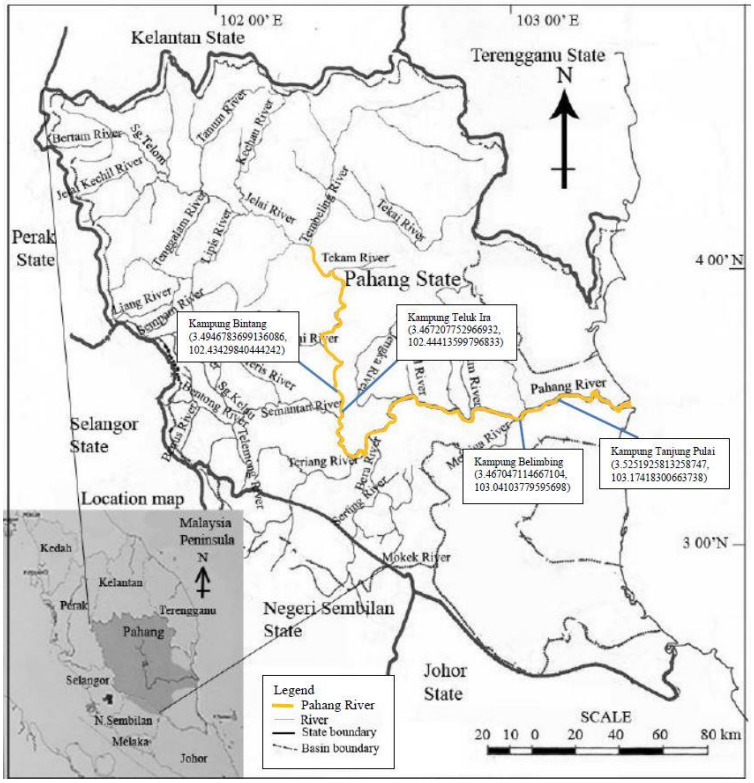

Fig 1 The map of Pahang State. Yellow line indicates Pahang River. Kampung Bintang and Kampung Teluk Ira were in Temerloh. Kampung Belimbing and Kampung Tanjung Pulai are located in Pekan

Fish sampling

Enteric septicemia of catfish (ESC), caused by the Gram-negative bacterium *Edwardsiella ictaluri*, is one of the most important diseases of farm-raised channel catfish (*Ictaluruspunctatus*), and now known to occur throughout the geographic range of the catfish industry. The source of the striped catfish fingerlings was obtained from the same supplier. On average, the size of the fish is about 3.5 inches to 4 inches during the

restocking phase. Early screeningfor each batch was done to detect the presence of *E.ictaluri* bacterium to ensure no infection occurred before the experiment. The total of samples throughout the study were 480 fish. At each farm, 30 samples were taken monthly from the two cages. Each sample was measured for their length and weight before examined for the external clinical signs. Fish were then pithed between its head and body segment to immobilize and kill the fish. Any internal clinical signs were observed and recorded. Spleen, liver, kidney, and brain were removed carefully and stored in -20°C for further used.

In Pekan at Kampung Tanjung Pulai, the size of the cage is 10x15x4 feet stocked with 4000 fingerlings. At Kampung Belimbing, the cage size was 10x9x5 feet were stocked with 3000 fish. In Temerloh, both Kampung Teluk Ira and Kampung Bintang were stocked with 4000 fisheach farm with cage size of 10x12x5 ft. These fish were reared until they were harvested at marketable size which consumed within seven to nine months.

Detection and identification of *E.ictaluri*

The collected tissue organ then were then proceeds with DNA extraction using DNA extraction kit (PrimeWay Genomic DNA extraction kit, Apical Scientific Sdn. Bhd., Malaysia).DNA fragment was checked qualitatively and quantitatively by using gel electrophoresis and Nanodrop. Polymerase chain reaction technique was used to confirm *E.ictaluri* as described inprevious study [11].

The primer set used are stated in the Table 1 below:

Table 1 Primers used to detect *Edwardsiella ictaluri*

Type	Primers	Size
Species specific for *Edwardsiella ictaluri* [11]	IVS (5'- TTA AAG TCG AGT TGG CTT AGG G-3')	2000bp
	IRS (5'-TAC GCT TTC CTC AGT GAG TGT C -3')	

The PCR mixture used were as in following Table 2:

Table 2 PCR reagents used

Reagent	Amount	Concentration
PCR buffer (Thermo Scientific)	2 µL	10x
MgCl2 (Thermo Scientific)	2 µL	25 mM
dNTP (Thermo Scientific)	0.5 µL	10 mM
IVS (NHK Bioscience, Kuala Lumpur, Malaysia)	0.5 µL	100 pmol μL^{-1}
IRS (NHK Bioscience, Kuala Lumpur, Malaysia)	0.5 µL	100 pmol μL^{-1}
DNA template	1 µL	50ng/µL minimum
Taq DNA Polymerase (Thermo Scientific)	0.5 µL	5 μL^{-1}
Distilled water	43 µL	
Total volume	50 µL	

The amplification was performed in thermocycler machine (Techne TC-3000 PCR ThermalCycler, GMI, US) with minor modification from the reference data [11] as in Table 3:

Table 3 Temperature and time setting up in PCR machine.

Step	Temperature	Time
Initial denaturation	94°C	4 min
Denaturation	94°C	1 min
Annealing	50°C	1 min
Extension	72°C	1 min
Final elongation	72°C	10 min

The cycle run for 35 cycles starting from denaturation to extension phase. The amplifiedDNA were then mixed with 6x loading dye (Thermo Scientific) and were distinguished by usinggel electrophoresis with stained 1% Agarose in 1x Tris/Borate/EDTA (TBE) buffer at 100V, 400A for 30 minutes. Any band appear were compared to the 1kb standard marker (Thermo Scientific).

For validation, *Edwardsiella ictaluri* (ATCC® 33202™) strain were used as positive control for each reaction. Fish samples that appeared band on the gel which in line with the positive control were considered positive with *E.ictaluri* strain.

Water quality sampling

In this study, water samples were collected monthly. The reading of *in-situ* physical datawas taken at three different depths at three different points along the farm. For water chemicaldata, the water sample were only collected from the surface at three different point. Water samples for chemical analysis were collected in sterilized 250ml high-density polyethylene (HDPE) bottles. These samples then were kept straight away in an insulated ice box and transported to the laboratory for immediate analysis.

The physical parameters that were measured in this study were temperature, pH, dissolved oxygen (DO) and total suspended solid (TSS) using a portable multiprobe (NKEInstrumentation WiMo multi-parameter sonde) and TSS were measured by using a colorimeter(HACH DR900, HACH Company, Loveland,CO, USA).

Meanwhile, the chemical parameters observed were ammonical-nitrogen (AN), sulfide, nitriteand iron. These parameters were assessed based on the standard procedures in the laboratory. The analytical determination of ammonical-nitrogen was using salicylate method, iron was using FerroVer method, nitrite used methylene blue method and sulfide were determined according to diazotization method. These methods required the usage of powder pillow and sulfide reagents for sulfide test and were used colorimeter (HACH DR900, HACH Company, Loveland,CO, USA) for measurement according to their respective wavelength.

Data analysis

The mean prevalence of *E.ictaluri* in reared pangasius was calculated and compared with growth rate by using Excel software. All data were analyzed by using IBM SPSS Statistics(Version 26). All data variables were arranged horizontally according to the timeline and the subject which are the farms were arranged vertically. Then, screening for multivariate outliersfrom the data was performed through Mahalanobis distance where this step is essential prior toperforming data analysis. The distance of a case is analyzed from the centroid of all cases in a data set which is conducted through multidimensional statistical techniques by Malahanobis distance. The centroid is a point in multivariate space where all means from all variables intersect which determine the value of Mahalanobis distance (the bigger the value of MD, the further away from the centroid the data point is).

Then each of the chemical and physical parameters were determined for their possible association with the prevalence of *E.ictaluri* through Spearman's rank-order correlation (ρ). Spearman's correlation measures monotonic relationships (whether linear or not) and if there are no repeated data values, a perfect Spearman correlation of +1 or −1 occurs when each of the variables is a perfect monotone function of the other.

Results and Discussion

Clinical signs of *Edwardsiella ictaluri*

During the sampling session, clinical signs observed were lethargy, inflame anus, patchy liver,white nodular spleen, black and rough nodular spleen, and puss kidney (Figure 2, Figure 3, Figure 4).

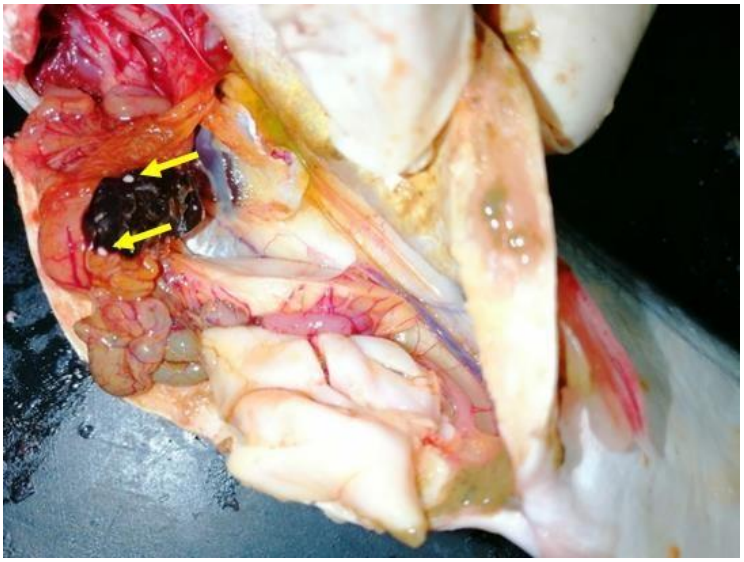

Fig 2 White nodular at spleen

Fig 3 Inflame anüs

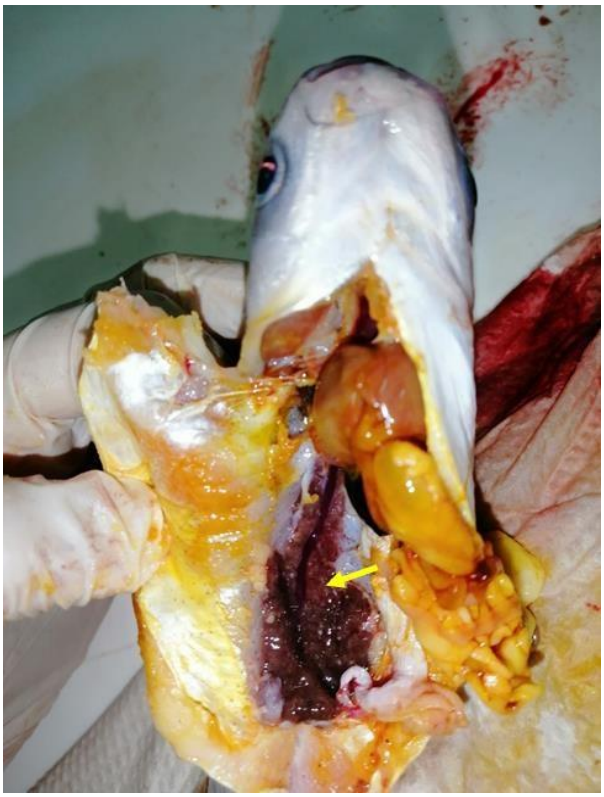

Fig 4 Puss kidney with straw-colour ascitic fluid

During the whole sampling session, there were neither acute nor chronic external clinical conditions presented rather than minor inflammation on the fin or anus. Acute externalclinical signs on affected catfish may have internal fluid accumulation that can lead to a swollen abdomen and exophthalmia, small red and white ulcers which cover the skin, pinpoint red spotsappearing under the lower jaw or belly region, and a raised or eroded red ulcer with inflammatory exudate protruding through the cranial foramen at the top of the skull, where chronic signs might show ulceration of the cranial foramen [1,7,12]. However, most of the affected fish had almost similar gross internal clinical signs such as such as pale areas of tissuedestruction (necrosis) or a general mottled red and white appearance in liver, white nodular spleen, black and rough nodular spleen, and puss kidney from the samples. Few of them showedclear, straw-colored, or bloody fluid (ascites) as per described by [12]. Liver, spleen, and kidneyare important for the defense systems in teleost. Kidney and spleen form an

extensive networkwhich is vital in trapping blood-borne substances. The populations of lymphocytes and macrophages in these organs can exhibit an immune response which is situated close to sites ofantigen trapping and frequently associated with accumulations of cell masses called melanomacrophages (MMC) [13]. The MMCs are histologically distinguishable within the tissue for presenting macrophages with distinct pigments such as melanin, hemosiderin and lipofuscins [14]. Melanin of MMC are more or less colored by yellow, brown or black pigmentswith its phagocytic characteristics which consume foreign particle, catabolic product or carbonparticle [15].

Bacteria identification from PCR

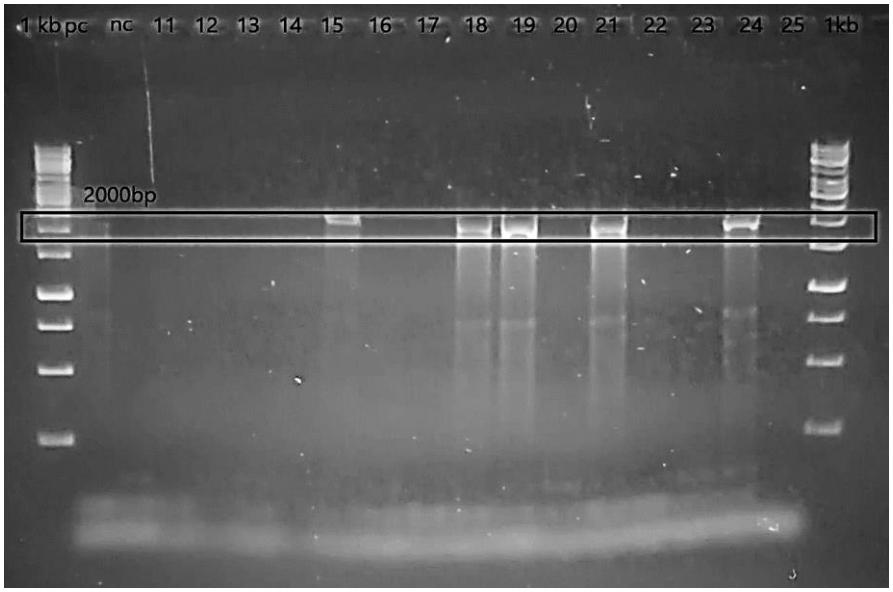

Fig 5 Samples from Kampung Tanjung Pulai during the fingerling size about 3.5 inches to 4 inches. From most left is 1kb ladder, followed by lane 2 (positive control; ATCC 33202), lane 3 (negative control), lane 4 until lane 18 (DNA samples from pooled organ), lane 19 (1 kb ladder). The positive samples with *E.ictaluri* were on the lane with sample numbered 15, 18,19,21 and lane 25 with the size of 2000bp referring to the positive control lane.

The pooled DNA of spleen, liver, kidney, and brain samples were tested with PCR by using IVS-IRS primer. Infected fish showed positive band with the size of 2000bp viewed from the gel electrophoresis of PCR product (Figure 5).

Prevalence Rate of *E.ictaluri*

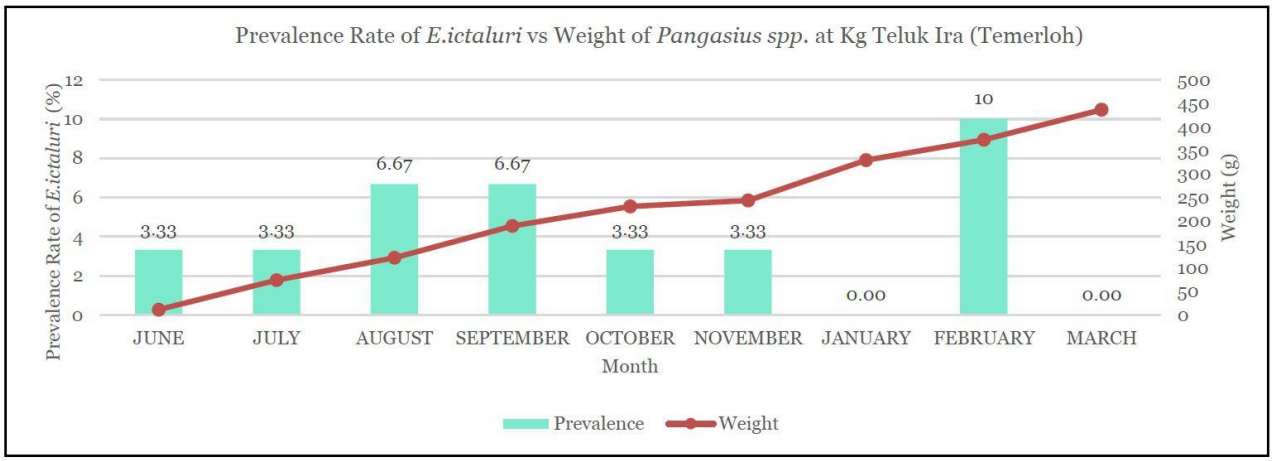

Fig 6 Prevalence Rate of *E.ictaluri* at Kampung Teluk Ira (Farm 1)

Throughout sampling session at Kampung Teluk Ira (Farm 1), *E.ictaluri* was observed in all months except in January and March. Meanwhile the highest prevalence of *E.ictaluri* (10%) was on February where the weight of the fish samples reached about 350g (Figure 6).

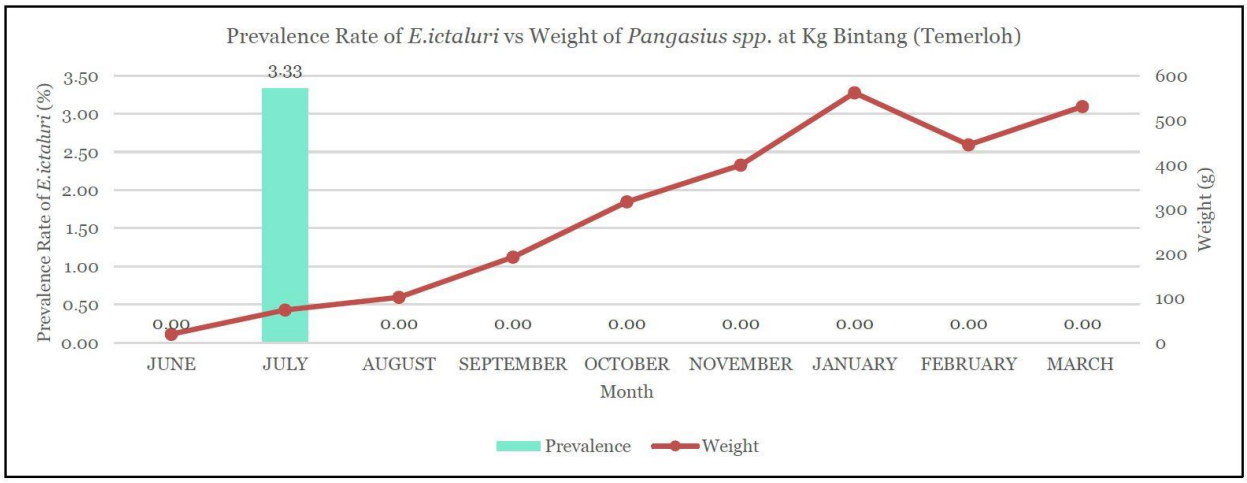

Fig 7 Prevalence Rate of *E.ictaluri* at Kampung Bintang (Farm 2)

The situation was reversible at Kampung Bintang (Farm 2) compared to Kampung Teluk Ira.Throughout the 9 months of sampling, prevalence of *E.ictaluri* was only in July with 3.33% (Figure 7).

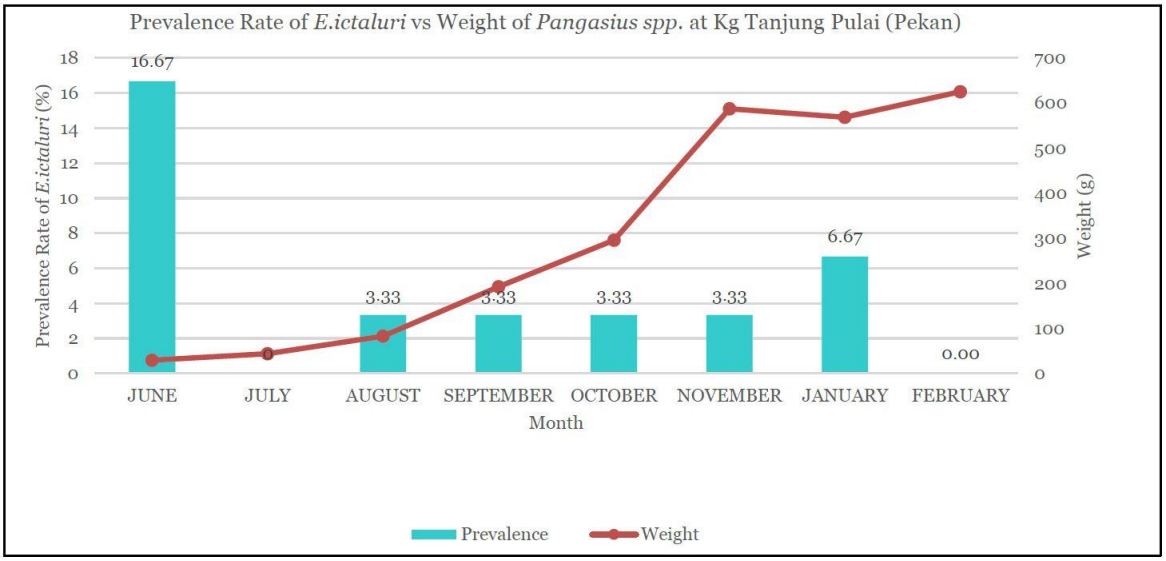

Fig 8 Prevalence Rate of *E.ictaluri* at Kampung Tanjung Pulai (Farm 3)

In Pekan at Kampung Tanjung Pulai (Farm 3), the highest prevalence was shown in June with 17.24%, where the average weight of the striped catfish at that time was less than 100g. The second highest prevalence was in January with 6.67% (Figure 8).

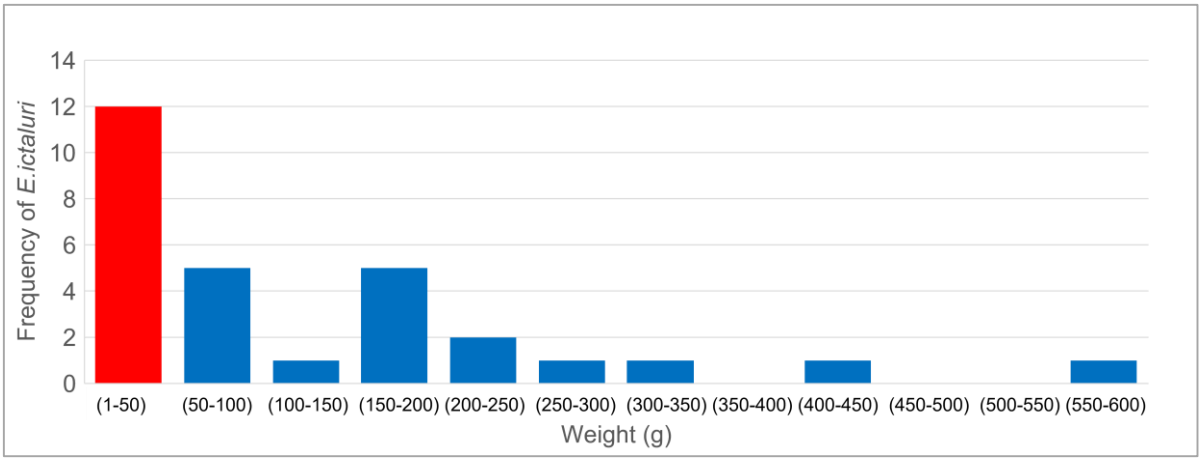

Fig 9 Susceptibility size of *E.ictaluri* in cage cultured striped catfish in Pahang River. The red bar chart indicate the most susceptible size of *Pangasius hypophthalmus* towards *E.ictaluri*. The blue bar chart show the frequency of *E.ictaluri* found in *P.hypopthalmus* according to their sizes throughout this study.

Compared to another farms, Farm 3 recorded the highest prevalence of *E.ictaluri* which obtained 16.67% where the average size of fish reached 29.5g during the first month of sampling. In overall, the susceptibility of this bacterium in stripe catfish is during the first month especially during the first restocking in the cage when the fish reach size 1g to 50g (Figure 9).

Fish are more dependent on theirnon-specific immune system for survival during the early stages of their embryonic life than higher vertebrates [16]. Several external and internal factors can influence the activity of innateimmune systems in teleost. Temperature changes, handling and crowding stress can have suppressive effects on innate parameters, whereas several food additives and immunostimulantscan enhance different innate factors [17]. In Vietnam, BNP in freshwater catfish (*Pangasianodon hypophthalmus*) may occur in fish of all ages, although especially fingerlings and juvenile fish seem to be more affected [18,19]. In general, fingerlings are high tendency to infection from *E.ictaluri* but adults may also succumb to the disease as well even though the immune status of individuals in the population may determine the outcome [12].

Edwardsiella ictaluri is susceptible to the striped catfish when environmental conditions are conducive to proliferation of the pathogen and stressful for the host. Such stressfulconditions include several factors such as netting, handling, over stocking density, improper diet and poor water quality like low oxygen or high levels of ammonia and nitrite [12]. There were significant ($p < 0.05$) positive correlations between the prevalence of *E.ictaluri* with concentration of ammonia, sulfide and total suspended solid (TSS) at Kampung Teluk Ira (Spearman's rank correlation test, rs = 0.55-0.56).

Ammonia is the main nitrogenous excretory product of aquatic animals, especially for feed-based aquaculture. Large stocking density without a proper management release more ammonia nitrogen into the culture system [20]. In tilapia, carp, and pufferfish, ammonia was found to cause tissue swelling and hydropic degeneration, increasing oxidative stress and apoptosis in liver tissues [21]. Meanwhile, the exposure of fish to high suspended solids can cause physical damage to gill structure of fish and clogging which leads to respiratory failure and mortality [22]. The same effect of total suspended solid in water may result such disturbancein migrations and spawning, movement pattern, reduced hatching rate as well as directmortality. Sulfide can be produced in the absence of oxygen in sediment by sulfate-reducing bacteria and enter the water column. Sulfide level which exceeds the optimum level may inhibitenergy metabolism by cells, which have the same effect as hypoxia. The condition in cage witha very low running water and lowered to the bottom of the riverbed might mimical to the condition in the pond which may lead to the deterioration of water quality within the cage itself. Previous study showed that striped catfish only exploited the upper-most 25% of the water column and frequently experienced severe hypoxia at night and at depths greater than 1 m belowthe surface, and with increasing hypoxia and anoxia towards the bottom [23]. Temperature values are varied at all sampling sites throughout the study, ranging from 25.88ºC to 30.43ºC which is very similar to previous study for the growth of *E.ictaluri* [24,25].

Conclusion

In conclusion, *Edwardsiella ictaluri* appear year-round in cultured *Pangasius hypopthalmus*. The factors which cause the disease are interrelated to each other. Improper stocking density adding the stress factor and thus decreasing the immune system in fish.

However, the susceptibility of this bacterium in stripe catfish is more prone during the first three month when the fish reach size 1g to 50g and 150g to 200g. The prevalence of *E.ictaluri*have strong relationship with ammonia, sulfide and total suspended solid (TSS). Deep investigation such as histology and study of cortisol level in fish is needed to determine the stress level of striped catfish with these correlated parameters. Further study on the co-infectionof *E.ictaluri* with another bacteria is also required to understand the epidemiological of this bacterium in natural environments.

Abbreviations

TSS: Total Suspended Solid; DO: Dissolved Oxygen; AN: Ammonical-nitrogen; PCR:polymerase chain reaction; ESC: enteric septicemia catfish; MMC: melanomacrophage

Funding

This study is funded by Department of Fisheries Malaysia in collaboration with Department ofMarine Science, Kulliyyah of Science, International Islamic University Malaysia.

Availability of data and material

Please contact the corresponding author for any data request.

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