UNIVERSITI PUTRA MALAYSIA

PATHOGENICITY STUDY OF EXTRACELLULAR PROTEINS (ECPs) AND CELLULAR MEMBRANE PROTEINS (CMPs) OF *Streptococcus agalactiae* AND ITS EFFECT AS IMMUNOMODULATOR IN AFRICAN CATFISH (*Clarias gariepinus*)

NOR ANISKIHA MAT YUNUS

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NOR ANISKIHA MAT YUNUS

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It is hereby certified that I have read this project paper entitled "Pathogenicity Study of Extracellular Proteins (ECPs) and Cellular Membrane Proteins (CMPs) of *Streptococcus agalactiae* and its effect as immunomodulator in African Catfish (*Clarias gariepinus)*", by Nor Aniskiha Mat Yunus and in my 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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Specially dedicated to my beloved parents, family and friends

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LIST OF ABBREVIATIONS

%	**Percent**
µl	**Micro liter**
mL	**Milliliter**
^{o} C	**Degree Celcius**
i.p	**Intraperitoneal**
hpi	**Hour post inoculation**
dpi	**Day post inoculation**
SPSS	**Statistical Package for the Social Sciences**
CFU/ml	**Colony forming unit per ml**
ECPs	**Extracellular Proteins**
CMPs	**Cellular Membrane Proteins**
AGPT	**Agar Gel Precipitation Test**

ABSTRAK

Abstrak daripada kertas kerja projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999- Projek Ilmiah Tahun Akhir Pelajar.

KAJIAN PATOGENISITI PROTEIN EKSTRASELULAR (PES) DAN PROTEIN MEMBRAN SEL (PMS) *Streptococcus agalactiae* DAN KESAN SEBAGAI IMUNOMODULATOR DALAM IKAN KELI AFRIKA (*Clarias gariepinus*)

Oleh

Nor Aniskiha Mat Yunus

2016

Penyelia: Prof. Madya Dr Hassan Hj. Mohd Daud

Penyelia bersama: Dr Mohd Fuad Matori

Protein Membran Sel (PMS) adalah protein permukaan bakteria yang boleh menjadi sumber immunogens manakala Protein Ekstraselular (PES) adalah produk yang dikeluarkan oleh bakteria yang dapat mengaktifkan tindak balas imun hos. Walau bagaimanapun, terdapat kekurangan dalam kajian sebelum ini untuk menilai tindak balas imun ikan keli terhadap PES dan PMS menggunakan AGPT. Kajian ini bertujuan untuk menilai kesan *in vivo* PES dan PMS bakteria *Streptococcus agalactiae* terhadap patogenisiti dan imuniti ikan keli Afrika (*Clarias gariepinus*).

PES dan PMS daripada *S.agalactiae* disuntik secara intraperitoneal (i.p) ke dalam ikan keli Afrika untuk menentukan sama ada bakteria produk dapat menyebabkan patogenisiti dan merangsang tindak balas imun ikan. Bakteria daripada kultur asal diambil menggunakan proses emparan pada 1800xg selama 15 minit untuk memisahkan PES dan PMS daripada larutan. Pencairan bersiri untuk PES dan PMS dilakukan pada 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, dan 10^{-5}. Morbiditi, mortaliti kumulatif dan peratusan kemandirian direkodkan setiap hari selama 7 hari. Sampel serum daripada ikan bagi setiap pencairan PES dan PMS serta kumpulan kawalan telah diambil melalui vena kaudal pedunkel pada hari ke-8 selepas suntikan. Tanda-tanda klinikal seperti tiada selera makan, lesu, berenang tidak menentu, kelegapan kornea dan eksoptalmia diperhatikan dan sera diuji untuk melihat tahap kehadiran antibodi menggunakan Ujian Presipitasi Agar Gel (AGPT).

Hasil kajian menunjukkan bahawa PMS lebih virulen daripada PES berdasarkan kematian, bagaimanapun, secara statistik tidak ada perbezaan yang signifikan di antara ikan disuntik dengan PES dan PMS pada $p < 0.05$. Keputusan juga menunjukkan bahawa tidak terdapat perbezaan yang signifikan di antara pencairan inokula dan peratusan kemandirian untuk kedua-dua PES dan PMS. Analisis serum melalui AGPT menunjukkan negatif untuk semua PES dan PMS dan ia menunjukkan bahawa tidak ada kompleks antigen-antibodi yang terbentuk bagi setiap pencairan.

Kata kunci: *Streptococcus agalactiae*, protein ekstraselular (PES), protein membran sel (PMS), ikan keli Afrika, imunomodulasi

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine as a partial fulfilment of requirement for the course VPD 4999- Final Year Project.

PATHOGENICITY STUDY OF EXTRACELLULAR PROTEINS (ECPs) AND CELLULAR MEMBRANE PROTEINS (CMPs) OF *Streptococcus agalactiae* AND ITS EFFECT AS IMMUNOMODULATOR IN AFRICAN CATFISH (*Clarias gariepinus*)

By

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2016

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Cellular Membrane Proteins (CMPs) are bacterial surface proteins that could be a source of immunogens while the Extracellular protein (ECPs) are bacterial secretory products that able to activate host's immune response. However, there is lack of previous studies done to evaluate the immune response of catfish against ECPs and CMPs using AGPT. This study aimed to evaluate the *in vivo* effect of the ECPs and CMPs of *Streptococcus agalactiae* on pathogenicity and immunity of the African catfish (*Clarias gariepinus*) fingerlings.

The ECPs and CMPs of *S.agalactiae* were intraperitoneally (i.p) injected into African catfish fingerlings to determine whether the bacteria products able to cause disease and also stimulate fish's immune response. Bacteria cells from pure culture were harvested by centrifugation at 1800xg for 15 minutes to separate ECPs and CMPs from bacteria suspension. Serial dilutions for ECPs and CMPs were done to give 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} diluents. The morbidity, cumulative mortality and percentage survivability of the fish were recorded within 7dpi. Pooled serum samples from fingerlings of each dilution of ECPs and CMPs injected fish as well as control group were taken via caudal peduncle vein at 8^{th} dpi. Clinical signs observed include anorexia, lethargy, erratic swimming, corneal opacity and exopthalmia. The sera were tested for antibody production by Agar Gel Precipitation Test (AGPT).

Results showed that CMPs was more virulence than ECPs based on mortality, however, statistically there was no significance difference between the fingerlings injected with ECPs and CMPs at $p<0.05$. Results also showed that there was no significant difference between the dilutions of the inocula and the percentage survivability of fingerlings for both ECPs and CMPs. Serum analysis via AGPT showed negativity for all ECPs and CMPs indicating that there was no antigen-antibody complex formed for every dilution.

Keywords: *Streptococcus agalactiae*, Extracellular Proteins (ECPs), Cellular Membrane Proteins (CMPs), African catfish, immunomodulation

1.0 INTRODUCTION

According to Department of Fisheries Malaysia, (2013) the total freshwater aquaculture in Malaysia reached about 132, 892.42 metric tonnes and about 38% from total production (50,533.79 metric tonnes) was contributed from freshwater catfish production from all culture system. Freshwater catfish production had the highest production compared to other fish species in Malaysia. Therefore, infectious diseases caused by pathogenic organisms such as *Streptococcus agalactiae* are an important issue that has caused a lot of financial loss in the aquaculture industry and it is increasingly being recognized as a potential constraint on aquaculture production and trade, and cause massive financial loss either through mortality or reduced meat quality, resulting in reduced profit margins (Smith *et al*., 2003).

Streptococcus agalactiae is a group B streptococcus, it was reported to cause neonatal pneumonia and meningitis in human (Brimil *et al*., 2006; Johri *et al*., 2006), mastitis in cows (Brochet *et al*., 2006; Yildirim *et al*., 2002) and streptococcal infection in fish (Toranzo *et al*., 2005).*Streptococcus agalactiae* also reported to cause such erratic swimming, loss appetite, exophthalmia and visceral cavity distension as main clinical signs in infected fish. According to Song *et al*. (2013), the development of vaccines is one of the solutions against the pathogen as a sustainable prevention method to control this emerging disease. Besides outer membrane proteins as a source of immune protective immunogens, recent increasing interests have been paid on extracellular secretory proteins The extracellular secretory proteins easily activate host's immune response since they are secreted out of cells and are easily contacted with the host (Zhang *et al*., 2012). Thus, investigation on protective immunogens from extracellular proteins may provide efficient candidates for development of vaccines (Song *et al*., 2013).

The objectives of this study were:

1) To identify the Extracellular Proteins (ECPs) and Cellular Membrane Proteins (CMPs) of *S. agalactiae*
2) To determine the clinical signs, mortality and Percentage Survivability in *Clarias gariepinus* fish when injected with ECPs and CMPs of *S. agalactiae*
3) To study the pathogenicity and immunity of *Clarias gariepinus* fish towards ECPs and CMPs of *S. agalactiae*.

Justification for this study:

Streptococcosis caused by *S. agalactiae* cause high morbidity and mortality in aquaculture industry primarily in cultured fish and cause great financial loss to the farmer. Therefore, the aim of the study is to evaluate the pathogenicity (clinical signs and mortality) and immune response of *Clarias gariepinus* fish when administered with Extracellular Proteins (ECPs) and Cellular Membrane Proteins (CMPs). Besides the host-agent immune response, the antigenic protein of the bacteria is important and potential for vaccine development. Other than that, there is also lack of study done on pathogenicity and immune response of African catfish fingerlings when challenged with ECPs and CMPs of *S. agalactiae*.

Hypothesis of this study were:

The hypothesis for this study is the Extracellular Proteins (ECPs) and Cellular Membrane Proteins (CMP) of *S. agalactiae* is able to produce pathogenicity (in terms of morbidity and mortality) and produce immunity in African catfish (*Clarias gariepinus*) fingerlings.

2.0 LITERATURE REVIEW

2.1 African catfish (*Clarias gariepinus*)

The African catfish (*Clarias gariepinus*) is locally known as Ikan keli Afrika and belongs to the family Clariidae. It is a native fish species in African countries and it has been introduced and commercially cultured in several countries in Europe (Netherlands, Germany, Belgium) and Asian countries (Indonesia, Thailand, Malaysia) and South America (Brazil). It is one among the highly demanded freshwater food fish and cultivar species in Malaysia and elsewhere due to its resistance to diseases, ability to tolerate a wide range of environmental conditions and high stocking densities under culture conditions, relative fast growth rate, and good quality meat. It can live in a variety of freshwater environments, including quiet waters like lakes, ponds, and pools. They are also very prominent in flowing rivers, rapids, and around dams. They are very adaptive to extreme environmental conditions and can live in pH range of 6.5-8.0. They are able to live in very turbid waters and can tolerate temperatures of 8-35 ^{o}C. Their optimal temperature for growth is 28-30 ^{o}C (Teugels, 1986). They are bottom dwellers and do most of their feeding there. They are also obligate air breathers, which mean they do spend some time on the surface. This species can live in very poorly oxygenated waters and is one of the last species to live in such an uninhabitable place (Pienaar, 1968).

2.2 *Streptococcus agalactiae*

Streptococcus agalactiae is the most commonly found species in hot climate, being associated to different fresh water, marine and estuary fish species (Evans et al., 2002). *S. agalactiae* is described as a Gram-positive, cocci-shaped bacterium which commonly occurs in pairs or in long chains. They produce small, translucent, round, and

slightly raised, pinpoint colonies, measuring 1-2 mm in diameter and appear yellowish to grey in colour when grown on solid agar (Plumb, 1999; Buller, 2004). Strains belonging to *S. agalactiae* are described as α-, β- or non-haemolytic (γ) when cultured on blood agar (Kitao *et al*., 1981; Buller, 2004). They are described as non-motile, non-capsulated, non-spore forming and are negative for the presence of oxidase and catalase enzymes. *Streptococcus agalactiae* was first reported in the captive freshwater shiners in 1966 (Robinson & Meyer, 1966).

2.3 Clinical signs of *S. agalactiae* infection

Streptococcal infections have been associated with significant morbidity and mortality among freshwater, estuarine and marine fish species. Pathogenesis in fish involves septicaemia and colonization of numerous organs such as the nares, brain, kidney and intestine. Clinical signs appear soon after infection, and include depression or excitability, anorexia, 'C' shaped body posturing, erratic swimming and whirling and death (Evans *et al*., 2002). Some of animal showed exopthalmia and corneal opacity. Clinical signs such as lethargy, erratic swimming, corneal opacity and caudal fin rot were observe during the experiment done by Abuseliana et al. (2011). In intraperitoneal (IP) trials, the affected fish showed behavioural changes such as lethargy, loss of appetite, grouping at the aquarium bottom and abnormal swimming. At the 3rd dpi some fish showed exopthalmia, corneal opacity and tail erosion (Abuseliana *et al.*, 2011). In this study also, clinical signs disappeared after the 8th dpi in IP challenge and this condition could be related to the ability of the immune system to resolve the infection in juvenile Red tilapia. Not all of these clinical signs are present in all of the affected fish and in some cases, the affected fish showed no obvious clinical signs before sudden death (Eldar *et al.,* 1995; Musa *et al*., 2009; Pretto-Giordano *et al*., 2010).

2.4 Pathogenicity of *S. agalactiae*

S. agalactiae has been increasingly recognised as pathogenic to fish especially for warm fresh water fishes (Plumb, 1999; Pretto-Giordano *et al.,* 2010). Experimentally, *S. agalactiae* has shown to be more infectious than any other environmental bacteria and the mortality in fish may reach up to 100% (Ferguson *et al.,* 1994). *S agalactiae* had caused 55% mortality in Nile tilapia within 10 days in intraperitoneal infection trials (Abuseliana *et al*., 2011).

2.5 Extracellular proteins (ECPs)

Extracellular proteins (ECPs) are extracellular secretory proteins which can activate host's immune response since they are secreted out of cells and are easily contacted with the host (Zhang *et al*., 2012). Extracellular products such as Streptolysins O and S, streptokinase, hyaluronidase, DNase enzyme, and cell-associated-proteins are important virulence factors for bacteria (Batt & Tortorello, 2014).

2.6 Cellular Membrane Proteins (CMPs)

Cellular Membrane Proteins (CMPs) are surface proteins that are expressed by many *Streptococcus strains* and serve as targets for protective antibodies (Lancefield *et al*., 1975). Several *S. agalactiae* virulence factors have been identified such as polysaccharide capsule, lipoproteins, superoxide dismutase and D-alanylated lipoteichoic acid. Polysaccharide capsule is important virulence factor. However, superoxide dismutase and D-alanylated lipoteichoic acid also play significant roles that contribute to virulence. There is also species specificity in the interactions between surface proteins and the infected host (Lindahl *et al.,* 2005).

3.0 MATERIALS AND METHODS

3.1 Experimental fish

About 300 apparently healthy African catfish (*Clarias gariepinus)* fingerlings were obtained from a commercial hatchery in Seri Kembangan, Selangor. Fish were randomly screened for disease infection prior to the experimental proper for at least 40% of the population to ensure that they were disease and pathogen free. The experimental proper used 120 fingerlings of *Clarias gariepinus* (size of 3-8 inches long) with duplicate. Prior to the experiment all tanks were cleaned and filled with dechlorinated tap water. The fingerlings were acclimatized for one week prior to the experiment and bathed with 0.2% $NaCl_2$. All fingerlings were maintained in two 500 L glass tanks. Later they were divided into 3 groups (control, group A and group B). The fish were fed daily with 3% body weight with commercial pellet feed.

3.2 Preparation of bacteria inoculum

S. agalactiae was originally isolated from a naturally infected tilapia. The organism was maintained in stock agar. A loopful of bacteria was recovered from stock culture and streak on TSA (tryptic soy agar) and bacteria was incubated at 28^{o}C for 24 hours. The purified and fresh bacteria colony was isolated and used for morphological characterization using gram staining and bacteria were identified as *S. agalactiae* group B using commercial identification kits (BBL Crystal GP ID kit) with 99.9% sensitivity of *S. agalactiae*. The bacteria were inoculated into 10ml tryptic soy broth (TSB) and incubated at 28 ^{o}C in incubator shaker (70rpm) for 24 hours. At this period, the cultures were in the stationary phase of growth. The bacterial optical density (OD) read at 600 nm was 1.994 using a spectrophotometer. The colony forming unit (CFU) was calculated

using spread plate technique and also read their $OD_{600nm.}$ The CFU was calculated at 8.53x 10^{11}/mL. The bacteria cultures were harvested from TSB by centrifuging at 1800xg (4500 rpm) for 15 minutes. From the centrifugation the cells pellet and supernatant were obtained. The supernatant was the Extracellular Proteins component while the pellet was the Cellular Membrane Proteins.

3.2.1 Preparation of Cellular Membrane Proteins (CMPs)

The cells pellet were washed 2x in phosphate buffered saline (PBS) using centrifugation, each time at 1000xg (3400 rpm) for 10 minutes. To obtained cellular membrane proteins (CMPs), the bacteria cells were boiled at 100°C for 10 minutes, to kill the bacteria and break the cells. After cooling, the bacteria cells component which is CMPs was pelleted again using centrifugation (1000xg for 10 minutes). The CMPs was diluted to desired concentration and stored chill prior to use. Ten fold serial dilutions (1:10) were made to give 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} diluents.

3.2.2 Preparation of Extracellular Proteins (ECPs)

The supernatant which is ECPs was filter-sterilised using 0.45-µm membrane filter. The ECPs was diluted to desired concentration and stored chill prior to use. Ten fold serial dilutions (1:10) were made to give 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} diluents.

3.3 Experimental infection trials

Fish were divided into three groups each group contained 50 fishes (Group A and Group B) and 20 fishes for control group. Group A and group B fish were further divided into five different glass aquarium (50L) contain 5 fingerlings each with duplicate. While, for the control group it was divided in two different glass aquarium also containing 5

fingerlings with duplicate. The temperature, pH and salinity were kept constant. For the experimental trial control group fingerlings were mock injected with 0.1 ml 0.85% normal saline intraperitonealy (i.p). While Group A fingerlings were i.p injected with 0.1 ml of each dilution of ECPs of *S. agalactiae* and Group B fingerlings were i.p injected with each dilution of CMPs of the bacteria.

3.4 Blood sample collection

Blood samples were collected at 8th days post inoculation (dpi) from naive and challenged fish. Firstly, the fish were anaesthetized with tricaine methane sulfonate (MS222) at a concentration of 50mg/mL Approximately 0.1mL of blood were drawn from each fish via the caudal peduncle vein using a syringe, with 25G needle and immediately transferred into plain tube and the blood was clotted to get the serum. The blood was pooled for each dilution of fish group.

3.5 Immunogenicity testing

3.5.1 Agar Gel Precipitation Test (AGPT)

The immunogenicity of CMPs and ECPs antigen and the serum dilution from fish were determined using AGPT following Ouchterlony technique (Ouchterlony, 2009). 0.9% agarose was prepared in normal saline solution. Agar was autoclaved at 15lb pressure for 15 minutes. The petri dish was placed on a flat surface and warm agarose (at 45-50 ^{o}C) was poured with a pipette. The agarose were allowed to cool and solidify. Wells were made in gel using gel punch and the excess gel was removed. A pattern of one central well surrounded by six peripheral well was used. Serum sample from each dilution of fish were loaded into five outer wells of the gel while the sixth well in the periphery of Petri dish contained serum from control fish. The antigen was loaded in central inner

well. The agar was then incubated in moist container at room temperature for 24 hours. The clear precipitin lines were observed after 24 hours and recorded as positive result showing antigen-antibody response.

3.6 Evaluation

The clinical signs, mortality and survivability of the fish injected with CMPs, ECPs and control fish were monitored and recorded daily for 7 days. The pooled serum sample from fish at different dilution injected with ECPs, CMPs and control fish were subjected for AGPT to see the immune response through precipitation line.

3.7 Statistical Analysis

Data were then subjected to statistical analysis, which involved summarizing the data using IBM SPSS version 20.0 followed by non parametric test Kruskal-Wallis test. Separation of means were tested using Duncan test significantly difference at level of $p<0.05$. Next, the data comparison between ECPs and CMPs on mortality was analyzed using Chi-square test at a significance difference level $p<0.05$.

4.0 RESULTS

4.1 Clinical findings and mortality

In this study, the fingerlings were observed after the injection procedure for a period of 7 days. Fingerlings in group A (ECPs) and group B (CMPs) showed similar clinical signs such as lethargy and off feed during 24 hours post inoculation (hpi). Clinical signs such as lethargy (Plate 1), inappetance (Plate 2), erratic swimming (Plate 3), corneal opacity (Plate 4), exopthalmia (Plate 5), and caudal fin rot (Plate 6) were observed during the experiment. All the fingerlings showed clinical sign of lethargy and inappetance and some showed erratic swimming behaviour. At 1st day post inoculation, the fingerlings showed clinical sign such as staying motionless on the aquarium bottom and lethargic. On the 3rd dpi, the infected fish were still lethargic but sudden erratic swimming. No clinical sign observed after the 7^{th} dpi and no S. *agalactiae* was isolated from the fish sample on 8^{th} dpi.

Mortality started after 48 hpi in CMPs group while in ECPs group mortality started after 72 hpi as shown in Table 1 and Table 2. The mortality occurred within 5 days post challenged. The accumulated mortality showed 8.33% mortality occurred in CMPs group fingerlings compared to ECPs group fingerlings with 6.67% accumulated mortality as in Figure 1. The result showed higher mortalities occurred in CMPs group compared to ECPs group.

The percentage survivability in different dilution tank of the fingerlings for CMPs and ECPs fingerlings were shown in Figure 2 and Figure 3. There was variation in pattern of mortality for both ECPs and CMPs injected fingerlings. The highest mortality was from both ECPs and CMPs groups occurred at Tank 5 which had highest dilution of

bacteria (10^{-5}) with a cumulative mortality of 20% and 30% respectively. Other mortalities occurred variably at Tank 1 from both CMPs and ECPs group and Tank 4 from ECPs group. There was no mortality observed from the rest of the tanks. The fingerlings from control group were active during the whole experiment and showed neither mortality nor clinical sign.

Tank	Dilution	No of dead fish (dpi)							Total no. dead fish	Cumulative mortality (%)	Percentage survivability (%)
		D1	D2	D3	D4	D5	D6	D7			
T1 R1	10^{-1}	0	0	0	0	0	0	0	0/5	20	80
T1 R2		0	0	0	1	1	0	0	2/5		
T2 R1	10^{-2}	0	0	0	0	0	0	0	0/5	0	100
T2 R2		0	0	0	0	0	0	0	0/5		
T3 R1	10^{-3}	0	0	0	0	0	0	0	0/5	0	100
T3 R2		0	0	0	0	0	0	0	0/5		
T4 R1	10^{-4}	0	0	0	0	0	0	0	0/5	0	100
T4 R2		0	0	0	0	0	0	0	0/5		
T5 R1	10^{-5}	0	1	1	0	0	0	0	2/5	30	70
T5 R2		0	1	0	0	0	0	0	1/5		
CxR1	-	0	0	0	0	0	0	0	0/5	0	100
CxR2		0	0	0	0	0	0	0	0/5		
Total									**5/60**	**8.33**	**91.67**

Table 1: Total cumulative mortality and percentage survivability recorded for fingerlings *Clarias gariepinus* intraperitoneally injected with CMPs of *S. agalactiae*

Tank	Dilution	No of dead fish (dpi)							Total no. dead fish	Cumulative mortality (%)	Percentage survivability (%)
		D1	D2	D3	D4	D5	D6	D7			
T1 R1	10^{-1}	0	0	0	0	1	0	0	1/5	10	90
T1 R2		0	0	0	0	0	0	0	0/5		
T2 R1	10^{-2}	0	0	0	0	0	0	0	0/5	0	100
T2 R2		0	0	0	0	0	0	0	0/5		
T3 R1	10^{-3}	0	0	0	0	0	0	0	0/5	0	100
T3 R2		0	0	0	0	0	0	0	0/5		
T4 R1	10^{-4}	0	0	0	0	0	0	0	0/5	10	90
T4 R2		0	0	1	0	0	0	0	1/5		
T5 R1	10^{-5}	0	0	0	1	1	0	0	2/5	20	80
T5 R2		0	0	0	0	0	0	0	0/5		
CxR1	-	0	0	0	0	0	0	0	0/5	0	100
CxR2		0	0	0	0	0	0	0	0/5		
Total									**4/60**	**6.67**	**93.33**

Table 2: Total cumulative mortality and percentage survivability recorded for fingerlings *Clarias gariepinus* intraperitoneally injected with ECPs of *S. agalactiae*

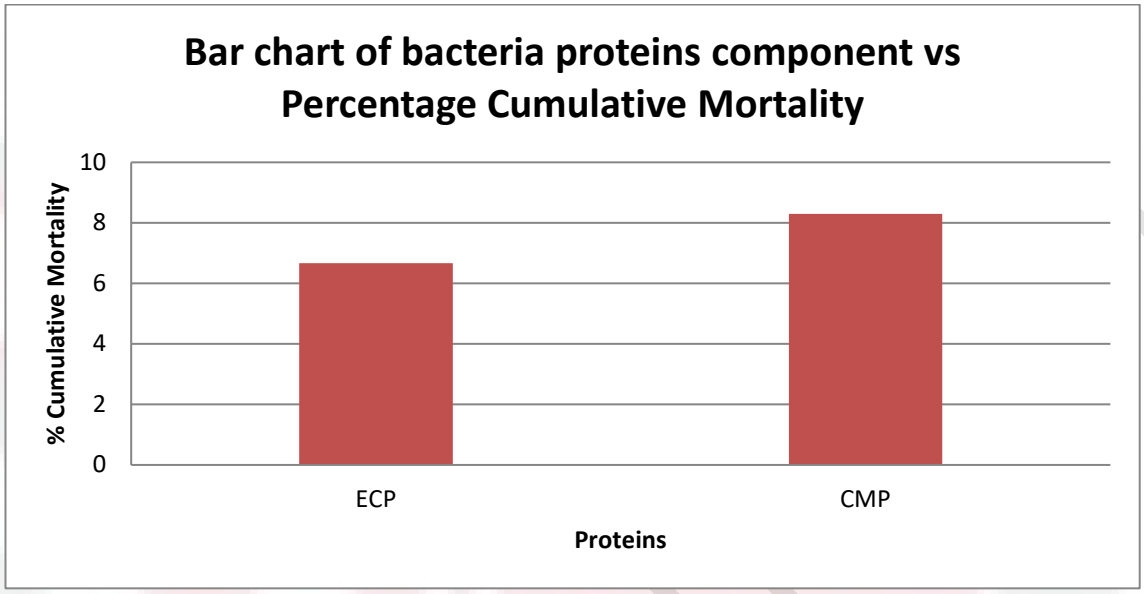

Figure 1: Graph shows relationship between ECPs and CMPs on cumulative mortality of fingerlings

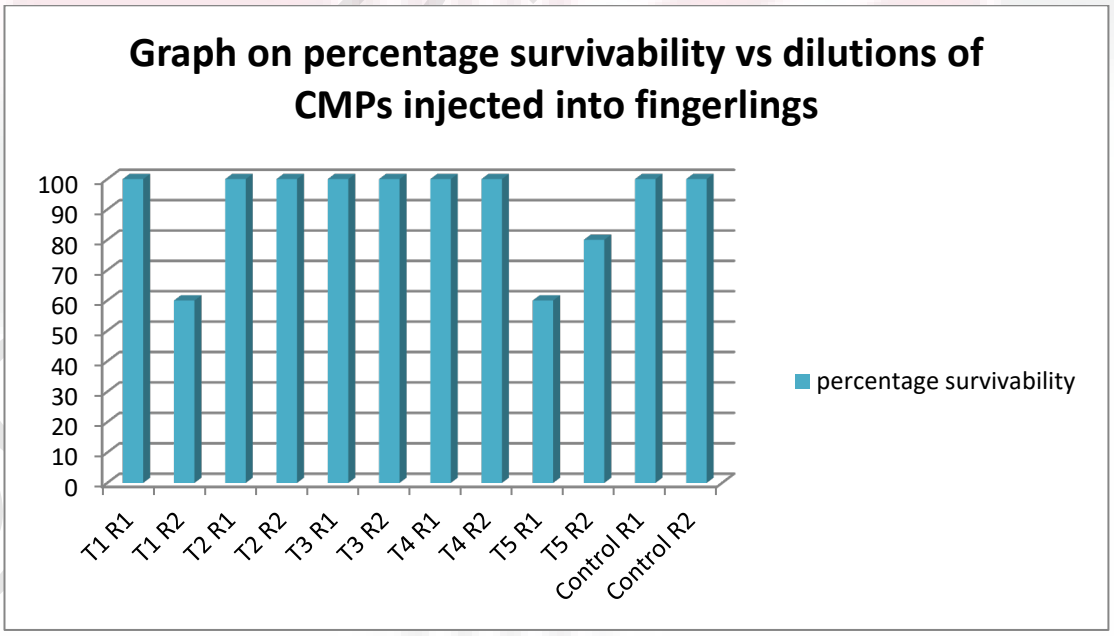

Figure 2: Percentage Survivability of CMPs injected fingerlings

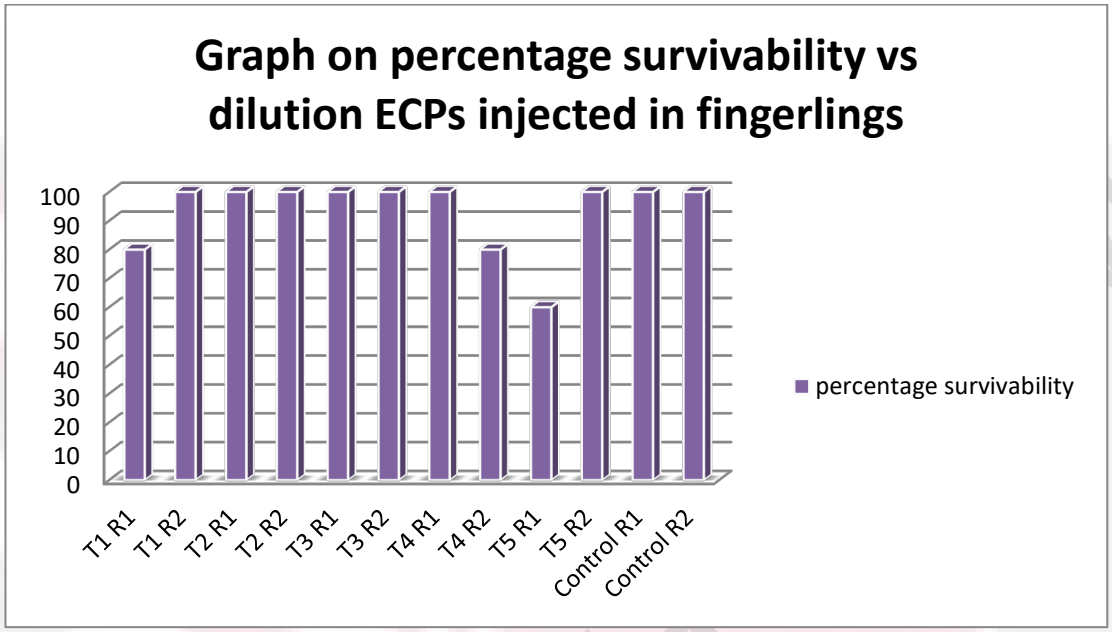

Figure 3: Percentage Survivability of ECPs injected fingerlings

4.2 Agar Gel Precipitation Test (AGPT)

The antigen-antibody response between antigen and serum were indicated by clear band between the wells. The result of the presence of the antigen-antibody response of CMPs group and ECPs groups were shown in Plate 7 and Plate 8. However, in this study there was no precipitation line observed in all groups indicated that there was no antigen-antibody response occurred.

4.3 Statistical Analysis

IBM SPSS version 20.0 was employed to analyze the percentage survivability of fish injected with different dilution of CMPs and ECPs. When the raw data were tested for normality test for normal distribution of the data, it was not normally distributed at $p<0.05$.So, the non parametric test which was Kruskal-Wallis being used. There was no significant difference between the different dilutions CMPs used with the percentage survivability of the fingerlings. There also no significance difference between the

different concentrations ECPs with the percentage survivability of fingerlings. Then we proceed with post hoc test for multiple comparisons between different concentrations of CMPs and ECPs from control. The result showed no significance difference between the different concentration compare to control.

As for comparison between the effect of CMPs and ECPs towards mortality of the fingerlings, Chi-square test were used at significance level $p<0.05$. There was no significance difference between effect of CMPs and ECPs towards mortality of fingerlings.

Bacteria products dilution	Kruskal-Wallis test (p-value)
CMPs	0.154
ECPs	0.593

Table 3: SPSS Output 1- The significant between dilution and percentage survivability for CMPs and ECPs injected fingerlings

Bacteria Products	Survivability		Chi-square test
	Alive	Dead	
CMPs	55 (91.67%)	5 (8.33%)	0.0729
ECPs	56 (93.33%)	4 (6.67)	

Table 4: SPSS Output 2- The significant between bacteria products on mortality of the fingerlings

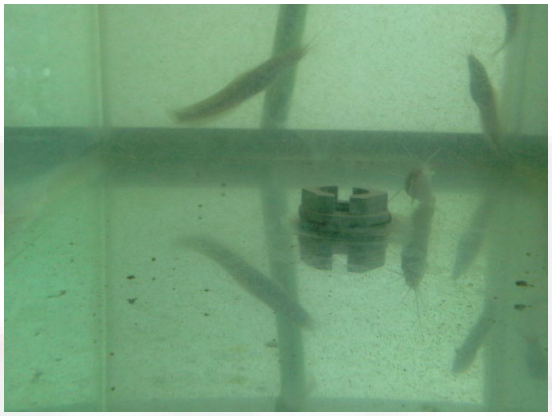

Plate 1: Lethargic fingerlings from ECPs and CMPs group seen at 24 hpi

Plate 2: Inappetance seen at 2nd dpi from ECPs and CMPs group

Plate 3: Erratic swimming behavior at 3rd dpi from ECPs group

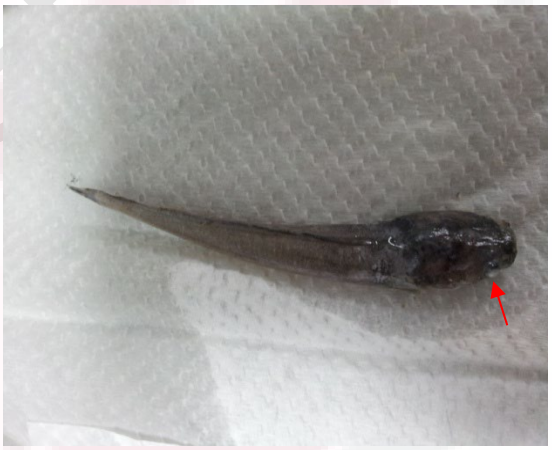

Plate 4: Corneal opacity at 2nd dpi from CMPs group

Plate 5: Exopthalmia seen on 3rd dpi from CMPs group

Plate 6: Caudal and dorsal fin rot at 3rd dpi from ECPs group

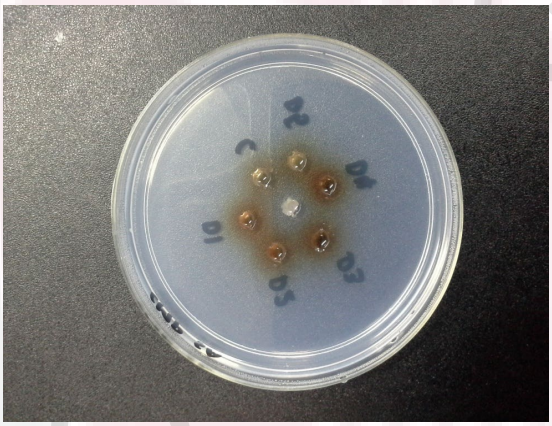

Plate 7: AGPT results of CMPs injected fish sera

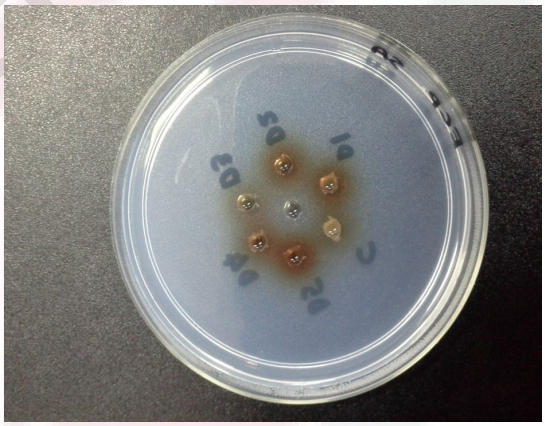

Plate 8: AGPT results of ECPs injected fish sera

5.0 DISCUSSION

Clinical sign such as lethargy, inappetance, erratic swimming, corneal opacity, exopthalmia and fin rot were observed for both CMPs and ECPs fingerlings and the onset of clinical sign could be seen after 24 hpi of injection with the bacteria products. The clinical sign observed were in agreement with previous study done by Abuseliana et al. (2011). A study done by Evans et al. (2002) reported that the onset of clinical signs were observed within 24 h after the i.p inoculation of *S. agalactiae* with a concentration of 1.0 x 10^7 CFU/fish and after seven days the accumulated mortality rate of 60% was observed. There was slight difference in the present study and the literature review on reported mortality. This could be due to several factors such as virulence of the bacteria products and ability of the bacteria strain to cause mortality. In this present study, the bacteria could only able to cause clinical signs but not to cause mortality. According to Pretto-Giordano et al. (2010), several factors that can contribute to differences in mortality including the concentration of inoculum, *S. agalactiae* strain or type, inoculation manner, observation period after challenge, age and weight of fish, and reactivation of pathogenicity.

The pattern of the mortality for ECPs and CMPs groups occurred was variable. Statistically there is no significant difference between concentration bacteria with survivability of the fish ($p<0.05$) for both ECPs and CMPs. However, this pattern of mortality can be explained probably due to individual fish response towards bacteria toxins. In this present study the ECPs and CMPs might able to cause lethal toxicity in some fish. Study done by Mekuchi et al. (1995) showed Japanese flounder fish ECP-injected died at maximum concentration of ECP diluted $1:2^{-3}$ However, the mortality occurred variably at different dilutions of ECP and CMP also could be explained due to position of the tanks that were not randomized and the different intensity of light affect

the water temperature. The effects of temperature on specific immune responses, especially the humoural response, have been studied by Avtalion et al. (1970). Experiments was done on carp immunized against bovine serum albumin and reported that primary antibody response is suppressed at low temperatures. Lower temperatures induce a delay in the peak of the primary response but have no effect on the magnitude of the response (Rijkers *et al*. 1980). Besides, the mortality occurred also could be due to stress such as stress handling and water quality that caused the fish to die.

The findings of the present study suggest that ECPs and CMPs had no effect on fish's immune response. This is could be due to concentration of the bacteria that were not able to increase the immune response to the fish and only caused weak immune response. According to Amrullah et al. (2014) correct doses is able to increases non-specific cellular immune response in fish optimally. Khushiramani et al. (2007) reported specific antibody production increased in fish receiving booster dose of antigen as compared to fish receiving single dose of antigen. According to Kreutz et al. (2014) re-inoculation of antigens is important since antibody titre is believed to be short lived in fish.

Besides, the immune response is not present in this study also could be due to short observation time after the challenge that the antibody production still in early stages. Previous study done by Boesen et al. (1997) on immune response of rainbow trout showed serum antibody titres started to rise 2 weeks after vaccination, and reached maximum at 4, 6 or 8 weeks after vaccination depend on antigen preparation tested as for ECPs it peak on 4 weeks after vaccination and CMPs peak after 6 to 8 weeks. Study done by Mekuchi et al. (2015) on Japanese flounder reported there was increased in agglutinating antibody levels in the blood of immunized fish two weeks after vaccination. However, study done

by Kreutz et al. (2014) reported that antibody can be seen as early as 7 days post inoculation but antibody is short lives. Therefore, these literature findings support the present study on the absence of the immune response in fish at 8th days post injected with ECPs and CMPs.

Other than that, no precipitation lines were observed from AGPT in our study could be due to concentration of antibody presence in the fingerlings is low. It is supported by Arun et al. (1991) where precipitate will form a visible white line between the two wells when antibody and its specific antigen meet one another and are at the proper concentrations. Antibody concentration and persistence in serum can differ according to species, age, sexual maturity and physiological events (Takahashi *et al*., 2012).

6.0 CONCLUSION AND RECOMMENDATIONS

In conclusion, clinical signs observed in ECPs and CMPs injected fish were similar to naturally infected fish however low mortality was recorded. ECPs is a better immunostimulant than CMPs based on the mortality recorded. Besides, water temperature also affects on survivability and antibody production of the fish in this study.No antibody was detected in ECPs and CMPs injected fish sera using AGPT could be due to several factors such as doses of antigen administered, low concentration of antibody and short observation time after challenged.

The recommendation for this study is to extend the duration of the experiment to properly evaluate the immune response through humoral antibody production of the fish. In addition, we also recommended using bigger size of fish because the age and weight of fish might contribute to the different results and increase sample size to get proper result. Besides, the control, ECPs and CMPs injected fingerlings previously should be re-challenged with live bacteria *S. agalactiae* and the relative percentage survival (RPS) of the fish should be calculated to know the efficacy of the bacteria antigen injected.

Furthermore, the study also can be improved by measuring the lethal dose at 50% end-point of the bacteria for the catfish first before preparations of the ECPs and CMPs as the level of sensitivity and toxicity for fish species might be different. In addition, before preparation of the ECPs and CMPs from the bacteria, the pure culture bacteria from stock agar should be passaged in the fish at first so that we can know the virulence property of the bacteria since bacteria virulence is weakened after being cultured on artificial medium for a longer period of time. Other method that is more sensitive in detecting antibody such as ELISA and SDS-PAGE should be use. Lastly, further study

should be done on purified bacteria using western blot (protein immunoblot) to detect specific proteins in sample.

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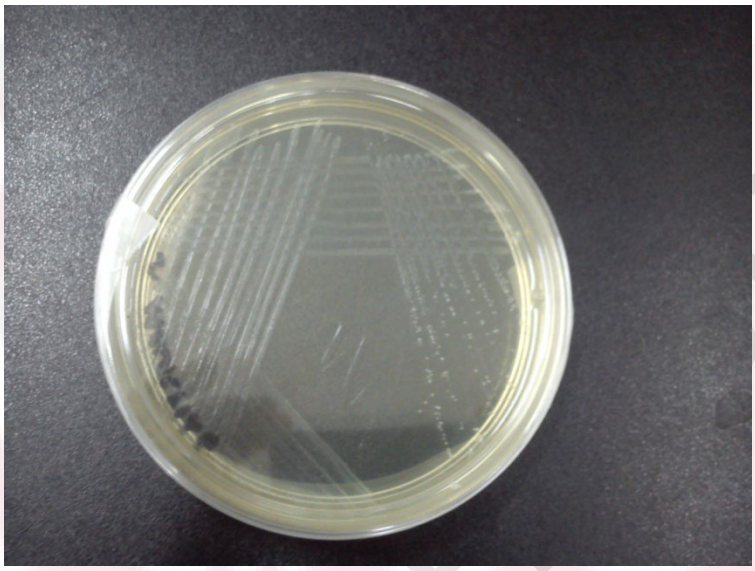
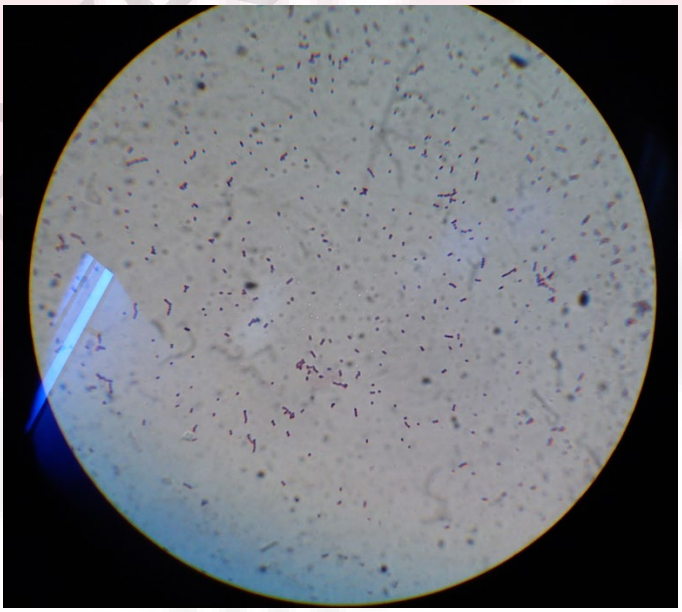
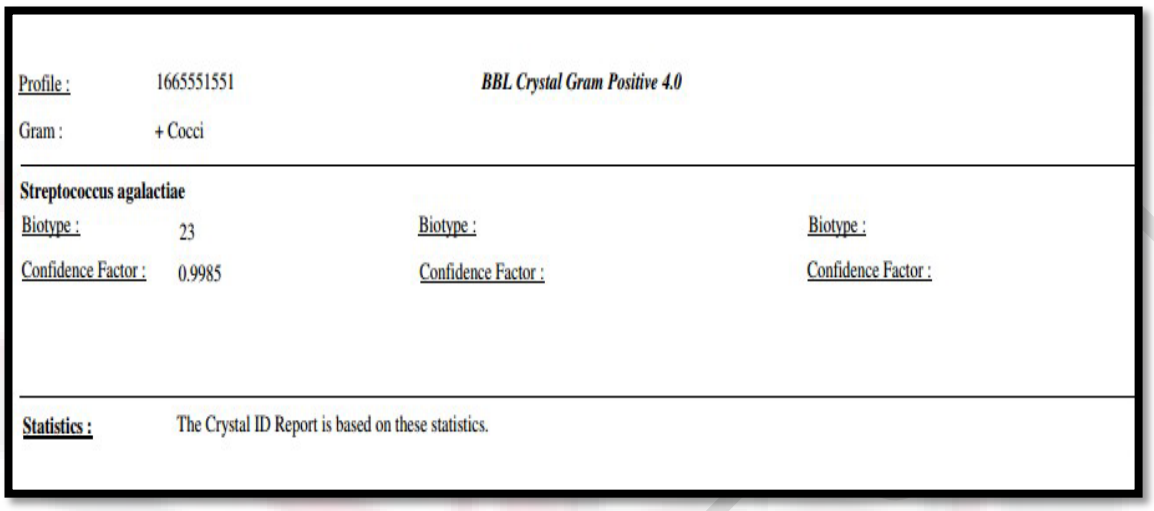
8.0 APPENDICES



Streptococcus agalactiae bacteria colony on Tryptic Soy Agar (TSA) incubated at 28℃, 24 hours



Cells morphology of *Streptococcus agalactiae* with Grams staining



Profile :	1665551551	*BBL Crystal Gram Positive 4.0*	
Gram :	+ Cocci		
Streptococcus agalactiae			
Biotype :	23	Biotype :	Biotype :
Confidence Factor :	0.9985	Confidence Factor :	Confidence Factor :
Statistics :	The Crystal ID Report is based on these statistics.		

Bacteria identification result using BBL Crystal