UNIVERSITI PUTRA MALAYSIA

PATHOGENICITY ASSESSMENT OF CELLULAR MEMBRANE PRODUCTS (CMPs) AND EXTRACELLULAR MEMBRANE PRODUCTS (ECPs) OF *Aeromonas hydrophilia* TOWARDS AFRICAN CATFISH *(Clarias gariepinus).*

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SERDANG SELANGOR

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A project paper submitted to the Faculty of Veterinary Medicine

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In partial fulfilment of the requirement for the degree of

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It is hereby certified that we read this project paper entitled "Pathogenicity Assessment of Cellular Membrane Products (CMPs) And Extracellular Membrane Products (ECPs) of *Aeromonas hydrophilia* towards African Catfish *(Clarias gariepinus)",* by Humairak binti Shariruzi and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999-Project.

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4990-Projek.

PENILAIAN PATOGENISITI PRODUK MEMBRAN SEL (PMS) DAN EKSTRASELULAR (ES) DARIPADA *Aeromonas hydrophilia* TERHADAP IKAN KELI AFRIKA *(Clarias gariepinus)*

HUMAIRAK BINTI SHARIRUZI

MAC 2016

Penyelia utama: Prof. Madya Dr Hassan Hj. Mohd. Daud

Penyelia bersama: Dr Mohd Fuad Matori

Produk membran sel bakteria adalah pelet yang terdiri daripada struktur permukaan yang paling banyak, dikenali sebagai lapisan-S. Produk ekstraselular pula adalah supernatan bakteria terdiri daripada *haemolysin*, *enterotoxin*, *cytotoxin* dan *protease*. Pada masa ini, kajian patogenisiti terhadap ikan produk membran sel dan produk ekstraselular amat kurang. Tujuan kajian ini dijalankan adalah untuk menemtukan sama ada produk membran sel dan produk ekstraselular daripada *A. hydrophilia* samada boleh menyebabkan kesan patogenik terhadap ikan keli Afrika *(Clarias gariepinus). Aeromonas hydrophilia* telah dicambah dalam Tryptic Soy Broth (TSB) dan dituai dengan menggunakan kaedah emparan pada kelajuan 1800xg selama 15 minit bagi memisahkan produk membran sel dan produk ekstraselular dalam bancuhan bakteria. Kemudian, produk membran sel dan produk ekstraselular dicairkan ke cairan 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dan disimpan sejuk sebelum digunakan. Setiap cairan produk membran sel dan produk ekstraselular akan disuntik kepada ikan melalui intraperitoneal dan air salin normal disuntik sebagai kawalan. Serum dikumpulkan pada hari kelapan selepas suntikan. Kehadiran antibodi dalam serum ikan dikesan melalui ujian *Agar Gel Precipitation Test* (AGPT). Tanda-tanda klinikal seperti kelesuan, pereputan sirip, kekelabuan kornea, mata tersembul, bola mata pecah dan hakisan lapisan epidermis diperhatiakan. Berdasarkan jumlah kematian kumulatif, produk membran sel lebih patogenik daripada produk ekstraselular. Secara statistic walau bagaimanapun, tiada perbezaan signifikan dalam kematian kumulatif di antara produk membran sel dan produk ekstraselular. Walaupun tiada tanda reaksi Ab-Ag berlaku dalam ujian AGPT, dipostulat bahawa produk membran sel dan produk ekstraselular dapat mendorong tanda-tanda klinikal yang ketara dan merangsang tempoh imuniti yang singkat.

Kata kunci: *Aeromonas hydophilia*, Produk sel membrane, Produk ekstraselular, *Clarias gariepinus*, patogenisiti.

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement of the course VPD 9999- project.

PATHOGENICITY ASSESSMENT OF CELL MEMBRANE PRODUCTS (CMPs) AND EXTRACELLULAR PRODUCTS (ECPs) ISOLATED FROM *Aeromonas hydrophilia* FOR TOWARDS OF AFRICAN CATFISH *(Clarias gariepinus)*

HUMAIRAK BINTI SHARIRUZI

MARCH 2016

Supervisor: Assoc. Prof. Dr Hassan Mohd. Hj. Daud

Co-Supervisor: Dr Mohd Fuad Matori

Cell membrane products (CMPs) are made of bacterial cell pellet which consisted of most abundant surface structure termed S-layers. While extracellular products (ECPs) are bacterial supernatant made of haemolysin, enterotoxin, cytotoxin and protease. However, there was lack of previous study done on the effect of CMPs and ECPs towards pathogenicity of fish. This study aimed to determine whether CMPs and ECPs of *A. hydrophilia* stimulate the pathogenicity of African catfish (*Clarias gariepinus)*. *Aeromonas hydrophilia* was cultured in Tryptic Soy Broth (TSB) and harvested by centrifugation at 1800 xg for 15 minutes to separate the CMPSs and ECPs of bacterial suspension. They were diluted into 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} suspension and stored chill prior to use. The CMPs and ECPs were then injected into fingerlings intraperitoneally, including normal saline as a control. The fish sera were collected at 8 dpi from caudal peduncle vein. Presence of antibody in the fish's sera were detected using Agar Gel Precipitation Test (AGPT). Clinical signs such as lethargy, severe fin rot, corneal opacity, exophthalmia, eye-ball rupture and erosion of epidermis were noticed. CMPs was more virulence than ECPs based on cumulative mortality. Statistically, there was no significant different in cumulative mortality between CMPs and ECPs. There was no Ab-Ag reaction in AGPT test indicated CMPs and ECPs unable to stimulate immunity in fish at 8 dpi. In a conclusion, it was postulated that the CMPs and ECPs were able to induce marked clinical signs and stimulate short period of immunity.

Keywords: *Aeromonas hydophilia*, Cellular Membrane Products (CMPs), Extracellular Membrane Products (ECPs), *Clarias gariepinus*, pathogenicity

1.0: INTRODUCTION

Background:

African catfish also known as *Clarias gariepinus* (Burchell, 1822). The fish is characterised by having an elongated body tapered posteriorly. The head is flattened and highly ossified skull bone with smooth scaleless skin and generally darkly pigmented (Graaf & Janssen, 1996). They are also equipped with a unique accessory air breathing organ, which help them to survive in a wide environmental tolerance (Graaf & Janssen, 1996). According to Marimuthu in 2010, this species inhabits in a wide range of water bodies like swamps, lakes and rivers. They are hardy and are able to thrive in harsh environmental conditions in muddy, turbid and oxygen depleted water bodies with the help of their accessory air-breathing organ (labyrinth organ) that allows them to breathe atmospheric oxygen. It is an important species in the aquaculture industry throughout the world (Mukai & Lim, 2011) especially among the farmers due to its good taste, fast growth, resistance to low oxygen and ease in farming (Nieuwegiessen *et al*., 2009). Bacterial disease is known to be the main infections towards catfishes (Al-Dohail *et al*., 2009). *Aeromonas* which is a gram negative, motile, facultative anaerobe, non-spore forming and rod shaped bacterium (Pridgeon *et al*., 2012; Roberts *et al*., 1996) is considered as one of the most important fish pathogens (Shayo *et al*., 2012). *Aeromonas hydrophila* is usually found in aquatic habitats (Angka, 1994) and also the cause of zoonotic diseases or food borne infections (Kirov, 1993; Krovacek *et al*., 1995; Daskalov, 2006). Molecular and phenotypic characterization of *A. hydrophila* isolates from diseased fishes will be helpful for determining sources of pathogens to control the spread of diseases outbreaks (Ye et al,. 2013).

Overall Objective:

1. To identify the ECPs and CMPs of *A.hydrophilia.*
2. To determine the Percentage Survivability of *Clarias gariepinus* fish after experimental challenge with ECPs and CMPs obtained from *A.hydrophilia.*
3. To study the presence of antibody in *C. gariepinus's* serum after challenge with ECPs and CMPs of *A. hydrophilia.*

Justification:

Aeromonas hydrophilia group 2 was known to cause high morbidity and mortality in aquaculture industry primarily in cultured fish and cause great financial loss to the farmer. A scientific study on the effects of CMPs and ECPs of *A. hydrophilia* towards immunity of African catfish *(C. gariepinus)* is still in infancy. Therefore, this study is important and potential for vaccine development for purpose of disease management. Hence, this study aimed to obtain basic information of motile *Aeromonas* virulency by determining the presence of active proteins in CMPs and ECPs and their clinical effects as well as their host immune response in African catfish..

2.0: LITERATURE REVIEW

2.1: *Clarias gariepinus* production in Malaysia

The African catfish *(Clarias gariepinus)* is locally known as "Ikan keli" belongs to the family of Clariidae (Marimuthu, 2010). African catfish is also a native fish species in African countries that has been introduced and commercially cultured in Asian countries such as Malaysia ((Uys, 1989). According to Department of Fisheries Malaysia in 2013, the statistic showed the total catfish production was about 50,533.79 tonnes which is the highest among the other fish species production and contributed to thirty-eight percent of the total of Aquaculture Production from Freshwater Culture System. Besides that, it is consider as the important species in the aquaculture industry throughout the world (Mukai & Lim, 2011) including Malaysia. This is due to its resistance to diseases and ability to tolerate a wide range of environmental conditions such as high stocking densities under culture conditions and resistance to low oxygen condition besides its good taste, fast growth, and high availability of fry as well as ease in farming make them famous among the farmers (Nieuwegiessen *et al*., 2009; James, 2003; Kechik, 1995). In particular, production of catfish (12,708.11 metric tonnes) and red hybrid tilapia (14,567.74 metric tonnes) together represented 61.77% of the total freshwater aquaculture production (Kechik, 1995).

2.2: *Aeromonas hydrophilia* infection in African catfish (*C. gariepinus)*

Now, *A. hydrophilia* outbreaks has a major impact in aquaculture (Vivas *et al*., 2004). It is a normal inhabitant of the gastrointestinal tract which could affects freshwater fish such as catfish (Ullal *et al.* 2008) and several other freshwater species. As reported by Freshwater Fisheries Research Centre in 2004, the pathogens from genus *Aeromonas* such as *A. hydrophilia* which is about 69.6% were commonly found in freshwater fishes in Malaysia. In a study conducted by Ashiru *et al*. (2011), *A. hydrophila* and *A. sobria* were predominant in catfish. Besides that, this bacteria is known as an opportunistic pathogen which are able to produce the disease if given the chance (Swann & White, 1991) and the presence of it, is not an indicative of the disease caused by it (Carraschi., 2012). According to Cipriano *et al*. (1984), stress presented in fish farming is the principal factor responsible for the outbreak of this pathogens and was supported by Swann & White (1991) as they also stated that the common occurrence of this disease is usually relates to stress factor such as mishandled, overcrowded, transported under poor conditions, poor level of nutrition and poor water quality. *Aeromonas hydrophilia* infection in fish is usually associated with skin condition or surface ulcerations on fish called Motile aeromonas septicaemia (MAS), Red sore, Red pest and Haemorrhagic septicaemia (Anyanwu *et al*., 2015; Poobalane, 2008) and among anaerobic bacterial organism that impede African catfish (C. *gariepinus*) production (Anyanwu *et al*., 2015). Previous study showed that, clinically fish that infected with motile aeromonad septicaemia (MAS) was observed to be stagnant and swimming closer to surface, reluctance to eat, darkening of the skin, hyperemia of the fin bases as well as skin over the pectoral fins and fin rot may also be observed (Banu & Aydin, 2011). In addition, according to study done by Laith AR & Najiah M in 2013, the clinical signs of the diseased fish showed symptoms of increased respiration and lethargy with skin lesions such as white discoloration, shallow haemorrhagic ulcers or deep ulcers with exposed underlying muscle. Some fish showed marked haemorrhages on the base of the fins and vent. Others were dropsy, kidney congestion and enlargement, pale liver and gills, or gall-bladder enlargement with the accumulation of yellowish fluid in the body cavity. The pathogenicity and virulence of *A. hydrophilia* depend on the bacteria ability to produce factors associated with gastroenteritis (Kirov, 2003). These factors are exotoxins, cytotoxins, endotoxins, siderophores, invasins, adhesins, S-layers and flagella (Daskalov, 2006).

2.3: Cellular Membrane Products (CMPSs) and Extracellular Membrane Products (ECPs)

Cellular Membrane Products (CMPs) of an *Aeromonas hydrophilia* was the bacterial cell pellet with various surface components. According to Todar (2016), the surface properties of a bacterium can be determined by the exact molecular composition of its membrane and cell envelope. Some structural features associated with *Aeromonas* species related with its virulency including pili, flagellum, lipopolysaccharides (LPS) and outer membrane proteins (OMPs) (Janda, 1991). Usually, the molecular weight of the CMPs protein varying from 17kDA to 59kDA and of the most common surface structures on bacteria are monomolecular crystalline arrays of proteinaceous subunits termed surface layers or S-layers (Sleytr *et al*., 1999) and it represent one of the most abundant cellular proteins (Sara *et al*., 2000). For examples, Dooley in 1988, reported that the members of the high-virulence group of *A. hydrophila* produced tetragonal array S-layers composed primarily of a 52kDa protein subunits and was also the major surface protein antigen on intact *A.hydrophilia* TF7 cells.

Extracellular Membrane Products (ECPs) defined by Pallock in 1962 was the medium which exists around the cell, having originated from the cell without any alternation to cell. The molecular weight varying from 49kDA to 96kDA. The virulency of the ECPs of *A. hydrophilia* depends on the toxins and other enzymes elaborated which might contribute to the higher degree of virulence (Angka *et al*., 1995) of *Aeromonas* spp. For example are haemolysins (Yadav *et al*., 1992), aerolysin (Chakraborty *et al*., 1986), cytotoxin (Boulanger *et al*., 1977) enterotoxin (Antos *et al*., 1988) and cytolytic enterotoxin (Chopra, 1993) protease, amylase and chitinase (Janda, 1991). However, the main virulence factors that have effect on pathogenicity of *Aeromonas* spp. are enterotoxin, aerolysin and haemolysin (Daskalov, 2006) with aerolysin to be assumed as virulence genes which exhibiting both haemolytic and cytolytic properties (Rabaan *et al*., 2001). In a conclusion, according to Janda *et al*. in 1994 and Esteve *et al.* in 1995, surface components (CMPs) and ECPs play an important role in their pathogenesis in order to evade the defence mechanism of the fish thus allowing the infection to take place.

3.0: MATERIALS AND METHODS

Experimental study

3.1: Preparation of Fish

One hundred and twenty apparently healthy fingerlings of African catfish (*Clarias gariepinus)* with size ranging from 3 to 8 inches long were obtained from commercial farm in Seri Kembangan, Selangor. At least 40% of fish were randomly screened prior to the trials in order to ensure that they are disease and pathogens free. The trial used 60 fingerlings with duplicate. Prior to the experiment, glass tanks were cleaned and filled with dechlorinated tap water. After that, all fingerlings were bathed in 0.2% $NaCl_2$ and acclimatized for 1 week prior to the experiment. They were maintained in 500 L glass tanks.

The fingerlings were divided into three groups [control, group A (CMPs), and group B (ECPs)]. Then, Group A and B were divided into five different tanks and with 5 fingerlings in each tank with duplicate. While the control group was divided into two tanks each represents control group for CMPs and ECPs and total of 5 fingerlings were put in each tank with duplicate. The fish was fed daily with 3% body weight with commercial feed.

The temperature, pH and salinity were kept constant.

3.1: Preparation of inoculum/ bacteria

Isolation and Identification of *Aeromonas hydrophila* group 2:

Aeromonas hydrophilia group 2 was originally isolated from a naturally infected African catfish. The organism was maintained in stock agar broth and a loop full from broth tube was streaked onto the TSA agar. Then, the bacteria was incubated at 28ºC for 24 hours. The isolate was sub-cultured on TSA plates at 28ºC for 24 hours.

The purified and fresh bacteria colony was isolated and used for morphological and biochemical identifications.

Morphological Characterization of *Aeromonas hydrophilia* group 2:

A loopful of pure and fresh bacteria isolated from a single bacterial colony was smeared onto clean glass slide and stained with Gram's stain, catalase and oxidase test. After that, the slide was examined under the light microscope at 100x magnification.

Biochemical Identification:

The test bacteria was identified as A. *hydrophilia* group 2 using commercial API20E (Analytical Profile Index 20E) identification kit and performed according to manufacturer's instruction. The bacteria was identified as 99% as A. *hydrophilia* group 2.

After that, pure and fresh A. *hydrophilia* group 2 was inoculated into 10 ml Tryptic Soy Broth (TSB) and incubated at 28°C in incubated shaker (70 rpm) for 24 hours. At this time, the bacteria culture was in the stationary phase of growth. A bacterial culture of Optical density (OD) at 600nm = 1.972, measured using spectrophotometer was then transferred into sterile normal saline. Ten-fold serial dilutions were done to obtained A. *hydrophilia* group 2 concentration of 5.2 x 10^{10} to 5.2 x 10^{6} cfu mL^{-1}. After that, serially diluted A. *hydrophilia* group 2 was plated onto TSA plates immediately and incubated at 28°C for 24 h. The concentration of the suspension was calculated using colony-forming unit (cfu), spread-plate technique and also read by optical density (OD) value using spectrophotometer at 600 nm.

The bacteria grown in TSB culture were centrifuged at 1800xg for 15 minutes to get bacterial pellet and supernatant. The supernatant will become the extracellular products (ECPs) components, while the pellet will become the bacteria cells or Cellular Membrane Products (CMPs). The bacterial pellets were washed twice with Phosphate Buffered Saline (PBS) via centrifugation, each time at 1000xg for 10 minutes. To obtained cell membrane products (CMPs), the bacterial pellets will be boiled at 100°C for 10 minutes in order to kill the bacteria and breaks the cells. After cooling, the bacteria pellets or CMPs was again re-suspended in PBS and centrifuge at 1000xg for 10 minutes. The CMPs was stored chill and later diluted to desired concentration prior to use. The supernatant or ECPs was filter-sterilised using 0.45-μm membrane filter and stored similarly.

Ten folds serial dilutions were made to give 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} diluents.

3.2: Experimental infection

The fish were divided into three groups (control, Tank A, Tank B). Then, 25 fishes in Group A with duplicate were inoculated intraperitonealy with 0.1ml of serially diluted bacterial pellet or CMPs. A total of 25 fishes in Group B were divided into five different tanks and with 5 fingerlings in each tank with duplicate. They were inoculated intraperitonealy with 0.1ml of serially diluted bacterial supernatant or ECPs. While the control group was divided into two tanks representing controls for CMPs and ECPs, with 5 fingerlings in each tank with duplicate. For the experimental trial, fingerlings in control groups were mock injected with 0.1 ml of 0.85% normal saline intraperitonealy (IP). While Group A fingerlings were injected with 0.1 ml of Extracellular products (ECPs) of *A. hydrophilia* group 2 and Group B fingerlings were injected with Cells Membrane Products (CMPs) of the bacteria. Each dilution of ECPs and CMPs were injected into 10 fingerlings.

3.3: Blood sample collection

Blood samples were taken at 8th day post-inoculation. Firstly, the fish were anaesthetised with tricane methane sulfonate (MS-222) at a concentration of 50mg/L. Approximately, 0.1mL of blood samples were withdrawn from each fish via caudal vertebral vein using 1mL syringe and 25G needle. The collected blood were transferred into normal plain tube and pooled into one tube for blood drawn from the fish in similar dilution tank. Serum was collected after clotting.

3.4: Immunogenicity *in vitro* test

Agar Gel Precipitation Test (AGPT)

On 8th day post-inoculation, the immunogenicity of CMPs and ECPs was determined using AGPT according to Ouchterlony method, 2009. 1% of Agarose was prepared in Normal Saline Solution (NSS) and autoclaved for 15 minutes. After that, 3.5 ml of warm agarose solution (45ºC- 50 ºC) was pour into clean petri plate placed on flat surface and allowed the agarose solution to cool and solidify. After that, the wells on the gel were made using gel punch and pattern of one central well surrounded by six pheripheral wells was used. A 10µl of serum of the fish from ECPs and CMPs groups with different serial dilution were loaded into the each well on the AGPT plates. After that, the plates was put into a moist container and incubated at room temperature, 28ºC for 24 hours. The line of precipitation observed indicating presence of antibody (immunity) in the fish due presence of antigen and antibody complex reaction.

3.5: Data Analysis

After the fingerlings were injected, they were observed for 7 days. Daily morbidity, mortality and survivability were recorded until 7th day post inoculation. Data were analyzed using SPSS version 20, Pearson Chi Square, Kruskal-Wallis H test.

RESULTS

Clinical findings

The clinical signs observed throughout this experiment were lethargy in almost 100% of them. Usually, the fish were found close to the wall and floor of the aquarium. This behaviour could be seen after 24 hours post-inoculation with CMPs and ECPs of *A. hydrophilia*. Besides that, most of the fish also showed signs of fin rot especially at the dorsal fin. In addition, some of the fish showed inappetance and reduction in the amount of the feed ate. By the 7th day pi, some of the fish showed reddening of the skin. All the clinical signs were similar for both the CMPs and ECPs group tanks. There was also no difference in the severity across the dilution. No clinical signs were seen in non-infected fish. All the clinical findings were shown as in Figure 1 to Figure 5.

In addition, the morbid fish showed obvious clinical signs of severe fin rot and necrosis of barbels. Corneal opacity and exophthalmia were also observed, where the condition found to affect one eye or both of the eye. Somehow, the orbit was ruptured and caused blindness which lead to death. The dermal lesions with focal haemorrhage and inflammation was also seen. Both dermis and epidermis were eroded, however the underlying muscle layer was still intact. All the findings were shown in Figure 6 and Figure 7.

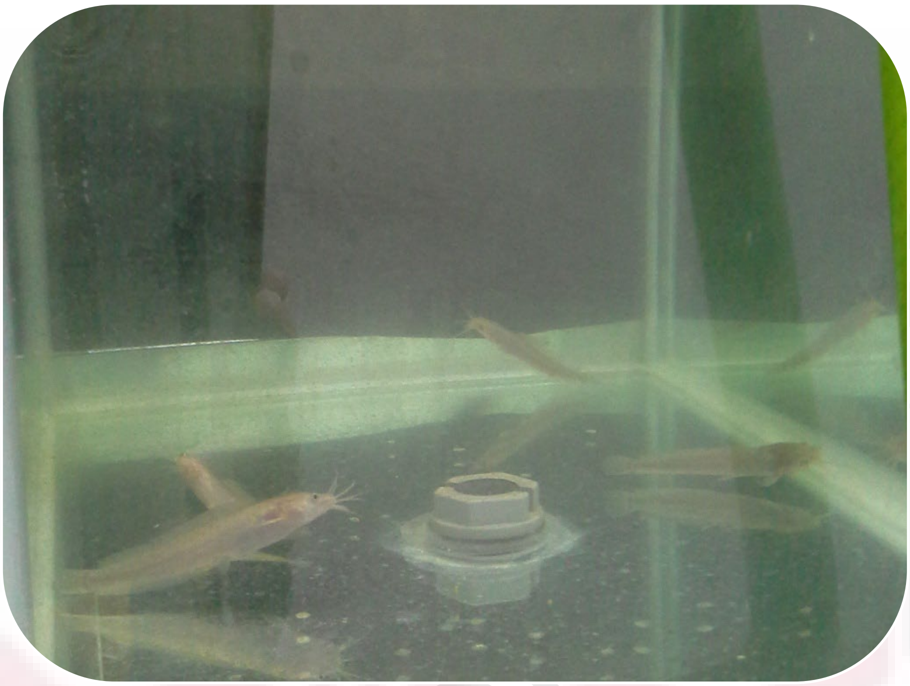


Figure 1: Fingerlings from group (T2R1) ECP-injected fish were seen to stay at the bottom of aquarium, at 1 dpi.

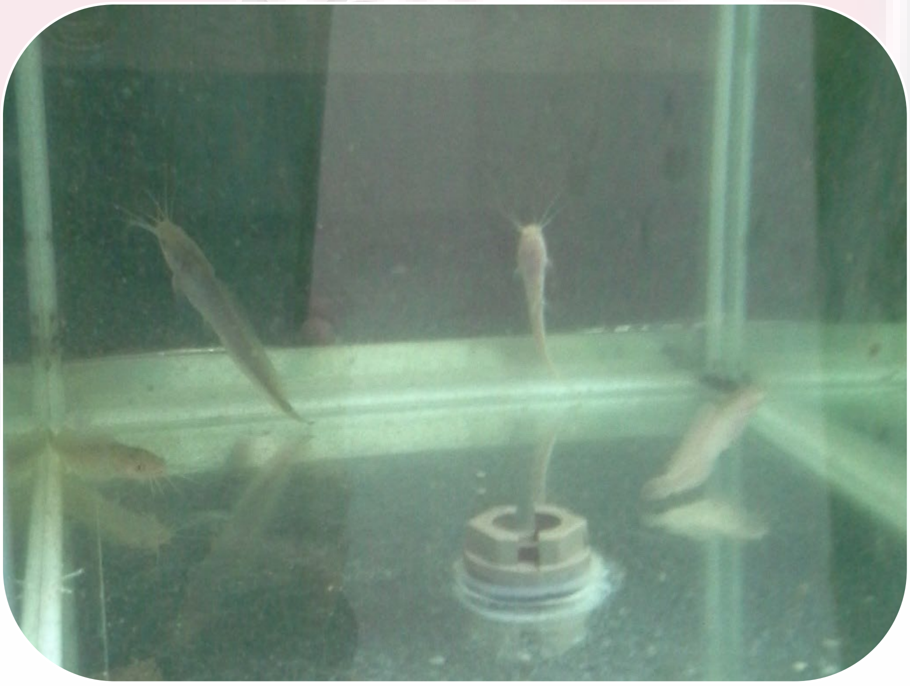


Figure 2: Fingerlings found close to the wall of the aquarium in group (T1R2) at 1 dpi.

Figure 3: Fin rot was observed on the dorsal fin of the fingerling in group (T2R1), CMP-injected fish at 1 dpi.

Figure 4: Fingerlings found stagnant at the water surface in group (T2R2) ECP-injected fish at 6 dpi.

Figure 5: Reddening of the skin of fingerlings in group (T1R3) at 8 dpi.

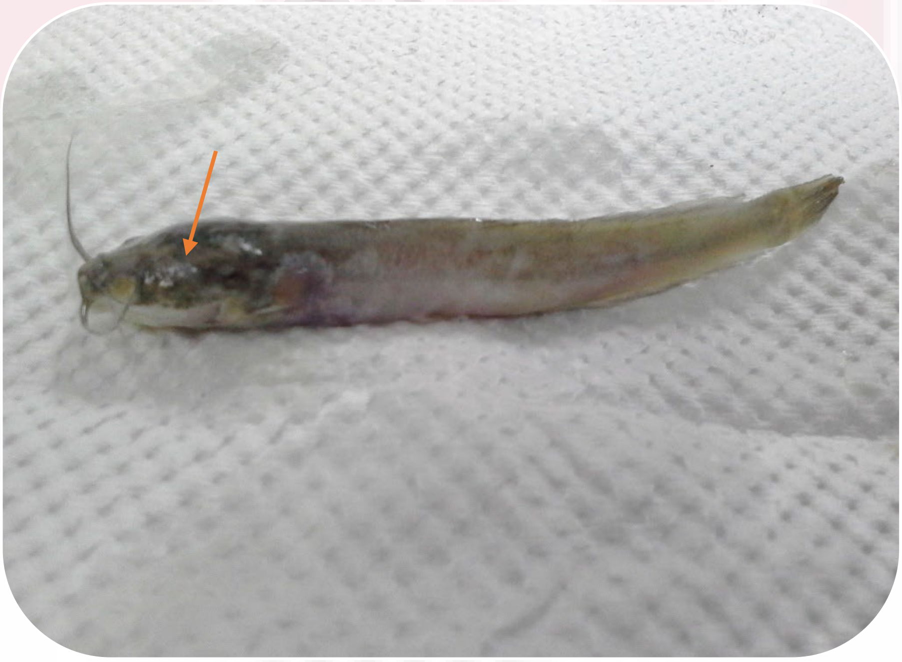


Figure 6: Fish showed severe dorsal, ventral and pectoral fin rot, skin ulceration and eyeball rupture (red arrow) of fingerlings from group (T3R2) CMP-injected fish at 1 dpi.

Figure 7: Fingerling showed corneal opacity (red arrow), loss of barbels from group (T3R2) ECP-injected fish at 6dpi.

Figure 8: Fingerlings showed exopthalmia of the eyeball from group (T5R1) ECP-injected fish at 3 dpi.

<u>Mortality and Percentage of Survivability</u>

Mortality of the fish in CMPs group was seen by the first day of post-inoculation. While, in the ECPs group the mortality of the fish begin by third day of post-inoculation. The mortality pattern for both groups was seen to be randomly distributed and not as expected to be decreasing in the number of total mortality across the dilutions. Moreover, the mortality recorded mostly at the lowest bacterial dilution both in the ECPs and CMPs group. All the data were collected in the Table 1 and Table 2.

Table 1: Mortality and Percentage of Survivability at 7th days post inoculation with Cell Membrane Products (CMPs)

Tank	CFU/ml	Number of mortality/ day							Total mortality	% of Cumulative Mortality	% of Survivability
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7			
Control R1	-	0	0	0	0	0	0	0	0/5	0	100
Control R2		0	0	0	0	0	0	0	0/5	0	100
T1R1	5.2×10^{10}	0	0	0	0	0	0	0	0/5	0	100
T1R2		0	0	0	0	0	0	0	0/5	0	100
T2R1	5.2×10^{9}	0	0	0	0	0	0	0	0/5	0	100
T2R2		1	0	0	0	0	0	0	1/5	20	80
T3R1	5.2×10^{8}	0	0	0	0	0	0	0	0/5	0	100
T3R2		1	0	0	0	0	0	0	1/5	20	80
T4R1	5.2×10^{7}	0	0	0	0	0	0	0	0/5	0	100
T4R2		0	0	0	0	0	0	0	0/5	0	100
T5R1	5.2×10^{6}	0	0	0	1	0	0	0	1/5	20	80
T5R2		0	0	0	1	0	0	0	1/5	20	80
Total									4/60	80 (6.67%)	1120 (93.33%)

Table 2: Mortality and Percentage of Survivability at 7th days post inoculation with Extracellular Products (ECPs)

Tank	CFU/ml	Number of mortality/ day							Total mortality	% of Cumulative Mortality	% of Survivability
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7			
Control R1	-	0	0	0	0	0	0	0	0/5	0	100
Control R2		0	0	0	0	0	0	0	0/5	0	100
T1R1	5.2 x 10^{10}	0	0	0	0	0	0	0	0/5	0	100
T1R2		0	0	0	0	0	0	0	0/5	0	100
T2R1	5.2 x 10^{9}	0	0	0	0	0	0	0	0/5	0	100
T2R2		0	0	0	0	0	0	0	0/5	0	100
T3R1	5.2 x 10^{8}	0	0	0	0	0	0	0	0/5	0	100
T3R2		0	0	0	0	0	1	0	1/5	20	80
T4R1	5.2 x 10^{7}	0	0	0	0	0	0	0	0/5	0	100
T4R2		0	0	0	0	0	0	0	0/5	0	100
T5R1	5.2 x 10^{6}	0	0	1	0	0	0	0	1/5	20	80
T5R2		0	0	0	0	0	0	0	0/5	0	100
								Total	2/60	40 (3.33%)	1160 (96.67%)

Pattern of mortality and survivability in each dilution for both groups could be explained by Figure 1 and Figure 2.

Figure 1: Mortality pattern of ECPs-injected fish for each dilution.

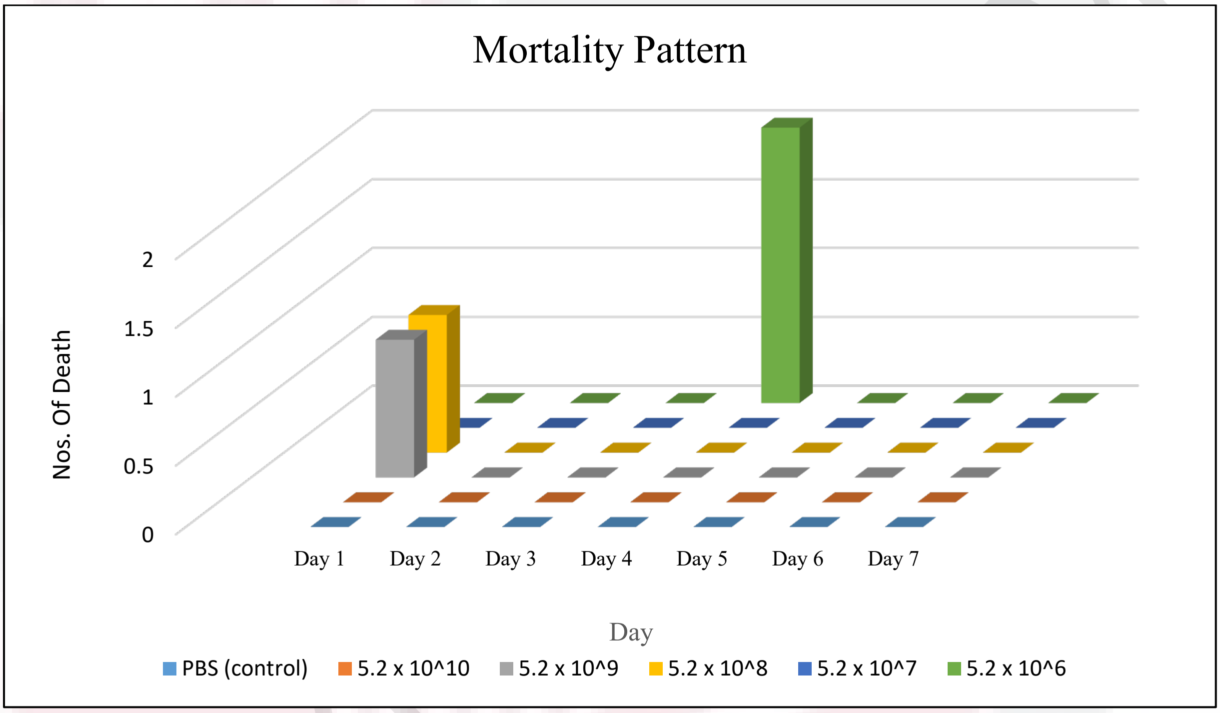
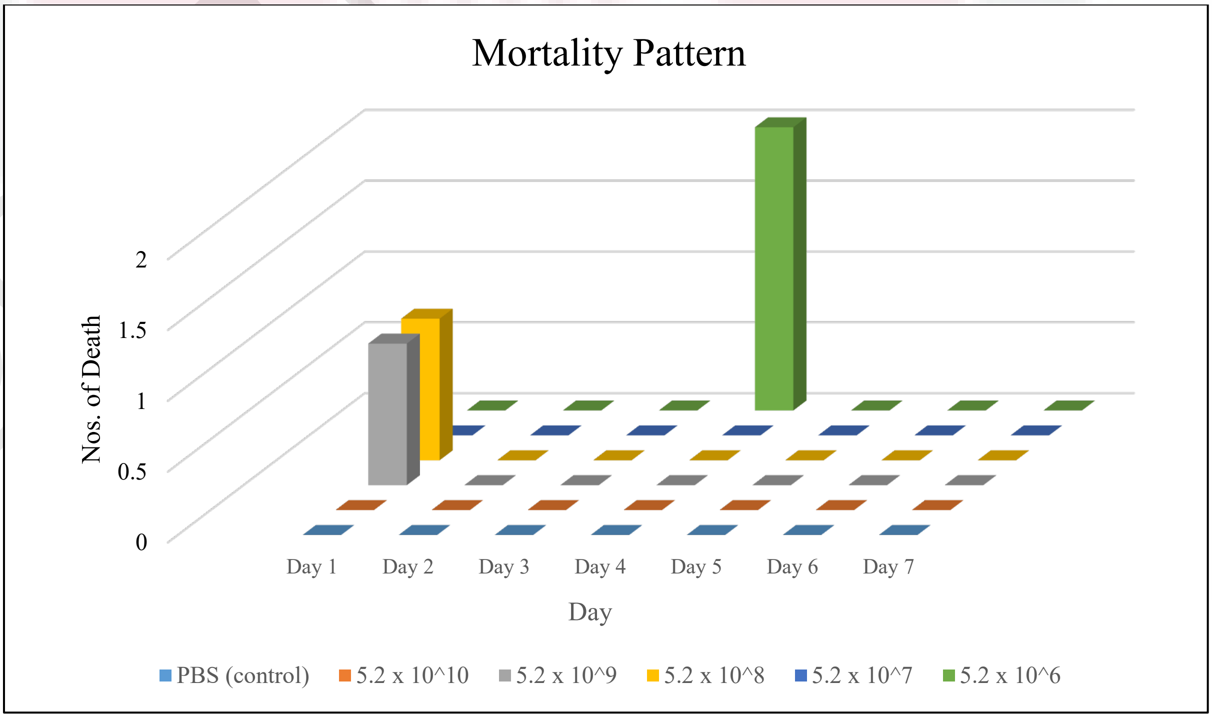


Figure 2: Mortality pattern of CMPs-injected fish for each dilutions.



Figures 3 and 4 showed cumulative mortality for ECPs and CMPs group respectively at 7 days post-inoculation. The result showed no significant different of total mortality across the dilution for both ECPs and CMPs group as well as no reducing in the number of mortality towards the lowest bacterial products concentration.

Figure 3: Cumulative mortality of ECPs-injected fish for each dilution

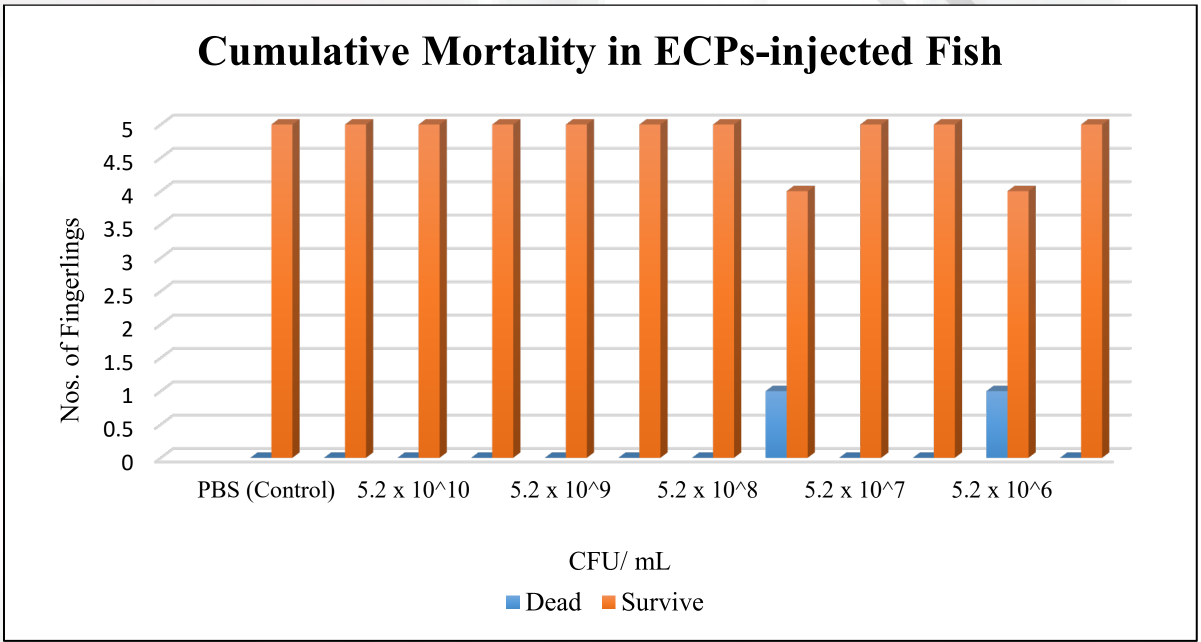
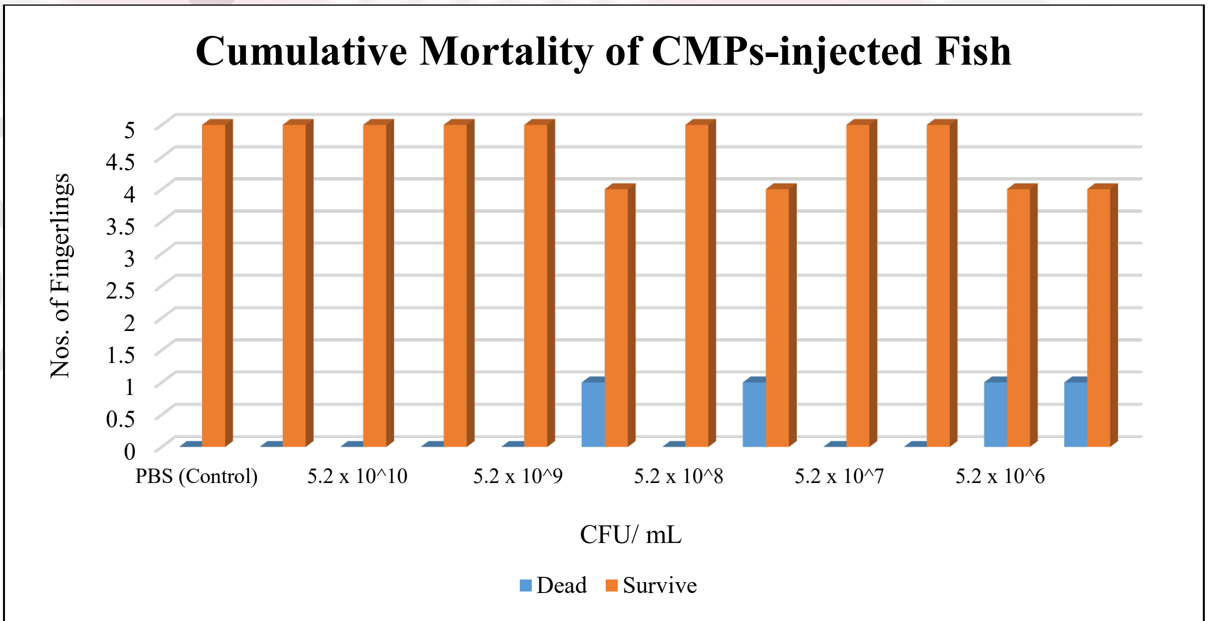
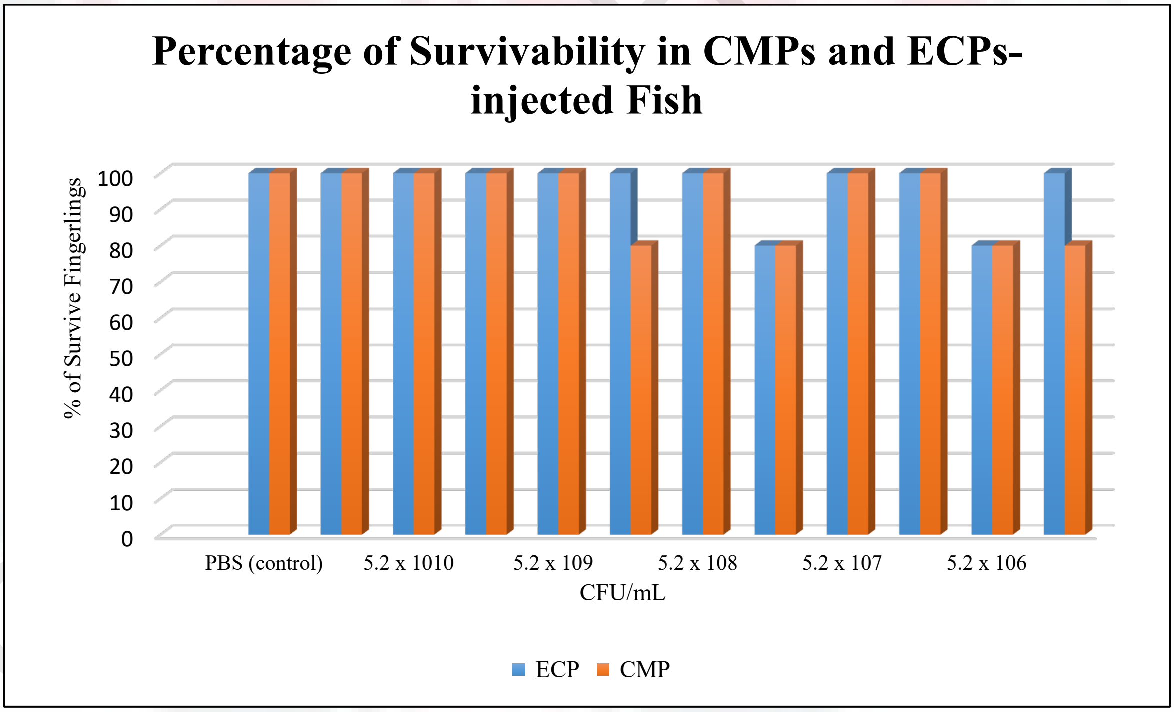


Figure 4: Cumulative mortality of CMPs-injected fish for each dilution.



Percentage of survivability for both group were high in each of the bacterial product dilutions. From the graph of Figure 5, the survivability of the fingerlings in both group showed no significant different across the dilution for both ECPs and CMPs group as well as no increasing in the number of survive fingerlings towards the lowest bacterial products concentration.

Figure 5: Percentage of survivability of fingerlings in CMPs and ECPs-injected fish groups for each dilution.



Hence, the statistical analysis using SPSS version 20, Kruskal-wallis H test was done to see the significant of these assumptions. From the SPSS output 1, statistically it proved that there was no significant difference of the total mortality as well as survivability in CMPs and ECPs group. Similarly, SPSS version 20, post hoc Duncan test was done to find the association of different tank with different bacterial dilution within CMPs and ECPs group. Based on SPSS output 2 and 3 for CMPs and ECPs respectively, statistically both group showed no significant different or association of total mortality and survivability between different tank with different bacterial dilution to the control within CMPs and ECPs group.

SPSS Output 1: The significant different of the number of mortality and survivability in CMPs and ECPs injected fish.

Products	Mortality/ Survivability		Kruskal-wallis H test
	Dead	Alive	
Cellular Membrane Protein (CMPs)	3.33%	96.67%	0.230
Extracellular Membrane Protein (ECPs)	6.67%	93.33%	0.493

SPSS Output 2: Association of total mortality and survivability of fish within CMPs group.

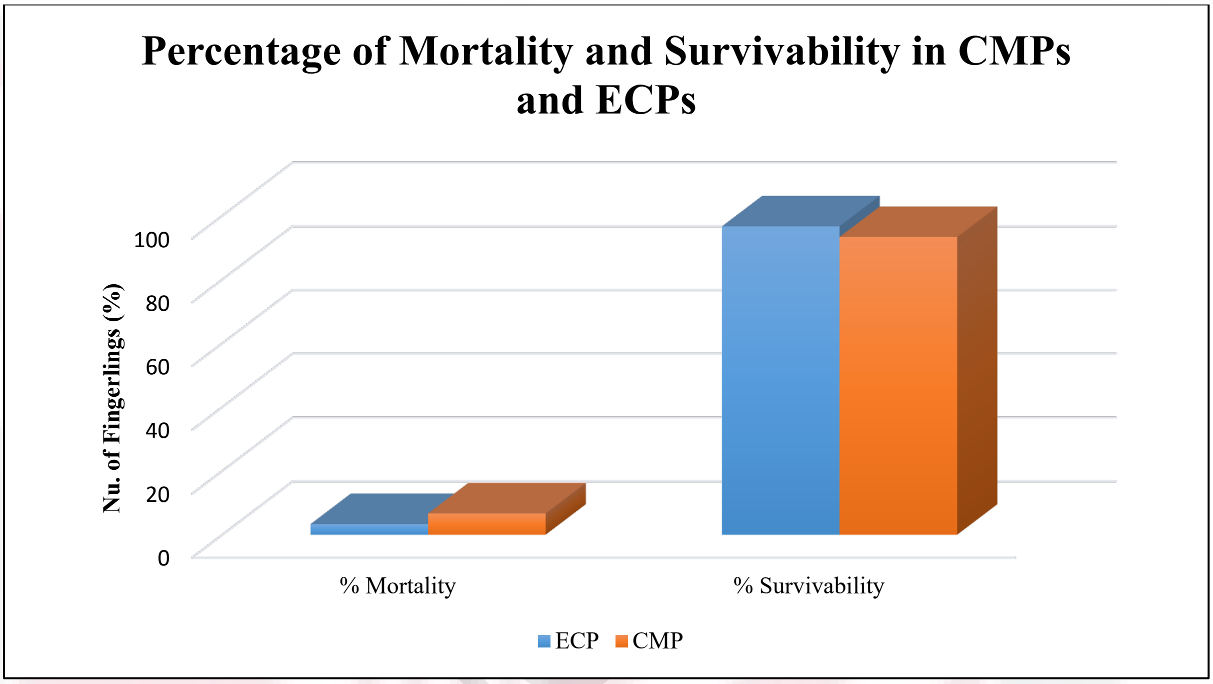
Dilution Tank	n	Sample Mean
Control	2	100.00[a]
Tank 1 (5.2 x 10^{10} CFU/mL)	2	100.00[a]
Tank 2 (5.2 x 10^{9} CFU/mL)	2	90.00[a]
Tank 3 (5.2 x 10^{8} CFU/mL)	2	100.00[a]
Tank 4 (5.2 x 10^{7} CFU/mL)	2	100.00[a]
Tank 5 (5.2 x 10^{6} CFU/mL)	2	80.00[a]
Sig.	-	0.061

SPSS Output 3: Association of total mortality and survivability of fish within ECPs group.

Dilution Tank	n	Sample Mean
Control	2	100.00[a]
Tank 1 (5.2 x 10^{10} CFU/mL)	2	100.00[a]
Tank 2 (5.2 x 10^{9} CFU/mL)	2	100.00[a]
Tank 3 (5.2 x 10^{8} CFU/mL)	2	90.00[a]
Tank 4 (5.2 x 10^{7} CFU/mL)	2	100.00[a]
Tank 5 (5.2 x 10^{6} CFU/mL)	2	90.00[a]
Sig.	-	0.284

Besides that, overall total of mortality and survivability of CMPs higher than ECPs as shown in Figure 6. However, both factors have not much different in terms of percent mortality or survivability between the two groups. Therefore, statistical analysis was done by using SPSS version 20, Pearson Chi-Square test. Based on statistical output 4, it showed no significant different between CMPs and ECPs for both factors. Hence, it could be conclude that the CMPs and ECPs of an *A.hydrophilia* cause similar pathogenic effect towards *C.gariepinus* in terms of mortality and survivability.

Figure 6: Comparison of percentage mortality and survivability of fish between ECPs and CMPs group.



Statistical output 4: The significant different of the number of mortality and survivability between CMPs and ECPs injected fish.

Products	Survivability		Total	Pearson Chi-Square test
	No	Yes		
CMPS	4	56	60	0.402
ECP	2	58	60	
Total	6	114	120	

<u>Agar Gel Precipitation Test</u>

All results were tabulated as in Table 3. No precipitation line was observed in all of the fingerlings' serum collected in every dilution for both ECPs and CMPs groups as showed in Figure 8 and Figure 9. Thus, it could be conclude that, it was no antibody and antigen reaction happened by 8^{th} day of post-inoculation in the fish body system.

Table 3: Agar Gel Precipitation Test of serum collected on 8^{th} day of post-inoculation with CMPs.

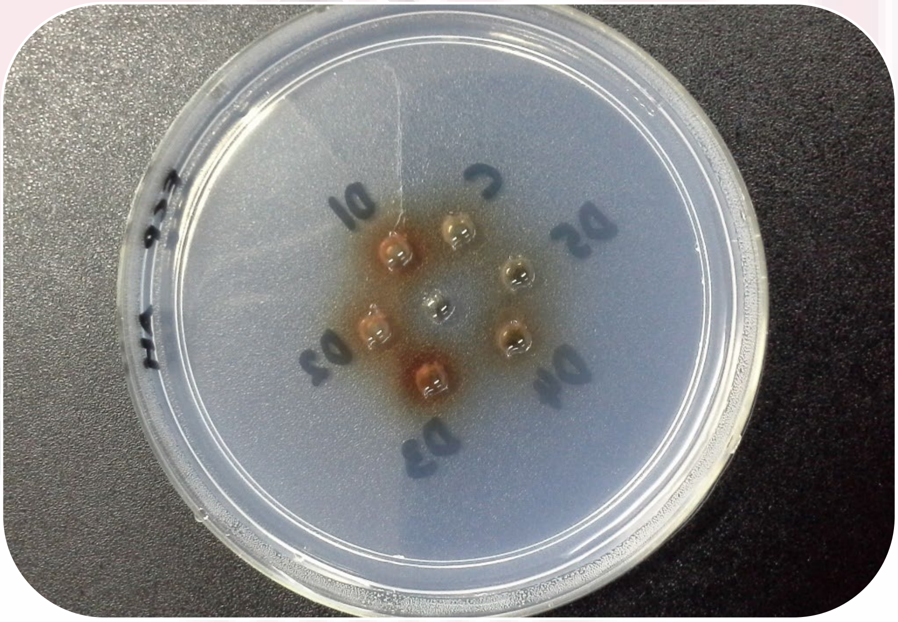
Tank	CFU/ml	Precipitation line
Control R1 Control R2	PBS	Absent
T1R1 T1R2	5.2×10^{10}	Absent
T2R1 T2R2	5.2×10^{9}	Absent
T3R1 T3R2	5.2×10^{8}	Absent
T4R1 T4R2	5.2×10^{7}	Absent
T5R1 T5R2	5.2×10^{6}	Absent

Table 4: Agar Gel Precipitation Test of serum collected on 8th day of post-inoculation with ECPs.

Tank	CFU/ml	Precipitation line
Control R1 Control R2	PBS	Absent
T1R1 T1R2	5.2×10^{10}	Absent
T2R1 T2R2	5.2×10^{9}	Absent
T3R1 T3R2	5.2×10^{8}	Absent
T4R1 T4R2	5.2×10^{7}	Absent
T5R1 T5R2	5.2×10^{6}	Absent

Statistically, the data cannot be computed as all the results for both groups were constant. Therefore, from the results tabulated, it can be suggested that, CMPs and ECPs isolated from *A.hydrophilia* unable to produce immunity of the fingerlings.

Picture 8: No precipitation line formed for each of CMPs dilution.



Picture 9: No precipitation line formed for each of ECPs dilution.

DISCUSSION

In this study, *Clarias gariepinus* challenged with CMPs and ECPs of *A.hydrophilia* had showed various clinical signs resembling a natural infection of *A.hydrophilia* as reported by Banu and Aydin (2011), Afifi *et al.* (2000), Arda *et al.* (2005), Yambot and Inglis (1994), Baran *et al.* (1980) and Faktorovich (1969). The clinical signs showed such as lethargic, being close to the wall and floor of the aquarium, fin rot, inappetance and reddening of the skin were observed. Besides that, the clinical signs begin after 24 hours post-inoculation in agreement with Bach (1978) and Huizinga (1979), where in experimental infection the incubation period of the disease range between 8 to 48 hours. However, the incubation period of the disease may varies between the fish species and resistance, environmental conditions and the season (Banu & Aydin, 2011). All the clinical findings were observed except for luminal shaped or free mucus defecation that might be due to inappetance.

Corneal opacity and exophthalmia were also observed in one or both eye causing opaqueness of morbid fish resembling the findings reported in the study by Afifi *et al*. in 2000. Severe fin rot was identified which was the evident of severity as the time pass while the mildness of the dermal lesions was due to the visceral organ based acute infection whereas in chronic infection the underlying musculature will become severely necrotic (Banu & Aydin, 2011). Moreover, all the clinical signs showed by the fish inoculated with CMPs and ECPs have no difference in term of gross lesion. It could be because all the virulent factors which lead to the development of clinical signs in fish was originated from same bacteria species.

In terms of severity, CMPs induced higher mortality than ECPs. Total mortality of the fingerlings in CMPs higher than in ECPs were recorded and CMPs assumed to be more virulence than ECP. In the study done by Pridgeon and Klesius (2011) stated that, the bacterial cells were highly toxic than their ECPs which produced *in vitro*. They also found the catfish fingerlings exposed with filter-sterilized ECPs recorded no mortality due to filter-sterilized ECPs have no toxicity or low toxicity. However, if ECPs had been incubated at room temperature for at least 3 hours, it would allow some toxins activation during incubation period that would lead to dead of fingerlings. Besides that, based on their study the antigenic virulency of ECPs was observed slightly increase when the bacteria cell was injected in fingerlings together. Moreover, number of authors have reported that *A.hydrophilia* can alter its ECPs expression profiles when it is grown in different culture medium or at different temperature (Merino, Camprubi & Tomas, 1992). The statement also supported by Eley, Geary & Wilcox (1993) and Mateos *et al*. (1993), where expression of virulence factors, including haemolysins and proteases which may also probably enhance virulency of *Aeromonas* (Gosling, 1996) has been shown to be influenced by environmental temperature. Example of the toxin from ECPs which is sensitive towards the temperature is aerolysin or known as heat-labile haemolysin (Deen *et al*., 2014).

In this study, the mortality should be highest at the highest bacterial products dilution to the lowest bacterial products dilution. However, there is no mortality was recorded in the 1st tank for both CMPs and ECPs with the highest bacterial products concentration and the mortality pattern also randomly across the bacterial dilution. Thus, the bacterial products used in this study might not pathogenic enough to cause mortality in the catfish fingerlings. This findings might be due to the fish samples used in this study As stated in study by Schlotfeldt and Alderman (1995), effects of *A. hydrophila* in fish can vary according to the resistance to infection which depends on its immune status and species of the fish (Anyanwu, 2014).

As the fish samples taken from a source which was not certified as disease free farm, there was a possibility of fish being pre-infected before used in the experiment and possibly had developed their antibody towards *A.hydrophilia* infection. So, the inoculation of the antigens might rising their immunity towards the infection and produced no mortality in the fish. In the study done by Carraschi (2012), 1.5 x 10^8 CFU $^{mL-1}$ lead to the death of the fish within 24 hours of induction and considered as lethal dose (LD_{50}) concentration. However, although the bacterial concentration used is 5.2 x 10^6 CFU^{mL-1} quite high, however, LD_{50} done in pacu (*Piaractus mesopotamicus*) and not in *Clarias gariepinus*, so species variety might have host adaptive in order to evade host defence mechanism.

In addition, another factor that would influenced the pathogenicity of the bacteria could be due to bacteria itself. Next, as reported by Poobalane *et al.* (2008), different antigenic proteins may be expressed by the bacterium when the same bacterium grown *in vivo* compared to *in vitro* as bacteria grown inside the host might be able to modulate its virulence factors in such a way to allow the pathogen to evade the defence mechanism of the fish. Furthermore, strain of *Aeromonas* spp., dose of infective pathogen and route of administration also influenced the pathogenicity of the bacteria pathogens (Anyanwu, 2014). On the other hand, fish response and defence mechanisms to the challenged of microorganisms are highly diverse (Kreutz 2011). Hence, the ability of the fish to cope with the infecting pathogens relies on delicate balance between environment and the components of innate and acquired immunity.

Although the bacterial used did not able to cause mortality in fish, however the presence of mortality of the fingerlings in recorded in the tank 3, and tank 5 for ECPs group while mortality in the tank 2, tank 3 and tank 5 for CMPs group. Based on the observation, the result of mortality might be affected by different exposure to the intensity of the light where the intensity of light varies with the position of the tank and indirectly affect the water temperature as well. The intensity of the light reducing across the tank from tank 1 to tank 5 for both groups. So, tank 5 was located in an area with less intensity of light compared to other tanks which indirectly lowering the water temperature. According to Kreutz *et al*. (2014), generally antibody production in most fish species reduced in low water temperature and 15ºC of water temperature would decrease the immunoglobulin production in several fish species. But, there is no previous study stated the exact temperature for immunoglobulin production in African catfish (C. *gariepinus*). Indeed, the immunoglobulin production affected by water temperature but each fish species has their own temperature range which the immune response might be optimal (Kreutz *et al*., 2014). The mortality recorded might also being triggered by other stress factors such as handling, transportation and water quality.

Moreover, in the study done by Kreutz *et al*., in 2014, the specific serum antibodies will have sharp and significant increase as soon as 7 days of post-inoculation and will continue up steadily for up to 35 days in fish challenged with *A.hydrophilia* bacterin. However, the result of immunogenicity test for present study showed no antibody and antigen reaction happened by 8th day of post-inoculation in the fish body serum based on Agar Gel Precipitation Test (AGPT). Besides that, this finding concurrent with the findings of bacterial culture isolated from kidney and liver where it showed no bacterial growth on Trypticase Soy Agar (TSA) which could be due to fish antibody reaction towards the bacterial products injected earlier and at 8th day pi the antibody level had reduced. An antibody response in fish by Kreutz (2011), generally fish antibody titer was believed to be short-lived although the antibody response to bacteria-derived antigens can occurs within a few days. So, re-inoculation of antigen is a major concern to rise up the antibody titer in fish. In this study, the mechanism of antibody production activated through humoral immune response involving activation of B-cell to produce the antibody. But, if the inactivated whole bacteria of *A. hydrophilia* was being studied, T-cell might be activated and the reinoculation to raise antibody levels are not required (Kreutz *et al*., 2011). So, it is major concern in some experiments related to the requirement for antigen reinoculation. Sometimes, nature of the antigen with proper presentation to B-lymphocytes might influence the antibody secretion in this study.

CONCLUSION

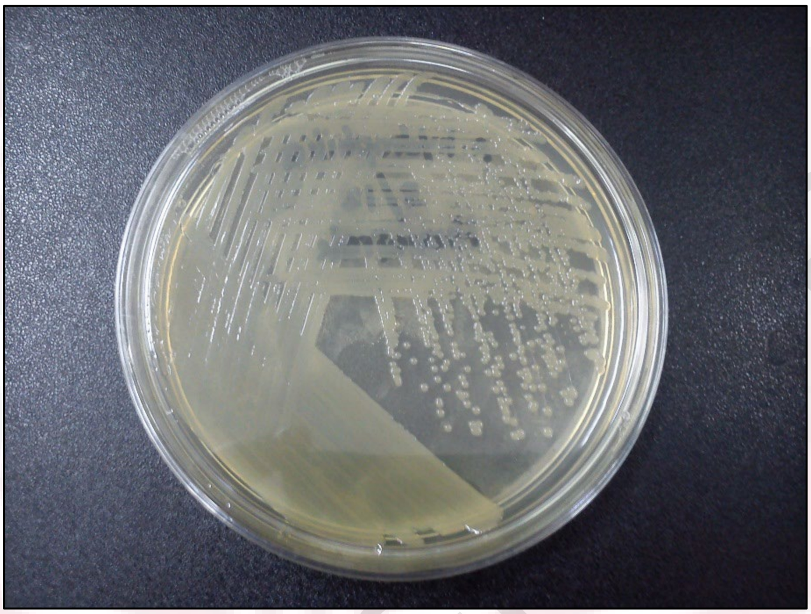
In conclusion, typical clinical signs were observed in the fish inoculated with Cellular Membrane Products (CMPs) and Extracellular Products (ECPs) of *A.hydrophilia*. There was no different in terms of gross lesion shown by the fingerlings inoculated either with CMPs or ECPs. However, based on the cumulative mortality which was higher in CMPs than ECPs, thus CMPs is assumed to be more virulent than ECPs. This could be associated with the expression of virulence factors especially ECPs which is very sensitive towards the environmental temperature and lead to the alterations of its antigenic properties to aid in the infection of the host. The pathogenicity of bacteria products with CFU of 5.2 x 10^{10} to 5.2 x 10^{6} mL^{-1} were capable to induce clinical signs in fish but unable to cause mortality in the *C.gariepinus*. But the presence of mortalities recorded in both CMPs and ECPs highly associated with the presence of stress factors such as temperature, water quality and handling which could lower the immunity and enhance the invasion of the pathogens.

Overall, we can conclude that CMPs and ECPs from *A.hydrophilia* are able to produce clinical signs but not pathogenic enough to cause mortality in fish and it is assumed to stimulate the immunity in a short period of time. Lastly, due to variable pattern of mortality and negative findings of antibody antigen reaction (immunity) from AGPT, hence this current study did not fulfil the requirement needed for vaccine development.

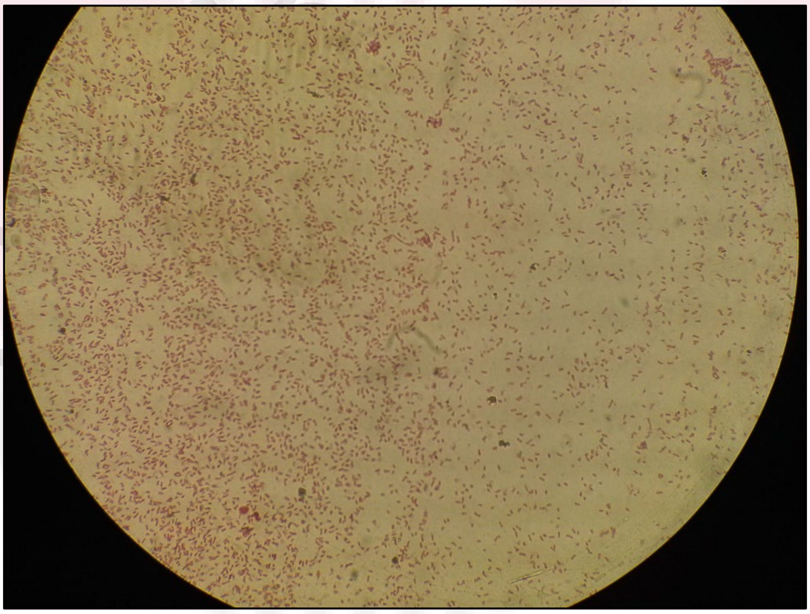
RECOMMENDATION

It is recommended to further this study by increasing the sample size and have more replicates in order to obtain more accurate and better result. Besides that, the pre-infection by bacteria should be taken into account and it is advisable to assess the presence of antibody or antibody status of the fish towards *A.hydrophilia* before the fish were used in the experiment. In addition, longer time is needed in this study to obtain a good result if to identify the antibody production towards bacterial products of *A.hydrophilia* in fish's serum as reinoculation of bacteria was recommended due to short-lived antibody titer in fish. Last but not least, it was really recommended to identify the lethal dose (LD_{50}) of the bacteria before it was used in this study to get a better pattern of mortality to be used as the target for highest immunostimulation. Further study by using other procedures such as SDS-PAGE and ELISA was highly recommended to identify molecular weight of the bacteria proteins and antibody titer if for complete study of vaccine development. Then, this study should be further proceed with identification of immunostimulation by rechallenged the injected fish with whole bacteria *(A. hydrophilia)* or its products.

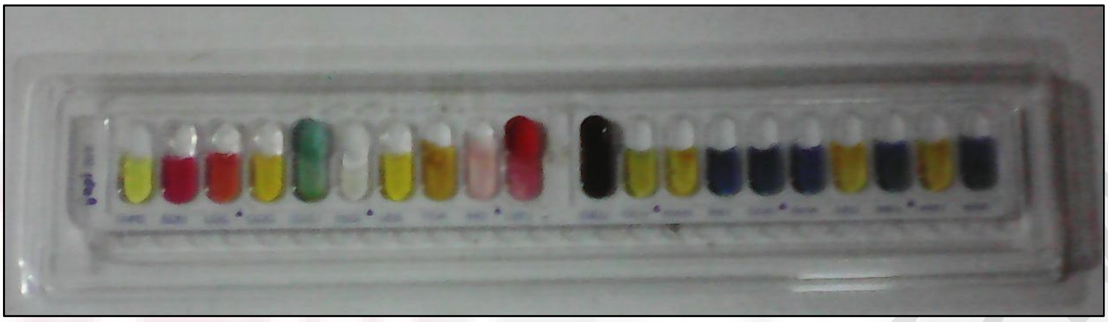
APPENDICES



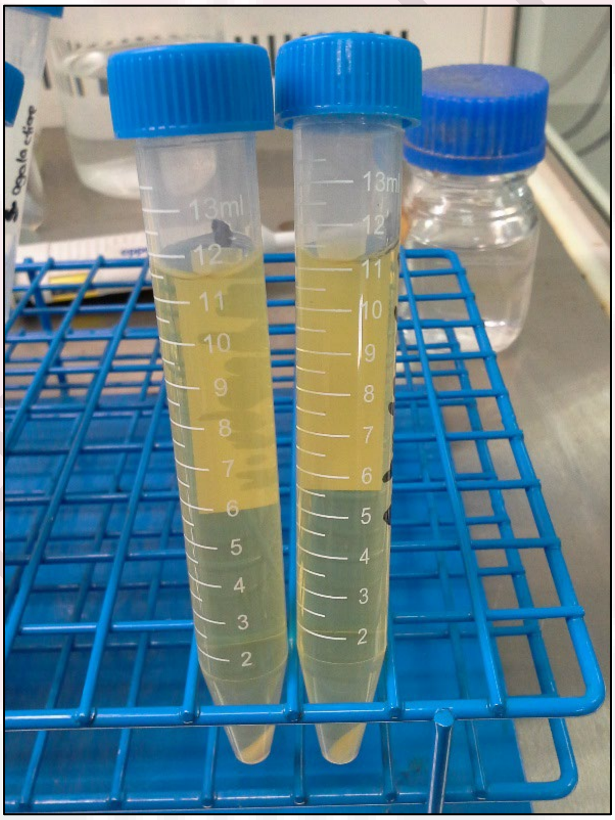
Pure bacterial culture of A. *hydrophilia* on Trypticase Soy Agar (TSA) incubated for 24 hours at 28ºC characterised by round, small about 1mm with smooth margin, flat to raised, shiny and smooth white appearance.



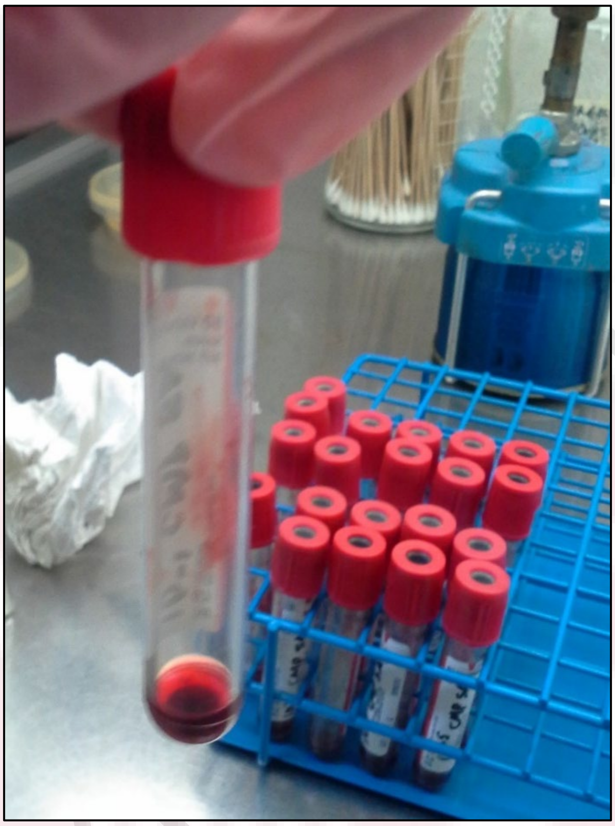
Gram stain of A. *hydrophilia* characterised by Gram-negative, rods with rounded ends at 1000x magnification.



API 20E microbiological identification kit for gram negative bacteria species identification and the result showed A.*hydrophilia* that was being isolated.



The pellet (CMPs) and supernatant (ECPs) of A. *hydrophilia* obtained after process of centrifugation.



Serum of the fingerlings obtained on 8th day post-inoculation.

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