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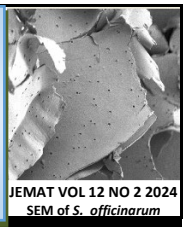

Characterization of *Streptococcus agalactiae* Strain TP540K and Its Potential as a Target for DNA Aptamer Development in the Preliminary Stage of Whole-Cell Based SELEX

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ABSTRACT

Streptococcus outbreak caused mainly by *Streptococcus agalactiae* leads to massive mortalities of freshwater aquatic organisms, especially cultured tilapias. This has caused a huge concern in the Malaysian aquaculture industry due to a lower aquaculture production affected by the outbreak. Thus, there is a need for a rapid and simple method to detect and monitor *S. agalactiae* in cultured fishponds. This study aims to investigate the characteristics of a local *S. agalactiae* strain TP540K and its potential as a target bacterium to develop DNA aptamer through the whole-cell based 'systematic evolution of ligands by exponential enrichment (SELEX)' procedure. The morphology and biochemical characteristics of *S. agalactiae* strain TP540K were studied. Furthermore, the preliminary stage of the SELEX process was carried out by incubating *S. agalactiae* strain TP540K with a single-stranded DNA library. In this study, *S. agalactiae* strain TP540K was confirmed to be spherical, gram-positive, catalase and oxidase-negative with beta-haemolytic characteristics. Additionally, the presence of bands with an estimated size of 80-90 base pairs on the agarose gel indicated the presence of DNA aptamer that could bind towards *S. agalactiae* strain TP540K during the SELEX process. The findings herein demonstrate that *S. agalactiae* strain TP540K has the potential to be utilised as a target for the production of DNA aptamers, and subsequent rounds of whole-cell based SELEX process can be performed to investigate the binding affinity and specificity for more accurate detection of *S. agalactiae* in a future application.

INTRODUCTION

In Malaysia, freshwater aquaculture is an important industry and serves as one of the backbones of Malaysia's economy. However, it was reported that the Streptococcus outbreak in cultured fishponds had affected negatively to production of fishes, especially tilapia [1]. According to Amin-Nordin et al. [2], the worst scenario is that the people who consumed the raw freshwater fish that were infected by *Streptococcus agalactiae* were reported to develop diseases such as septic arthritis and meningitis. To curb the infection and reduce the financial impact on fish producers, Maulu et al. [3] reviewed some potential preventive measures that can be considered such as selection of farm location, appropriate aquaculture farm methods, antibiotic use and appropriate immunisation programme. Based on their research, the use of vaccines has been proven to have the most potential among the currently available preventive methods.

In addition, it is also important to develop a rapid and efficient diagnostic tool for the early detection and constant monitoring of *S. agalactiae* present in fishponds to ensure the health and safety of consumers. Not only that, the prevalence and distribution of fish infected with *S. agalactiae* should be investigated as well to determine the most suitable way forward. To achieve this, there is the development of biosensor, a device which popularly utilises antibodies as the bioreceptors to detect the antigen on the bacteria. However, there are several significant limitations of this antibody-based biosensor. For example, the manufacturing process can be extremely costly and time-consuming. Furthermore, batch-to-batch variation, temperature sensitivity, irreversible denaturation, and a short shelf life are also some of the problems that must be addressed [4].

Alternatively, the development of DNA aptamer can be a way forward for this issue. DNA aptamer is an oligonucleotide (usually 25 to 100 nucleotides), which has high thermal stability and yet is reliable after long-term storage. The DNA aptamer is isolated through 'systematic evolution of ligands by exponential enrichment (SELEX)' procedure, a technique that screens random libraries of oligonucleotides in vitro using targets and then amplifies them by polymerase chain reaction (PCR). Among SELEX technologies, there is a whole-cell based SELEX procedure where whole live cells are employed as the targets. This allows the aptamers to specifically target the surface molecules on live bacteria cells in their original conformation, enabling the molecular profile of the cellular surface to be determined [5]. Besides, another advantage of DNA aptamer is its capacity to target and precisely identify microbial strains, without prior knowledge of the membrane molecules present on the bacteria. Hence, this allows the discovery of new cell biomarkers and the identification of various aptamers capable of recognising specific cell phenotypes [5, 6]. In short, the DNA aptamer that is isolated from this whole-cell based SELEX procedure will exhibit high selectivity and specific binding affinities towards live bacteria compared to those from other SELEX technologies.

A quick and easy approach is required for the on-site identification and monitoring of *S. agalactiae* to overcome Streptococcus outbreak in cultured fishponds. For instance, DNA aptamer could be selected via whole-cell based SELEX procedure to 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 characterised using morphology and biochemical tests. Furthermore, the possibility of *S. agalactiae* strain TP540K as a potential target by DNA aptamer was examined through whole-cell based SELEX procedure.

MATERIALS AND METHODS

Characterisation using morphology and biochemical tests

Morphology test and several biochemical identification tests were conducted on the *Streptococcus agalactiae* strain TP540K, including Blood agar plates (BAP), Gram staining, catalase test and oxidase test.

Blood agar plates (BAP)

Firstly, a loop of bacteria was obtained from the thawed glycerol stock using a sterilised loop. Secondly, the loop with the bacteria was streaked on the BAP. Next, the BAP was incubated at 37°C for 24 h, then the zone surrounding the colonies formed were observed by holding the BAP up to a light source with the light coming from behind. The haemolytic reaction of the bacteria was determined as either alpha-haemolysis, beta-haemolysis, or gamma-haemolysis [7].

Gram staining

A single bacteria colony from BAP was first spread in a very thin film on the surface of the slide. Secondly, the slide was passed briefly through the flame of a Bunsen burner to fix the bacteria and prevent them from washing away during the staining steps. Thirdly, several drops of crystal violet (primary stain) were added to the smear and let sit for 1 min. The slide was then rinsed with water. Next, several drops of iodine (mordant) were added and let sit for 1 min. Again, the slide was rinsed with water. The decolourisation step was performed by adding ethanol drop by drop until the runoff was clear. The slide was then rinsed with water. After that, several drops of safranin (counterstain) were added to the smear and let sit for 1 min. The slide was rinsed with water and blotted dry. The slide was observed under a light microscope [8].

Catalase test

The tube method was performed. Firstly, 4–5 drops of hydrogen peroxide were added to a test tube. Secondly, a single bacteria colony from BAP was picked and immersed into the hydrogen peroxide solution using a sterile loop. Thirdly, the test tube was placed against a dark background to observe for immediate bubble formation [9].

Oxidase test

The oxidase test was performed according to the protocol [10]. Firstly, a small piece of filter paper was first soaked in 1% Kovács oxidase reagent and let dry. Secondly, a single bacteria colony from BAP was picked and rubbed onto the treated filter paper using a loop. Thirdly, the colour changes were observed within 10 s.

Whole-cell based systematic evolution of ligands by exponential enrichment (SELEX)

Whole-cell based SELEX was performed according to the protocols as described by several studies with some modifications [11-14]. Major modifications were made on polymerase chain reaction (PCR) in terms of the annealing temperature (Ta), the number of amplification cycles, the time for PCR steps, the concentration of primer and the volume of DNA template. This was done to determine the optimal condition for the designed forward and reverse primers during PCR amplification. The detailed procedures of whole-cell based SELEX were described as below.

Initial single-stranded DNA (ssDNA) library preparation
2 nmol of ssDNA library consisting of a 45-nt randomised region (5' – ATC CAG AGT GAC GCA GCA – N_{45} – TGG ACA CGG TGG CTT AGT – 3') was added to 350 µL of binding buffer which consists of Phosphate Buffered Saline with Tween 20 (PBST) + 1% Bovine serum albumin (BSA). The mixture was resuspended and heated at 95°C for 5 min. It was then snap-cooled on ice and stored at -20°C. The ssDNA library was thawed on ice whenever ready to use [11].

Washing of bacterial cells by centrifugation
S. agalactiae was cultured overnight at 37ºC in brain heart infusion broth (Oxoid, United Kingdom) and harvested during the log phase with a minimum OD_{600} of 0.3 [12]. One mL of harvested cell culture was centrifuged at 5,000 g for 10 min at 4ºC using a refrigerated benchtop centrifuge (Sorvall Legend XTR). Next, the supernatant was removed, and the cell pellet was washed with wash buffer PBST. This step was repeated twice. Finally, the cell pellet was collected and suspended in 200 µL of binding buffer [11].

Incubation of bacterial cells with ssDNA library
Exactly 200 µL of cells that were suspended in binding buffer was mixed with 2 nmol of ssDNA library. The mixture was shaken with gentle agitation using VortexGenie® 2 Vortex (MO BIO Laboratories, Inc.) at room temperature for 1 h. At this step, the binding of random ssDNA to the molecules on the target cell surface happened [11].

Washing step
After incubation, the mixture was centrifuged at 5,000 g for 5 min at 4°C using a refrigerated benchtop centrifuge (Sorvall Legend XTR). The supernatant containing unbound sequences was removed carefully using a transfer pipette to avoid cell loss. The cell pellets were then resuspended in 1 mL of wash buffer. The mixture was centrifuged again at 5,000 g for 5 min at 4°C. This step was repeated twice for a total of three washings [11].

Elution of bound sequences
Exactly 500 µL of nuclease-free water was added to the cell pellet to elute the bound sequences. The cells were resuspended, and the cell suspension was transferred into a 1.5 mL microcentrifuge tube. The cell mixture was heated at 95°C for 10 min, and centrifuged at 5,000 g for 5 min at room temperature [11]. The supernatant containing eluted DNA was collected and concentrated by ethanol precipitation [13].

Polymerase chain reaction (PCR)
The DNA template used in PCR was 81 bases with the sequence of 5' – ATC CAG AGT GAC GCA GCA – N_{45} – TGG ACA CGG TGG CTT AGT – 3' [11]. Besides, a forward: 5' – ATC CAG AGT GAC GCA GCA – 3' and a reverse: 5' – ACT AAG CCA CCG TGT CCA – 3' primer set was used to amplify the targeted sequence. All of them were purchased from Integrated DNA Technologies (IDT).
Before setting up the PCR reaction, a master mix that contains all of the PCR ingredients except for the DNA template was prepared. Calculations were made to determine the volume of each reagent in the master mix. Ultimately, the master mix for PCR was prepared by adding the reagents into a tube according to the sequence in **Table 1** below, except for the DNA template. The master mix prepared was then pipetted into PCR tubes, and 5 µL of DNA template was lastly added, except for the negative control. For negative control, 5 µL of nuclease-free water was added to make up the volume to 50 µL [14].

Table 1. Reagents involved in master mix preparation for PCR.

Reagent	Initial concentration	Final concentration	Volume for one reaction (µL)
Nuclease-free water	-	-	36.75
10 x PCR buffer (1.5 mM $MgCl_2$)	10 x	1 x	5.0
Forward primer	10 µM	0.2 µM	1.0
Reverse primer	10 µM	0.2 µM	1.0
dNTP mixture	10 mM	200 µM	1.0
Taq polymerase	250 units	1.25 units	0.25
DNA template	-	-	5.0
TOTAL			50.0

After the preparation of 50 µL mixture in all PCR tubes, PCR was carried out according to the steps and conditions in **Table 2** below by referring to Lorenz's study with some modifications made [14]. For denaturation, annealing and extension steps, they were repeated for 10 cycles, with further increment up to 20 and 30 cycles to achieve the optimum amplification condition. The PCR products were kept on ice until proceeding to agarose gel electrophoresis (AGE).

Table 2. Condition of temperature and time for each step in PCR.

Step	Temperature (ºC)	Time (s)
Initial denaturation	95	150
Denaturation	95	30
Annealing	56.3	30
Extension	72	30
Final extension	72	180

Agarose gel electrophoresis (AGE)
Firstly, a standard 3% agarose gel was prepared by mixing 3 g of agarose powder into 100 mL of 1 x Tris-acetate-EDTA (TAE) buffer in a conical flask. The mixture was then heated in a microwave for 2–5 min until the agarose powder was completely dissolved. Next, 2 µL of staining dye was pipetted into the mixture and the conical flask was swirled to get a homogenous mixture. The mixture was lastly poured into an AGE mould and let cool for 15 min to solidify. The agarose gel samples were prepared as shown in **Table 3** below [11].

Table 3. The volume of each reagent for sample, negative control and DNA ladder.

Reagents	Sample (µL)	Negative (µL)	control DNA ladder (µL)
Loading dye	2	2	2
PCR product	10	-	-
PCR negative control	-	10	-
Ladder (100 bp)	-	-	1
Nuclease-free water	-	-	9
TOTAL	12	12	12

The samples were loaded in lanes and electrophoresis was performed at 100 V for 40 min. Next, the gel was visualised under UV light.

RESULTS AND DISCUSSION

Characterisation of *Streptococcus agalactiae* using morphology and biochemical tests
In this study, *Streptococcus agalactiae* strain TP540K, a local strain that was isolated from red hybrid tilapia fish in Malaysia (Kedah) was investigated. To ensure that the isolated strain is a pure culture, morphology test and biochemical tests were performed. First of all, morphology test or blood agar plates (BAP) test was carried out. The *S. agalactiae* strain TP540K from glycerol stock was firstly streaked on BAP and incubated at 37ºC for 24 h.

BAP was used as it is a differential media and three different types of haemolysis can be seen. Thus, haemolytic bacteria can be detected and differentiated [15].

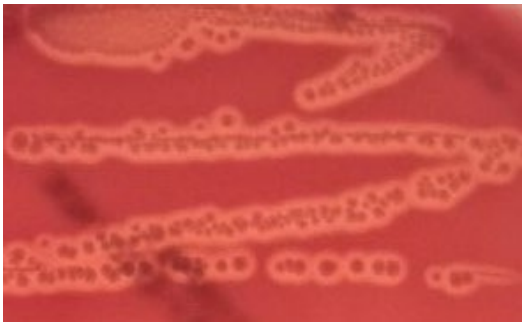

Fig. 1. Streak plate of *S. agalactiae* strain TP540K on BAP.

Fig. 1 above shows the morphology of *S. agalactiae* TP540K strain on BAP. It could be observed that the bacteria appeared spherical and formed grey-white colonies with a narrow zone of beta-haemolysis as shown in **Fig. 1**. As there was a narrow transparent zone surrounding the colonies, this indicated beta-haemolysis or complete haemolysis [16]. In addition, the isolate was also subjected to biochemical tests to further support the result, which included Gram staining test, catalase test and oxidase test.

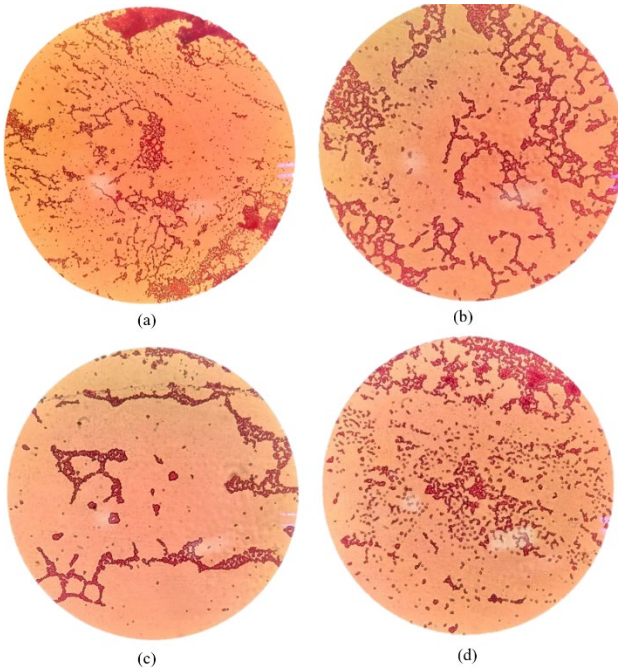

Fig. 2. Result of Gram staining under light microscope at (a) 40 x, (b) 100 x, (c) 400 x and (d) 1000 × magnification.

As shown in **Fig. 2**, the bacteria were stained purple for Gram staining test. This indicated that the bacteria were gram-positive bacteria as the presence of a thick layer of peptidoglycan in their cell walls could retain the crystal violet [8]. Under 1000 × magnification using a light microscope, the bacteria appeared in gram positive spherical shape and mostly occurred in pairs or chains as shown in **Fig. 2(d)**. Apart from that, no bubble formation was observed for catalase test. This explained the absence of catalase enzyme in the bacteria which could catalyse the decomposition of hydrogen peroxide, producing water and oxygen [17]. Hence, this proved that the bacteria were catalase-negative. Besides, there was no colour change for oxidase test, indicating the absence of cytochrome c oxidase (CcO). As a result, the reagent was reduced and remained colourless. In contrast, if CcO was present, the reagent would be oxidised and turned purple [10]. In essence, these few tests confirmed that the local bacteria strain isolated from fish was *S. agalactiae* which is gram-positive, catalase-negative and oxidase-negative.

Whole-cell based systematic evolution of ligands by exponential enrichment (SELEX)

As the name suggested, whole-cell based SELEX procedure was supposed to be an iterative screening procedure. To give an instance, it comprises repeated processes of (1) separating aptamers that are bound to the target from those that are not, and (2) amplifying aptamers that are bound to the target using the polymerase chain reaction (PCR) [18]. In this study, the first round of SELEX was conducted to discover the potential of *S. agalactiae* strain TP540K as a target in this whole-cell based SELEX.

At the last step in the first round of SELEX, *S. agalactiae* was washed to elute any bound oligonucleotides on the surface of the bacteria. The eluted solution, which was the final selected DNA pool from the first round of SELEX was collected and precipitated using ethanol to concentrate the highly diluted DNA sample. The concentrated DNA was separated into 3 tubes, namely 1A, 1B and 1C as triplicates. Next, the concentrated DNA in tubes 1A, 1B and 1C was amplified by PCR and examined using agarose gel electrophoresis together with 100 base pairs (bp) DNA ladder.

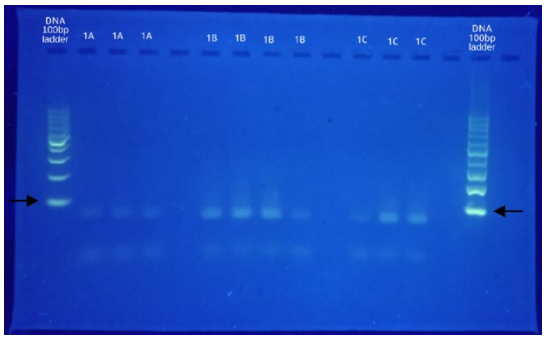

Fig. 3. Agarose gel electrophoresis image showing the products of the first SELEX round selected DNA pool amplification with 100 bp ladder. Lanes 1 and 15: 100 bp ladder; lanes 1A, 1B and 1C: replicates of tubes 1A, 1B and 1C respectively. The black arrow indicates the ladder with a molecular weight of 100 bp.

As shown in **Fig. 3**, the presence of bands on the gel for lanes 1A, 1B and 1C indicated that there were DNA aptamers that could bind to the target bacteria, *S. agalactiae* during SELEX process. In contrast, the first round of SELEX would have to be repeated if there was no band at all for lanes 1A, 1B and 1C. The size of the DNA was estimated to be 80 to 90 bp after comparing to the 100 bp DNA ladder, which is the expected aptamer size [19, 20]. However, based on **Fig. 3**, smearing of the bands and some faint bands at the bottom of the gel were also observed. This pointed out several problems such as non-specific amplification and formation of primer dimers [14]. Hence, PCR optimisation was performed to tackle these problems. The details of the PCR optimisation are discussed below.

Polymerase chain reaction optimisation
PCR optimisation was performed to optimise a few factors, including the annealing temperature (Ta), the number of cycles, the time for PCR steps, the concentration of primer and the volume of DNA template.

Optimisation of the annealing temperature
The forward primer and reverse primer used in the PCR were as follows:
Forward primer: 5' – ATC CAG AGT GAC GCA GCA – 3' (Tm = 56.5ºC)
Reverse primer: 5' – ACT AAG CCA CCG TGT CCA – 3' (Tm = 56.2ºC)
There are three common methods to determine the optimal annealing temperature (Ta). First, the rule of thumb for annealing temperature (Ta) is theoretically 3ºC or 5ºC lower than the melting temperature (Tm), which can be calculated using the formula [14]:
Ta = Tm - 3ºC or Ta = Tm - 5ºC
Ta = 56.2 - 5 = 51.2ºC
Ta = 56.5 - 3 = 53.5ºC
Thus, the estimated annealing temperature (Ta) was between 51.2ºC and 53.5ºC if using this theoretical method.

Second, Tm calculator available online can also be used to estimate Ta for primers. For example, Tm calculator by Thermo Fisher Scientific and New England Biolabs Inc. was used in this study as a reference. The calculated Ta by Thermo Fisher Scientific is 57.7°C whereas the calculated Ta by New England Biolabs Inc. is 53.0°C. It is noted that the calculated annealing temperature (Ta) using the two methods above is meant as a starting reference. Thus, it requires further optimisation to enhance the specificity and accuracy, here comes the third method. Third, the annealing temperature (Ta) can be determined or further confirmed using gradient PCR. In this method, the annealing temperature (Ta) was set in a gradient pattern from 51.0ºC to 60.0ºC across the thermal cycler blocks. Hence, different annealing temperatures (Ta) can be accessed simultaneously. Next, the temperature that could yield the brightest band indicates it is the optimal annealing temperature (Ta). The corresponding annealing temperature (Ta) for each lane in the thermal cycler was as follows in **Table 4**.

Table 4. The set annealing temperature (Ta) for each lane in the thermal cycler.

Lane	1	4	6	8	9	12
Annealing Temperature (ºC)	51.0	53.3	55.1	56.5	57.6	60.0

Fig. 4. Agarose gel electrophoresis image showing the products of the first SELEX round selected DNA pool amplification for 10 cycles. Lane 1: 100 bp ladder. The black arrow indicates the ladder with a molecular weight of 100 bp.

After PCR, 10 µL of PCR product was mixed with 2 µL of loading dye. The mixture was then loaded into the AGE lane and duplicates were done for each temperature to compromise any pipetting errors. As shown in **Fig. 4**, it could be observed that after 10 cycles of PCR amplification, there were bands at the lane 51.0ºC, 53.3ºC and 56.5ºC respectively after visualising the gel under UV light. However, the bands were considered faint and there were primer dimers present at the bottom of the gel. According to Lorenz, there are several possible causes of the faint bands such as too few cycles were used, extension and annealing time was too short, and the denaturation time was too long [14]. On the other hand, primer dimers are some small molecules, which are resulted from the hybridisation of two primer molecules that have complementary bases. For instance, there are two types of primer dimers, namely self-dimer and cross-dimer. Hence, further optimisation in terms of other factors was carried out to get a brighter band, avoid the primer dimers and confirm the optimal annealing temperature (Ta).

Optimisation of the number of cycles
The same PCR steps were repeated for 20 cycles and 30 cycles of amplification respectively to determine the optimal number of cycles.

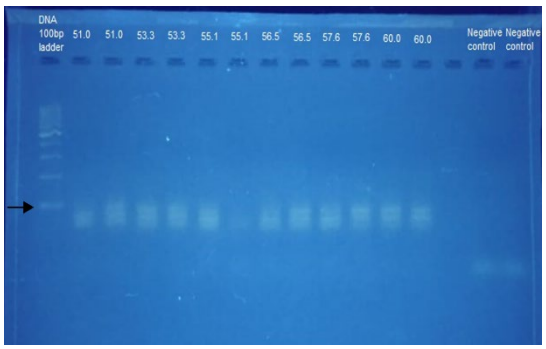

Fig. 5. Agarose gel electrophoresis image showing the products of cycle optimisation of the first SELEX round selected DNA pool amplification for 20 cycles. Lane 1: 100 bp ladder; lanes 15 and 16: negative control of PCR. The black arrow indicates the ladder with a molecular weight of 100 bp.

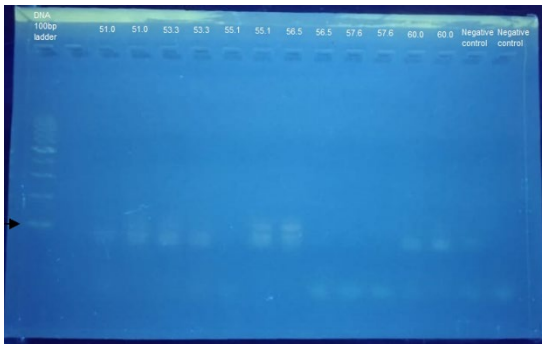

Fig. 6. Agarose gel electrophoresis image showing the products of cycle optimisation of the first SELEX round selected DNA pool amplification for 30 cycles. Lane 1: 100 bp ladder; lanes 15 and 16: negative control of PCR. The black arrow indicates the ladder with a molecular weight of 100 bp.

Based on **Figs. 5** and **6**, it could be seen that the bands shown in 20 cycles of amplification were brighter and more obvious compared to those in 30 cycles of amplification. Other than that, no primer dimer was observed in **Fig. 5**.

Hence, it could be said that 20 cycles of amplification was the best number of cycles compared to 10 cycles and 30 cycles. Other than that, 53.3ºC was confirmed to be the optimal annealing temperature (Ta) for the PCR with a cross-comparison of **Figs. 4, 5** and **6**, as the bands were the most obvious at that particular temperature in all of the figures above. However, there was still room for improvement as there were multiple bands formed.

Optimisation of the concentration of primer and the volume of DNA template

The PCR was repeated using Ta of 53.3°C with 20 cycles of amplification, but the concentration of primer and the volume of DNA template were adjusted to avoid the formation of multiple bands. There are a few possible causes for the formation of non-specific bands [14]. First, high concentration of primers was added. Second, high volume of DNA template added was also one of the causes for the formation of non-specific bands. This is because these scenarios increased the chance of primers binding to the non-specific sites on the DNA template. Thus, the concentration of primer was decreased from 0.2 µM to 0.1 µM whereas the volume of DNA template was decreased from 5.0 µL to 2.5 µL respectively in this optimisation.

Based on **Fig. 7**, it could be observed that there were still multiple bands on the gel even after the modification in terms of primer concentration and volume of DNA template. Still, this indicated the result of non-specific PCR amplification. In addition, the intensity of the bands for all conditions was similar. Hence, it could be said that both of these factors did not affect much on the results.

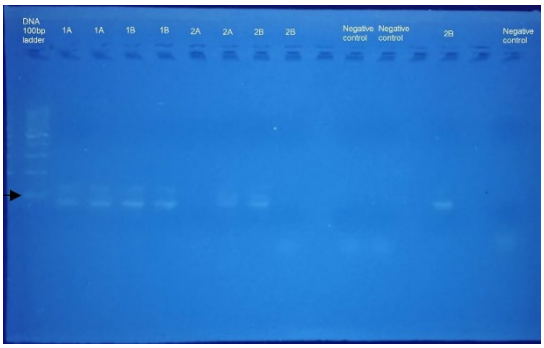

Fig. 7. Agarose gel electrophoresis image showing the products of concentration optimisation of the first SELEX round selected DNA pool amplification. Lane 1: 100 bp ladder; lane 1A: 0.2 µM primer, 5.0 µL DNA; lane 1B: 0.2 µM primer, 2.5 µL DNA; lane 2A: 0.1 µM primer, 5.0 µL DNA; lane 2B: 0.1 µM primer, 2.5 µL DNA; lanes 11, 12 and 16: negative control of PCR. The black arrow indicates the ladder with a molecular weight of 100 bp. Note: 1A is the original primer concentration and DNA template volume.

Optimisation of the time for PCR amplification steps

The time for PCR steps was optimised, especially for the steps including initial denaturation, extension and final extension to increase the intensity of the bands. The higher the concentration of PCR product, the brighter the band shown on the gel. Hence, it is crucial to determine the suitable duration for the steps mentioned above that could allow a higher amplification rate of the product.

For denaturation step, it is noted that DNA might be degraded if the denaturation time is too long, which may result in a faint band or no band formation at all. On the other hand, if the denaturation time is too short, the DNA template might not be completely denatured, and the amplification efficiency will be low as well. In addition, it is suggested that an extension time which is too short would lead to incomplete amplification of the target product [14]. Thus, in this study, we primarily shortened the initial denaturation from 150 s to 120 s as our early trial. At the same time, the extension time was increased from 30 s to 45 s whereas the final extension time was increased from 180 s to 300 s.

As shown in **Fig. 8**, it could be observed that the intensity of the bands was higher compared to that in **Fig. 5**, even when the number of cycles for both PCR runs was the same. In addition, the non-specific bands shown in **Fig. 8** were also dimmer, which indicated lesser non-specific amplification. Therefore, this demonstrated that the decrement of initial denaturation time, and the increment of extension time and final extension time were significant to increase the amplification of PCR products.

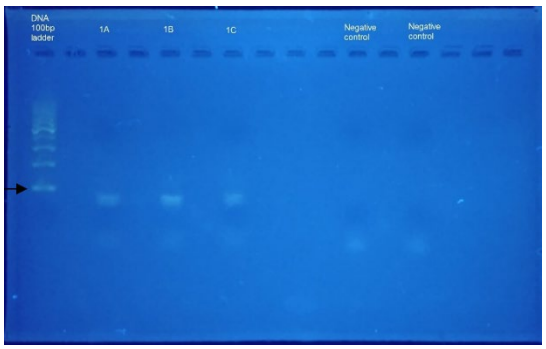

Fig. 8. Agarose gel electrophoresis image showing the products of time optimisation of the first SELEX round selected DNA pool amplification. Lane 1: 100 bp ladder; lanes 1A, 1B and 1C: triplicates of PCR reaction mixture; lanes 11 and 13: negative control of PCR. The black arrow indicates the ladder with a molecular weight of 100 bp.

Summary of PCR optimisation

In short, PCR optimisation was done on several factors such as the annealing temperature (Ta), the number of cycles, the concentration of primer, the volume of DNA template, and the time for PCR amplification steps. The modification on the concentration of primer and the volume of DNA template did not give significant improvement. In contrast, other factors did show improvement and the final conditions were summarised in **Table 5** below. For instance, annealing temperature (Ta) was decreased from 56.3ºC to 53.3ºC; initial denaturation time was decreased from 150 s to 120 s; extension time was increased from 30 s to 45 s; final extension time was also increased from 180 s to 300 s; and the optimum number of cycles for the steps (denaturation, annealing and extension) was found to be 20 cycles.

Table 5. Summary of the PCR condition after optimisation was made.

Step	Temperature (ºC)	Time (s)
Initial denaturation	95	120
Number of cycles		20
Denaturation	95	30
Annealing	53.3	30
Extension	72	45
Final extension	72	300

CONCLUSION

In conclusion, *Streptococcus agalactiae* strain TP540K was successfully characterised using both morphology and biochemical tests. For morphology test and blood agar plates (BAP) test, *S. agalactiae* strain TP540K showed morphology of spherical shape and formed grey-white colonies with a narrow

zone of beta-haemolysis. On the other hand, the bacteria were stained purple for Gram staining test (gram-positive); no bubble formation for catalase test (catalase-negative); and no colour change for oxidase test (oxidase-negative). Next, the whole-cell based 'systematic evolution of ligands by exponential enrichment (SELEX) procedure at the preliminary stage was performed. Based on the bands with an estimated size of 80-90 base pairs shown on the agarose gel, it was good evidence to prove that *S. agalactiae* strain TP540K has the potential to be used as a target for DNA aptamer development. For future recommendations, more genotyping studies should be conducted to understand the protein molecular profile on the cellular surface of *S. agalactiae*. The information can be utilised for a better selection of the aptamer with improved binding affinity, specificity and sensitivity. More rounds of SELEX and negative-SELEX using non-target bacteria can be performed to further improve the aptamer which can ultimately aid in the selective capture of *S. agalactiae*.

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