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Growth and Storage Study of *Streptococcus agalactiae* as a Potential Target in Whole-Cell Based 'Systematic Evolution of Ligands by Exponential Enrichment (SELEX)' Procedure

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ABSTRACT

Streptococcus agalactiae is a pathobiont that causes severe invasive infections, especially in fishponds or freshwater aquatic organisms. Streptococcus outbreak in cultured fishponds causes a negative impact on the production of fishes, especially tilapias. Therefore, it is essential to develop a reliable method for on-site monitoring of *S. agalactiae* in fishponds. This study aims to investigate a local *S. agalactiae* strain TP540K as a potential target for DNA aptamer in a whole-cell based 'systematic evolution of ligands by exponential enrichment (SELEX)' procedure. Before this procedure, the bacterial growth profile of *S. agalactiae* strain TP540K was studied. Besides, the viability of *S. agalactiae* strain TP540K was compared between different storage solutions (glycerol, phosphate buffer saline, distilled water) and temperatures (-20, 4 and 27 °C). In this study, *S. agalactiae* strain TP540K entered the log phase at 1.5 h of incubation time, thus the cells were collected at 2 h of incubation time with an OD_{600} value of 0.436 to proceed to SELEX process. Moreover, glycerol stock (20%, -20 °C) was the best storage condition for preservation of *S. agalactiae* strain TP540K until Day 84 with a cell viability of 2.48 x 10^7 CFU/mL. With the findings obtained, *S. agalactiae* strain TP540K could be utilised as a target for future study in DNA aptamer development through whole-cell based SELEX procedure, which could be useful for future investigations to overcome Streptococcus outbreak in the Malaysian aquaculture industry.

INTRODUCTION

According to Raabe and Shane [1], *Streptococcus agalactiae* also known as Group B Streptococcus (GBS) is a gram-positive bacterium that was first isolated from cattle with mastitis and was then identified by Lehmann and Neumann in 1930. Not only that, but it was also later discovered to be a part of the natural flora colonising many parts of the human body. Thus, *S. agalactiae* was identified to have two major permanent hosts: humans and cattle. It primarily colonises the gastrointestinal and genitourinary tracts in humans, and it is mostly found in the udder in cattle. In addition to humans and cattle, *S. agalactiae* also has been isolated from aquatic animals, especially fish. Although *S. agalactiae* is the normal flora of the human gut, it is classified as pathobiont which causes severe invasive infections in humans and animals, including aquatic species. Infections with *S. agalactiae* are becoming more common in aquaculture, with cases documented globally in a range of fish species, particularly

those that live in warm water [2]. According to Ndashe et al. [3], several factors contribute to Streptococcus outbreak in freshwater aquaculture, including high stocking densities, poor water conditions, and high temperatures. This is very concerning in Malaysia's context as freshwater aquaculture is one of the dominant industries for this country with an estimated production of 194 thousand tonnes at a value of USD 308 million [4]. For instance, the total freshwater aquaculture production is mainly contributed by Nile tilapia (*Oreochromis niloticus*) which accounts for 44.7%, followed by catfish (36.7%) and carp (10.08%). In terms of total production value, tilapia accounts for 49.37%, followed by catfish (37%) and carp (10%).

Apart from that, aquaculture has created a lot of job opportunities and it is the primary source of revenue for the bottom 40% of the household income (B40) community in Malaysia [5]. To overcome Streptococcus outbreak in cultured fishponds, a rapid and simple method is needed for the on-site detection and monitoring of *S. agalactiae*. This could be achieved through a whole-cell based 'systematic evolution of ligands by exponential enrichment (SELEX)' procedure by isolating DNA aptamer that can detect *S. agalactiae*. In this study, *S. agalactiae* strain TP540K, a local strain that was isolated from red hybrid tilapia fish in Kedah, Malaysia was investigated as a potential target in whole-cell based SELEX procedure.

The bacterial growth profile of *S. agalactiae* strain TP540K was studied to determine the log phase of the bacteria. Besides that, a reliable and more economical storage method for *S. agalactiae* strain TP540K in terms of viability among different storage conditions was evaluated to preserve the bacterial strain at a stable state and, as an intact whole-cell for the SELEX procedure.

MATERIALS AND METHODS

Bacteria broth culture preparation

Firstly, 0.74 g of brain heart infusion (BHI) powder (Oxoid, United Kingdom) was weighed and mixed with 20 mL of distilled water using a magnetic stirrer. Secondly, 6 mL BHI broth was placed into 3 universal bottles respectively where one of them as a negative control. 300 µL of *Streptococcus agalactiae* strain TP540K from the glycerol stock was pipetted and transferred into two of the 6 mL BHI broths respectively. The caps of the universal bottles were replaced and incubated at 37 ºC for 24 h in an incubator shaker with 150 rpm.

Glycerol stock solution preparation

The glycerol stock solution was prepared according to the protocol [6]. To prepare a 20% glycerol stock solution, 2 mL of 100% glycerol was pipetted into a 10 mL universal bottle. Next, 8 mL of distilled water was pipetted into the universal bottle as well. The mixture was vortexed to ensure homogeneity. The mixture was then sterilised at 121 ºC for 15 min in the autoclave. After autoclaving, the 20% glycerol stock solution was left cool. Next, 500 µL of 20% glycerol stock solution was pipetted into 20 microcentrifuge tubes. In addition, 500 µL of *S. agalactiae* was also pipetted from the overnight broth culture into each microcentrifuge tube respectively. The microcentrifuge tubes were stored at -20 ºC for future study.

Growth profile determination

Absorbance

300 µL of *S. agalactiae* was pipetted from the glycerol stock into 6 mL BHI broth. To revive the cells, the broth was incubated at 37 ºC for 24 h in an incubator shaker with 150 rpm. After 24 h, 1 mL of broth was inoculated into 100 mL BHI broth and incubated at 37 ºC in an incubator shaker with 150 rpm. On the other hand, a blank BHI broth was prepared to serve as a negative control. At every 30-min interval, 1.5 mL of sample was collected into a cuvette and its absorbance at wavelength of 600 nm was measured using UV/Visible Spectrophotometer (Secomam UviLine 9400). For this step, triplicate measurements were performed to ensure more accurate absorbance results. Lastly, a graph of absorbance reading against time was plotted [7].

Serial dilution and colony forming unit (CFU) counting

S. agalactiae (300 µL) was pipetted from the glycerol stock into 6 mL BHI broth. To revive the cells, the broth was incubated at 37 ºC for 24 h in an incubator shaker with 150 rpm. After 24 h, 1 mL of broth was inoculated into 100 mL BHI broth and incubated at 37 ºC in an incubator shaker with 150 rpm. On the other hand, a blank BHI broth was prepared to serve as a negative control. At every one-hour interval, a ten-fold serial dilution (10^1 to 10^7) and CFU counting according to the protocol by Blaize [8] were performed. To form a 1:9 dilution mixture, 100 µL of sample from BHI broth was transferred to a sterile microcentrifuge tube containing 900 µL of sterile distilled water.

The mixture was resuspended a few times to ensure homogeneity. The steps were repeated several times until 10^7 dilution factor was reached. After that, spread plate method was performed where 100 µL of each 10^5, 10^6 and 10^7 dilution factor was pipetted and spread on their respective blood agar plates (BAP) using a sterile glass hockey. Duplicates were made for each of the dilution factors. The BAP was incubated at 37 ºC for 24 h. The countable colony in the range between 30 and 300 colonies was calculated and recorded. The CFU/mL was calculated with the formula below and finally, a graph of CFU/mL against time was plotted.

$$\text{CFU/mL} = \frac{\text{Number of colonies x Dilution factor}}{\text{Volume of sample pipetted (mL)}}$$

Storage and preservation

Three different storage solutions were prepared, which included 20% glycerol stock, sterile distilled water and phosphate buffer saline (PBS) (pH 7.2). For the preparation of 20% glycerol stock, it was described in the previous paragraph. For the preparation of sterile distilled water, 1 L of distilled water was placed in a Scott bottle and sterilised by autoclaving at 121°C for 15 min. It was then let cool and stored at room temperature. For the preparation of PBS, 0.8 g of NaCl, 0.02 g of KCl, 0.024 g of KH_2PO_4 and 0.144 g of Na_2HPO_4 were weighed using an electronic balance. Next, 100 mL of sterile distilled water was added and mixed with the chemicals.

The PBS was adjusted with 0.1 M NaOH until the pH was within the range of 7.2 to 7.4. Lastly, the PBS was filtered using a syringe filter with 0.2 µm pore size [9]. 500 µL of each storage solution was pipetted into 9 microcentrifuge tubes respectively. Hence, there would be a total of 27 microcentrifuge tubes. Next, 500 µL of *S. agalactiae* was pipetted from the glycerol stock into each tube. The microcentrifuge tubes were then properly labelled and gently vortexed. The microcentrifuge tubes with different storage solutions were also stored at different storage temperatures (-20 ºC, 4ºC and 27ºC). To determine the best storage condition in terms of storage solution and temperature, the observation of bacterial growth was done for every two-week interval. For the bacterial growth observation, a ten-fold serial

dilution (10^1 to 10^5) and CFU counting were performed for each of the storage conditions [8].

Statistical analysis

Results were an average of triplicate measurement with the coefficient of variation for each data is less than 20%. Comparison between mean was carried out using a t-test assuming unequal variance whilst comparison for more than two means was carried out using one-way ANOVA.

RESULTS AND DISCUSSION

Growth profile determination of *Streptococcus agalactiae*

In order to proceed to the whole-cell based 'systematic evolution of ligands by exponential enrichment (SELEX)' procedure, bacteria in log phase (minimum OD_{600} of 0.3) had to be harvested as the majority of the population's cells are healthy and actively dividing [10]. Hence, a growth profile for *S. agalactiae* strain TP540K was constructed to determine the log phase. At the same time, two *S. agalactiae* strains from the American Type Culture Collection (ATCC), namely *S. agalactiae* strain 12386 and *S. agalactiae* strain 13813 were used for positive control and comparison. The OD_{600} of these three strains in brain heart infusion (BHI) broth cultures at 37 ºC was determined at every 30-minute interval.

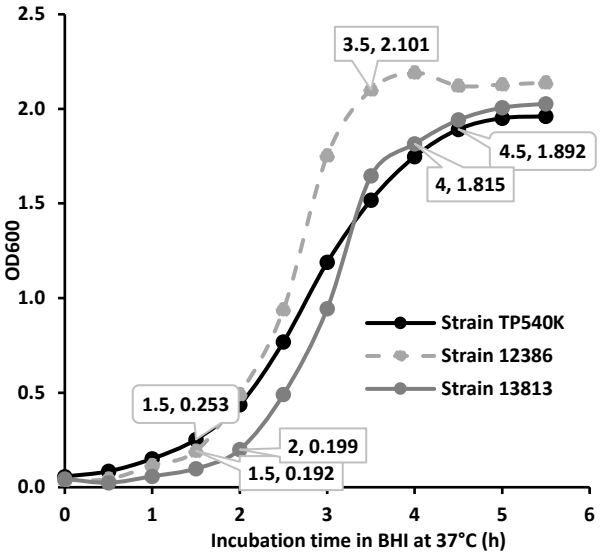

Fig. 1. The growth profile of *S. agalactiae* for three different strains in BHI.

The growth profiles of three different *S. agalactiae* strains were compared. Based on **Fig. 1**, both strain TP540K and strain 12386 entered the log phase at a faster rate (1.5 h) compared to strain 13813 (2 h). However, strain 12386 entered the stationary phase at the fastest rate which was after 3.5 h whereas strain 13813 entered the stationary phase after 4 h.

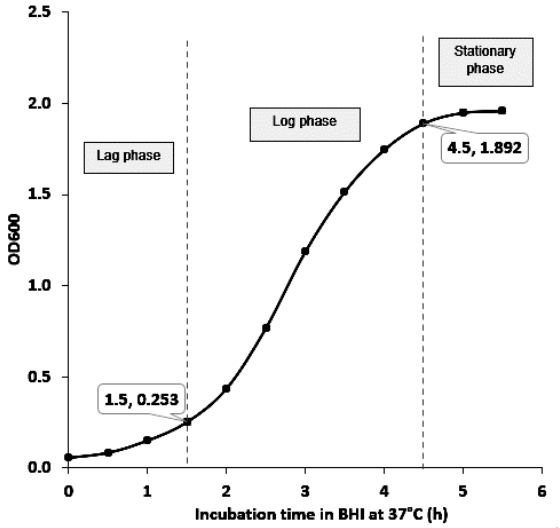

Fig. 2. The growth profile of *S. agalactiae* strain TP540K in BHI at 37 °C.

On the other hand, the growth profile of strain TP540K with its respective phases is shown in **Fig. 2** above. It was a smoother curve with a longer period of log phase from 1.5 h to 4.5 h, and strain TP540K entered stationary phase after 4.5 h. According to Duan et al. [10], the cells should be harvested during log phase with a minimum OD_{600} of 0.3. This is because at that phase, most of the cells would be in their active state and an intact whole-cell could be used in the SELEX procedure. In contrast, cell lysis may occur when the cells are in the stationary phase or death phase. Since the log phase of strain TP540K started at 1.5 h as shown in **Fig. 2**, the cells were harvested at around 2 h with an OD_{600} value of 0.436 for the preparation of SELEX.

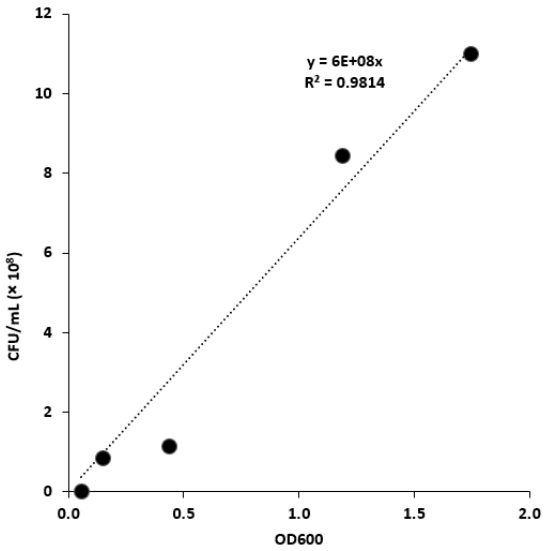

Fig. 3. The correlation graph of CFU/mL against OD_{600} for *S. agalactiae* strain TP540K.

At the same time, a correlation graph between OD_{600} and CFU/mL for strain TP540K was also plotted as shown in **Fig. 3**. The r^2 of 0.9814 in the graph was above 0.95 and near to 1, which indicated that the data had a good fit. The equation of $y = 6 \times 10^8$ (x) could also be used to estimate the value of CFU/mL in future by substituting OD_{600} reading as x.

Determination of the most effective storage condition for *Streptococcus agalactiae*

In this study, the most effective storage condition for *S. agalactiae* strain TP540K in terms of viability and cellular structure was determined. There are a few reasons behind this. Firstly, this is because the availability of authentic microbial strain is crucial for research study to ensure significance and accuracy. Secondly, it is always a goal to determine a cost-effective and simple way for the preservation of bacteria. Usually, the ideal long-term storage method for cultures will be lyophilisation or freeze-drying. It is undeniable that this method is excellent to use, but it requires specialised equipment which can be difficult to access [6]. Last but not least, an intact whole cell is required for the whole-cell based SELEX in this study. Therefore, it is vital to store or preserve the bacterial strain in a genetically stable state.

As the aim is to find a convenient method of preservation, the storage solutions and storage conditions used in this study could be easily obtained. For instance, the three different storage solutions used were 20% glycerol stock, sterile distilled water and phosphate buffer saline (PBS) (pH 7.2). There are a lot of studies that utilise glycerol with a final concentration ranging from 10% to 20% as a storage solution [6,11]. This is because the formation of ice crystals is always a critical problem when cultures are stored at low temperatures. Hence, glycerol can act as a cryoprotectant to avoid cell dehydration and denaturation under freezing conditions. Nonetheless, PBS might be another alternative in this study as it could also act as a protectant that prevents cells from rupturing or shrivelling up due to osmosis, hence keeping the cells intact [12]. Apart from that, it is interesting to find out if distilled water can act as a new alternative for the storage of cells.

On the other hand, three different storage temperatures at -20 ºC, 4 ºC and 27 ºC had also been used. From several studies, -80 ºC is found to be the best temperature for storage and preservation, but the maintenance cost is higher and it is difficult to gain access to the specific freezing facility [6,11]. Hence, -20 ºC, 4 ºC and 27 ºC were chosen in this study as the refrigeration and freezing equipment were more accessible. In addition, we wish to find out the upper limit of these storage temperatures, and to what extent, in terms of duration, they can be used for storage. Thus, the cell viability of *S. agalactiae* strain TP540K under each storage condition was tested at every two-week interval. Based on **Fig. 4**, PBS seemed to be the best storage solution as the cell viability was considered high in all the storage temperatures on Day 14. For instance, it was 2.37 x 10^7 CFU/mL, 1.06 x 10^8 CFU/mL and 1.27 x 10^8 CFU/mL at 27 ºC, 4 ºC and -20 ºC respectively.

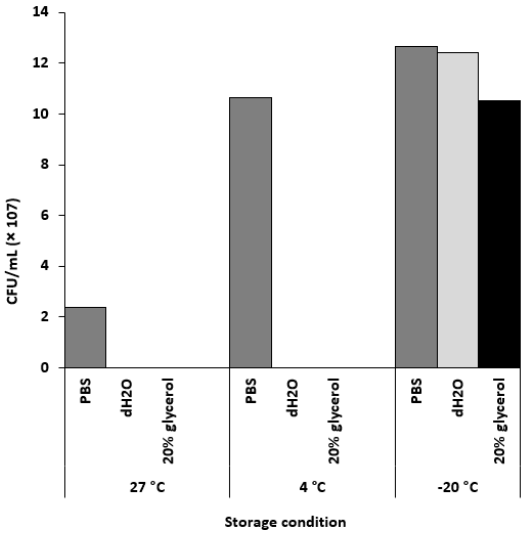

Fig. 4. The cell viability of *S. agalactiae* strain TP540K in CFU/mL under different storage conditions on Day 14.

Apart from that, distilled water and 20% glycerol stock were also the potential storage solution at -20 ºC, which maintained the cell viability at 1.24 x 10^8 CFU/mL and 1.05 x 10^8 CFU/mL respectively. Thus, these few storage conditions were being proceeded to Day 28. As shown in **Fig. 5**, the cell viability in PBS at 27 ºC and 4 ºC dropped below 2.00 x 10^7 CFU/mL. In addition, the cell viability in dH_2O at -20 ºC was also low, which was 6.80 x 10^6 CFU/mL. Thus, these conditions were not being proceeded with further storage study.

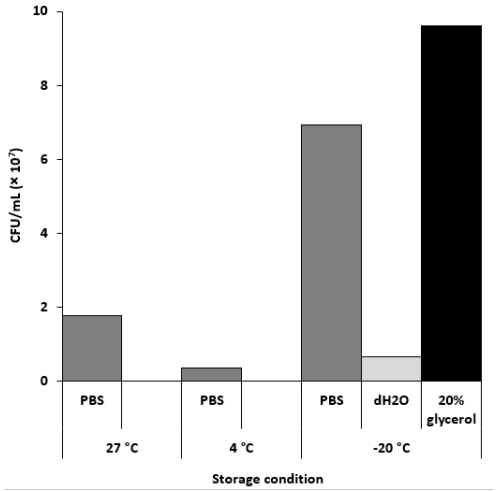

Fig. 5. The cell viability of *S. agalactiae* strain TP540K in CFU/mL under different storage conditions on Day 28.

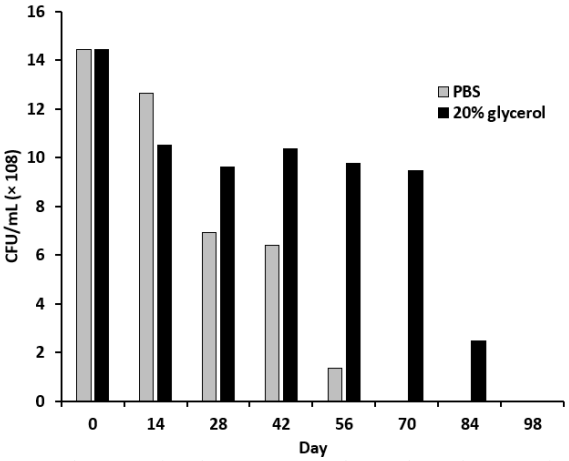

Fig. 6. The comparison between PBS and 20% glycerol at -20 ºC in terms of the cell viability of *S. agalactiae* strain TP540K in CFU/mL from Day 0 to Day 98.

In contrast, only PBS and 20% glycerol at -20 ºC managed to maintain the high cell viability, which was 6.95 x 10^7 CFU/mL and 9.61 x 10^7 CFU/mL respectively. Hence, both storage conditions had been compared until Day 98, which was 14 weeks in total. Based on **Fig. 6**, it was observed that 20% glycerol was a better storage solution compared to PBS at -20ºC as it managed to preserve the bacteria until Day 84 with cell viability of 2.48 × 10^7 CFU/mL. However, subculture of bacteria should be performed before Day 84 as well to continue the viability of bacteria as no growth could be observed on Day 98. In contrast, PBS only managed to preserve the bacteria until Day 56 with cell viability of 1.38 × 10^7 CFU/mL, and the bacteria lost its viability on Day 70. Hence, to preserve bacteria for a longer duration, 20% glycerol at -20ºC could be considered as a good option, given the simple and accessible materials and equipment.

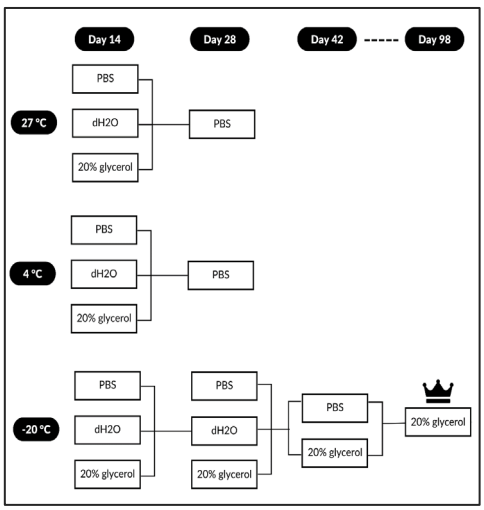

Fig. 7. The visualisation of storage condition selection into subsequent rounds.

A simple illustration of the storage condition selection into subsequent rounds is shown in **Fig. 7**. It could be seen that five out of nine storage conditions had been continued to Day 28. Next, from Day 42 to Day 98, only the cell viability in PBS and 20% glycerol at -20 ºC had been compared. Although the cell viability was lost in both storage conditions on Day 98, 20% glycerol at -20 ºC was claimed to be the best storage condition among all for *S. agalactiae* strain TP540K.

CONCLUSION

In conclusion, *Streptococcus agalactiae* strain TP540K was investigated in growth profile determination as well as storage and preservation study. The bacterial growth profile of *S. agalactiae* strain TP540K was compared with *S. agalactiae* ATCC strain 12386 and 13813. *S. agalactiae* strain TP540K entered log phase at 1.5 h of incubation time, hence the cells were harvested at around 2 h incubation time with an OD_{600} value of 0.436, which was at their most active state for the preparation of SELEX. The most effective storage condition for *S. agalactiae* strain TP540K in terms of viability was determined to be in 20% glycerol stock at -20 °C. It had managed to preserve the bacteria in an intact whole-cell form until Day 84 with cell viability of 2.48 x 10^7 CFU/mL. For future studies, *S. agalactiae* strain TP540K could be used as a target for DNA aptamer development through whole-cell based SELEX procedure, which could help to overcome *Streptococcus* outbreak in the aquaculture industry in Malaysia.

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REFERENCES

1. Raabe VN, Shane AL. Group B streptococcus (*Streptococcus agalactiae*). Microbiol Spect. 2019;7(2):10-128.
2. Geng Y, Wang KY, Huang XL, Chen DF, Li CW, Ren SY, et al. *Streptococcus agalactiae*, an emerging pathogen for cultured ya-fish, *Schizothorax prenanti*, in China. Transbound. Emerg Dis. 2012;59(4):369-75.
3. Ndashe K, Hang'ombe BM, Changula K, Yabe J, Samutela MT, Songe MM, et al. An assessment of the risk factors associated with disease outbreaks across tilapia farms in central and southern Zambia. Fishes. 2023;8(1):49.
4. Hashim M. National aquaculture sector overview-Malaysia. Food and Agriculture Organization of the United Nations. 2022.
5. Waiho K, Fazhan H, Ishak SD, Kasan NA, Liew HJ, Norainy MH, et al. Potential impacts of COVID-19 on the aquaculture sector of Malaysia and its coping strategies. Aquac Rep. 2020;18:100450.
6. Kumar S, Kashyap PL, Singh R, Srivastava AK. Preservation and maintenance of microbial cultures. In Analyz. Microb. 2013;135-52.
7. Lu J, Guevara MA, Francis JD, Spicer SK, Moore RE, Chambers SA, et al. Analysis of susceptibility to the antimicrobial and anti-biofilm activity of human milk lactoferrin in clinical strains of *Streptococcus agalactiae* with diverse capsular and sequence types. Front Cell Infect Microbiol. 2021; 905.
8. Blaize JF. Serial dilutions and plating: microbial enumeration. My J Vis Exp Corp. 2016.
9. Liao CH, Shollenberger LM. Survivability and long-term preservation of bacteria in water and in phosphate-buffered saline. Lett Appl Microbiol. 2003;37(1):45-50.
10. Duan N, Wu S, Chen X, Huang Y, Xia Y, Ma X, et al. Selection and characterization of aptamers against *Salmonella typhimurium* using whole-bacterium systemic evolution of ligands by exponential enrichment (SELEX). J Agric Food Chem. 2013;61(13):3229-34.
11. Sprouffske K, Aguilar-Rodríguez J, Wagner A. How archiving by freezing affects the genome-scale diversity of *Escherichia coli* populations. Genom Biol Evol. 2016;8(5):1290-8.
12. Martin NC, Pirie AA, Ford LV, Callaghan CL, McTurk K, Lucy D, et al. The use of phosphate buffered saline for the recovery of cells and spermatozoa from swabs. Sci. Justice - J. Forensic Sci. 2006;46(3):179-84.