ISSN 2989-0705 (Online) | Vol 2 | Issue 2 | Jul 2024

Development of a qPCR method for the detection of *Staphylococcus aureus* species

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Abstract

Rapid detection of *Staphylococcus aureus* is crucial because it causes food poisoning in living beings. *Staphylococcus aureus* bacteria are present on the skin surface and the mucous membranes of humans and animals. Quantitative PCR (qPCR) can be utilized to give fast and specific results for detecting *S. aureus*. Therefore, this research aimed to develop a qPCR method for detecting *S. aureus* species, in both non enriched and enriched medium. The DNA of pure *S. aureus* culture was extracted using the QIAGEN mericon food kit, followed by measuring the concentration of DNA using a fluorometer and performing qPCR using TaqMan chemistry for nuc gene of *S. aureus*. Artificially spiked meat samples were incubated for an 18-hour enrichment period. The collected samples both pre-enriched and enriched samples were then subjected to DNA extraction using the QIAGEN mericon food kit, followed by measuring the concentration using fluorometer and qPCR using TaqMan chemistry for the *S. aureus* nuc gene. This process was done in order to evaluate the accuracy of the qPCR method for the quantitative detection of *S. aureus*. The concentration of extracted DNA of *S. aureus* culture was 6.6 ng/µL. The concentration of the extracted DNA of pre-enriched sample and the enriched sample was 6.8 ng/µL and higher than the standard respectively. The five-times diluted enriched sample produces a DNA concentration of 42 ng/µL. In the qPCR results, *S. aureus* culture gave a Ct value of 16.961. The pre-enrich meat sample gave a Ct value of 27.119, the enriched meat sample gave a Ct value of 21.374. The aim of this study was achieved as the enriched sample produced a Ct value lower than the pre-enriched sample indicating the development of a qPCR method for the detection of *S. aureus* species.

Keywords: *Staphylococcus aureus*, pre-enrichment, enrichment, qPCR, Fluorometer, Ct value

1. Introduction

Staphylococcus aureus species are a division of the *Staphylococcus* genus which are anaerobic, non-motile, gram-positive cocci which colonize on the surface of the skin and the mucous membranes of humans and animals.[1] Figure 1 shows an image of the *S. aureus* species. *Staphylococcus aureus* is the most common coagulase-positive Staphylococci (CoPS) and is the foremost virulent.[2] The infection of *S. aureus* can cause most hospital- acquired diseases such as Methicillin Resistant *Staphylococcus aureus* (MRSA). Therefore, there's an urgent need to establish a rapid method to detect *S. aureus* species present in food.[3]

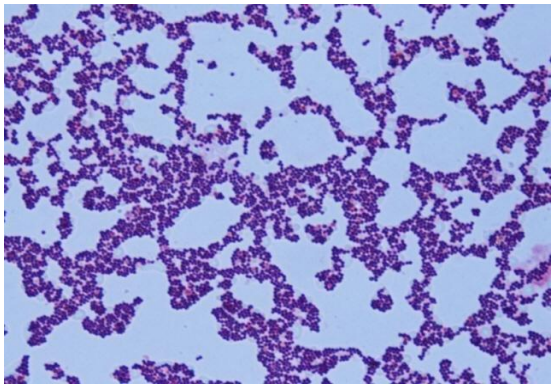

Figure 1. Gram staining of *S. aureus* species[4]

These organisms can grow in both aerobically and anaerobic conditions. They can also grow under diverse conditions such as heat. *Staphylococcus aureus* contamination can be minimized by heat treatment of foods. Symptoms of *S. aureus* include redness, swelling, and pain at the site of infection.[5] Approximately 30% of the human population in the world is colonized with *Staphylococcus aureus.*[6] In Sri Lanka, over 42% - 67% of hospitals are infected with MRSA. Figure 2 shows the percentage of MRSA isolates, by country, in recent years from 2011 to 2014.

qPCR is quantitative real-time PCR which is a PCR-based method in which the amplification of a target DNA sequence is combined with quantification.[14]

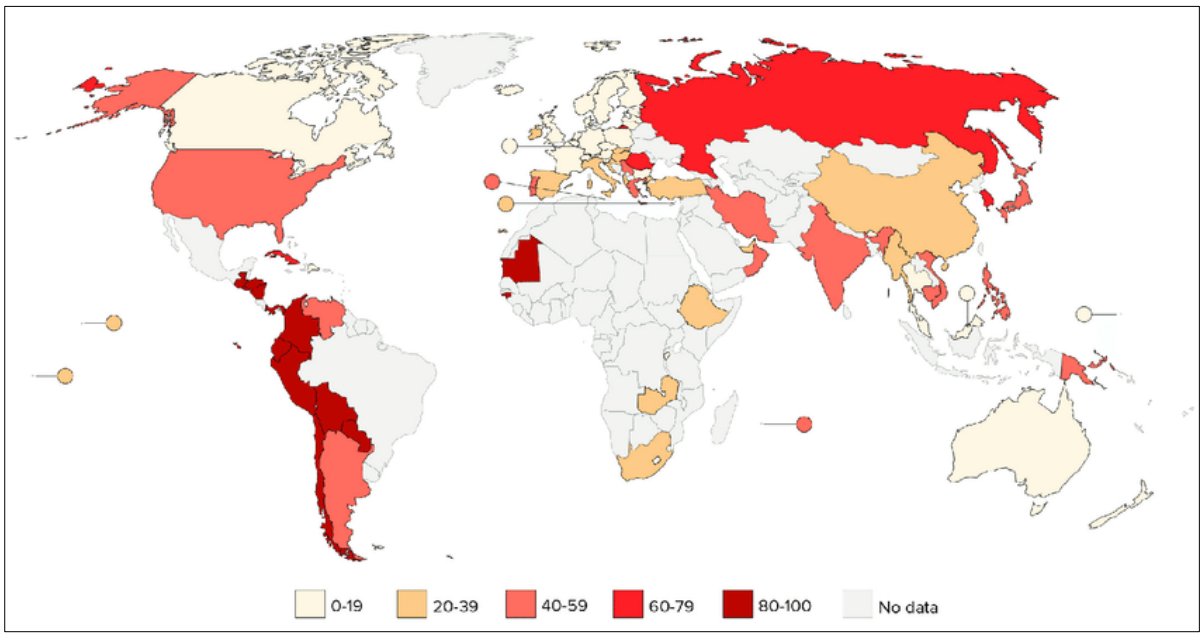

Figure 2: Percentage of MRSA isolates by country in recent years[7]

Staphylococcus aureus is detected using culture-based methods, ELISA and Aptasensors.[8] The disadvantages of using culture- based methods are they are time consuming and have a higher chance of contamination from occurring.[9] For the ELISA method an isolated pure culture is needed to obtain an accurate result.[10,11] Aptasensors also have disadvantages as the reagents are difficult to store as they have a poor stability, the catalytic activity can be affected by different conditions such as pH.[12] Therefore, this research focuses on the development of qPCR for the detection of *Staphylococcus aureus* species as this method is specific, takes less time compared to other methods and have a low chance of producing false positive results.[13]

This technique makes it possible to calculate the initial template concentration, making it a frequently utilized analytical tool when assessing the DNA copy number.[14,15] Methods of qPCR uses fluorescent dyes such as SYBR Green or DNA probes containing a fluorophore, such as TaqMan. Figure 3 shows the TaqMan-based assay chemistry.

The advantages of the TaqMan probe are they are labelled with different dyes, which allows amplification and detection of two distinct sequences in one reaction tube. Post-PCR processing is eliminated, which reduces material costs.[17] The primary disadvantage of TaqMan is that the synthesis of different probes is required for different sequences. The advantages of SYBR dyes are,

they can be used to monitor the amplification of any double-stranded DNA sequence, and no probe is needed.[17,18] The primary disadvantage of SYBR dye is that it may generate false positive signals.

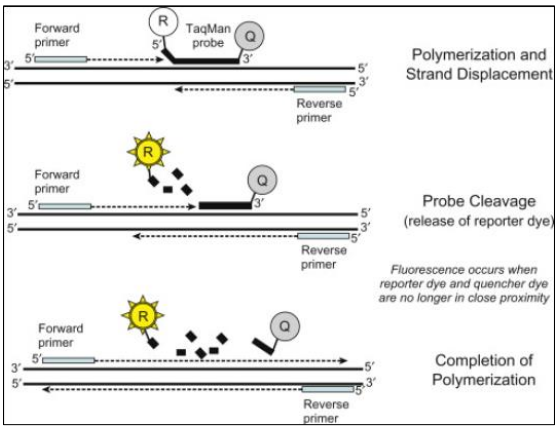

Figure 3. TaqMan-based assay chemistry[16]

Staphylococcus aureus is the most common CoPS and is considered the foremost virulent because it is the most typical hospital-acquired infection. Therefore, the detection of *Staphylococcus aureus* is crucial. qPCR can be used as a less time- consuming method to detect *S. aureus* species.[13] qPCR presents a fast and reliable method for the detection and quantification of *S. aureus* species.[19] Hence, this study aims to develop a qPCR method to detect *Staphylococcus aureus* species in both non enriched and enriched medium.

2. Methodology

2.1. Detection of S. aureus in the culture. *Staphylococcus aureus* culture was prepared by inoculating 5 mL of nutrient broth with *S. aureus* glycerol stock and was incubated at 37℃ overnight.

2.2. Extraction of DNA of S. aureus. Extraction of DNA from the overnight grown *S. aureus* culture was performed using QIAGEN DNeasy mericon Food Kit. Pelleted 1 mL of homogenized culture was placed in a 2 mL microcentrifuge tube then 1 mL of food lysis buffer and 2.5 µL of proteinase K solution was added and mixed thoroughly by using the vortex. Then the sample was incubated in a water bath at 60℃ for 30 minutes with constant shaking of about 1000 RCF. The sample was centrifuged at 2500 RCF for 5 minutes then 500 µL of chloroform was added to the microcentrifuge tube. Then 700 µL of clear supernatant obtained from earlier was added to the microcentrifuge tube containing chloroform. The microcentrifuge tube was vortexed thoroughly, then centrifuged at 14000 RCF for 15 minutes. 350 µL of buffer PB (Binding buffer) was pipetted into a fresh 2 mL microcentrifuge tube and then 350 µL of the supernatant were added and mixed thoroughly by using the vortext. Then the solution was pipetted into a QIAquick spin column placed in a 2 mL collection tube. The spin column is then centrifuged at 17900 RCF for 1 minute and the flow through is discarded and the collection tube is reused in the next step. 500 µL of buffer AW2 (Wash buffer 2) was added to the QIAquick spin column and the tube was centrifuged at 17900 RCF for 1 minute and the flow through was discarded. The collection tube is reused to centrifuge the sample again at 17900 RCF for 1 minute to dry the membrane.[18] 50 µL of elution buffer was used rather than 150 µL. The sample was stored at -20℃ until further use.

2.3. Quantification of DNA using Fluorometry. The blank sample, standard sample and DNA sample of extracted *S. aureus* culture was prepared according to the manufacturer's instructions.[20] The fluorometer was calibrated, and the concentration of the DNA was measured.

2.4. Amplification of DNA using qPCR. The PCR master mix was prepared with 0.25 µM of nuc forward primer, 0.25 µM of nuc reverse primer. 0.1 µM of the nuc probe and 1X applied biosystems master mix and 25 ng of DNA template. The nuclease free water was added as the negative control. The experiment was performed in dark conditions. The RT PCR computer software was set up using the following information in table 1 along with the PCR conditions given in table 2.

Table 1. The computer software set-up

Probe	NUC	
	Forward primer	Reverse primer
	5'-GGGTTGATACGCCAGAAACG-3'	5'-TGATGCTTCTTTGCCAAATGG-3'
Reporter	FAM	
Quencher	BHQ	
Fluorescence	TaqMan Reagents	
Run Mode	Standard	

Table 2. qPCR cycle

Step	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	95 °C	10 minutes	
Denaturation	95 °C	0.15 seconds	40 cycles
Annealing	60 °C	1 minute	
Extension	60 °C	1 minute	

2.5. Detection of S. aureus in the pre-enriched and enriched sample.

2.5.1 Spiking and Homogenization of the sample. A chicken piece of 25 g was measured using a weighing machine, and the chicken piece was added to the sterile filter bag along with 125 mL of nutrient broth and spiked with 1 mL of the *S. aureus* culture inside the laminar flow. The sample was homogenized using a stomacher blender at a speed of 4. Soon after the sample was homogenized, 2 mL was transferred to a microcentrifuge tube. This sample was labeled as pre-enrichment sample the remaining homogenized meat sample was incubated for 18 hours at 37℃. A volume of 2 mL of the homogenized enriched sample was obtained to microcentrifuge tube which was labelled as the enriched sample.

2.5.2 Extraction of pre-enriched and enriched sample DNA. Extraction of DNA was performed using QIAGEN DNeasy mericon Food Kit. Pelleted 1 mL of homogenized sample was placed in a 2 mL microcentrifuge tube. Then 1 mL of food lysis buffer and 10.0 µL of proteinase K solution were added and mixed thoroughly by vortexing. Then the sample was incubated in a water bath at 60℃ for 30 minutes with constant shaking of about 1000 RCF. The sample was centrifuged at 2500 RCF for 5 minutes then 500 µL of chloroform was added to the microcentrifuge tube. Then 700 µL of clear supernatant obtained from earlier was added to the microcentrifuge tube containing chloroform. The microcentrifuge tube was vortexed thoroughly, then centrifuged at 14000 RCF for 15 minutes. 350 µL of buffer PB was pipetted into a fresh 2 mL microcentrifuge tube then add 350 µL of the supernatant and mix thoroughly by using the vortex. Then the solution was pipetted into a QIAquick spin column placed in a 2 mL collection tube. The spin column is then centrifuged at 17900 RCF for 1 minute and the flow through is discarded and the collection tube is reused in the next step. 500 µL of buffer AW2 was added to the QIAquick spin column and the tube was centrifuged at 17900 RCF for 1 minute and the flow through was discarded. The collection tube is reused to centrifuge the sample again at 17900 RCF for 1 minute to dry the membrane.[18] 50 µL of elution buffer was used rather than 150 µL. The sample was stored at -20℃ until further use.

2.5.3. Quantification of DNA using Fluorometry. The samples (pre-enriched and enriched) were prepared according to the manufacturer's instructions.[20] The DNA from the enriched samples was diluted 5 times in order to get a measurable value for DNA concentration. The concentration of DNA was measured.

2.5.4. Amplification of DNA using qPCR. The PCR master mix was prepared with 0.25 µM of nuc forward primer, 0.25 µM of nuc reverse primer. 0.1 µM of nuc probe, 1X applied biosystems master mix and 25 ng of DNA template. The DNA from *Salmonella* was added as the negative control. The experiment was performed in dark conditions.

The RT PCR computer software was set up using the following information in table 3 along with the PCR conditions given in table 4.

Table 3. Computer software set up

Probe	NUC	
	Forward primer	Reverse primer
	5'-GGGTTGATACGCCAGAAACG-3'	5'-TGATGCTTCTTTGCCAAATGG-3'
Reporter	FAM	
Quencher	BHQ	
Fluorescence	TaqMan Reagents	
Run Mode	Standard	

Table 4. qPCR cycle

Step	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	95 °C	10 minutes	
Denaturation	95 °C	0.15 seconds	40 cycles
Annealing	60 °C	1 minute	
Extension	60 °C	1 minute	

3. Results and Discussion/Analysis

3.1 Quantification of DNA

The *S. aureus* culture demonstrated a value of 6.6 ng/µL. The pre- enriched culture produced a value of 6.8 ng/µL, the enriched culture produced a value higher than the standard. The five times diluted enriched culture produced a value of 42 ng/µL in the fluorometer.

3.2 Amplification of DNA

According to the amplification plot, the amplification of *S. aureus* can be confirmed using the qPCR method (Figure 4,5,6,7 and 8). Summary of the Ct Values are stated in the table 5.

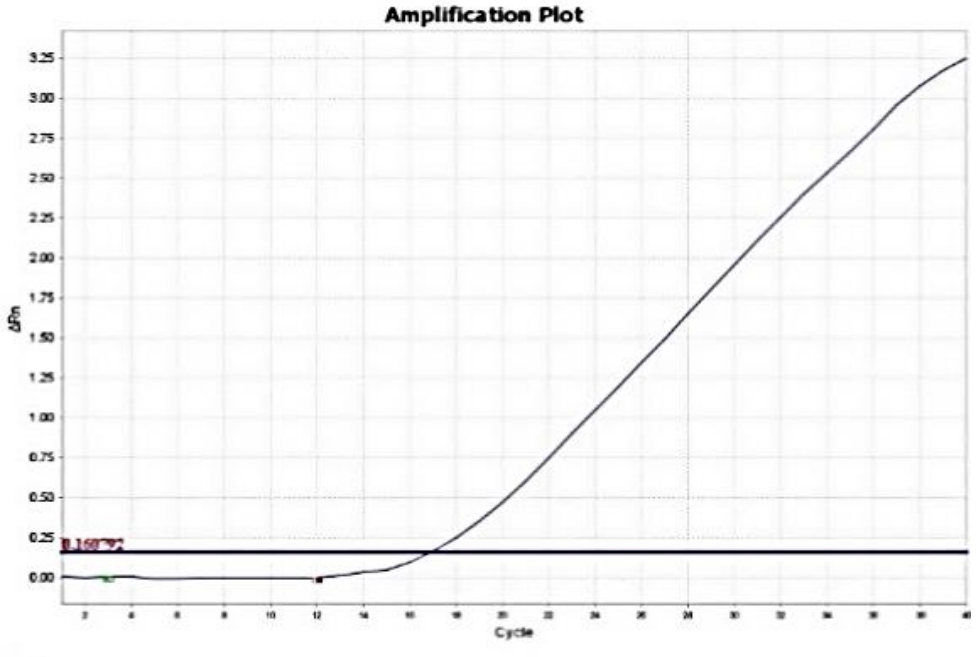

Figure 4. The amplification plot of *S. aureus* (Ct value – 16.961)

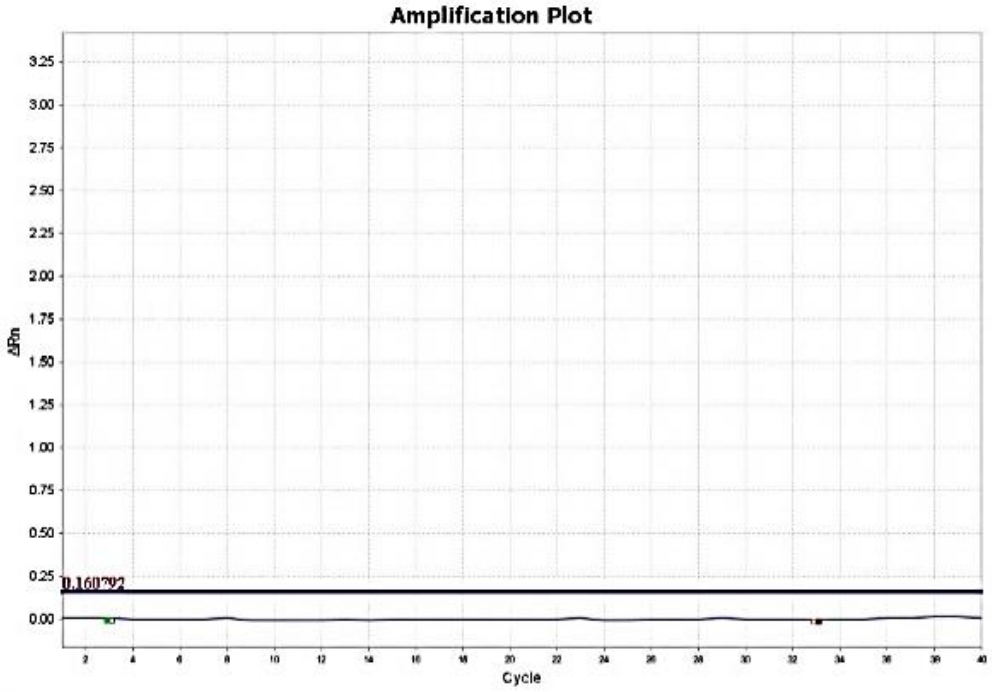

Figure 5. The amplification plot of the negative sample (nuclease free water)

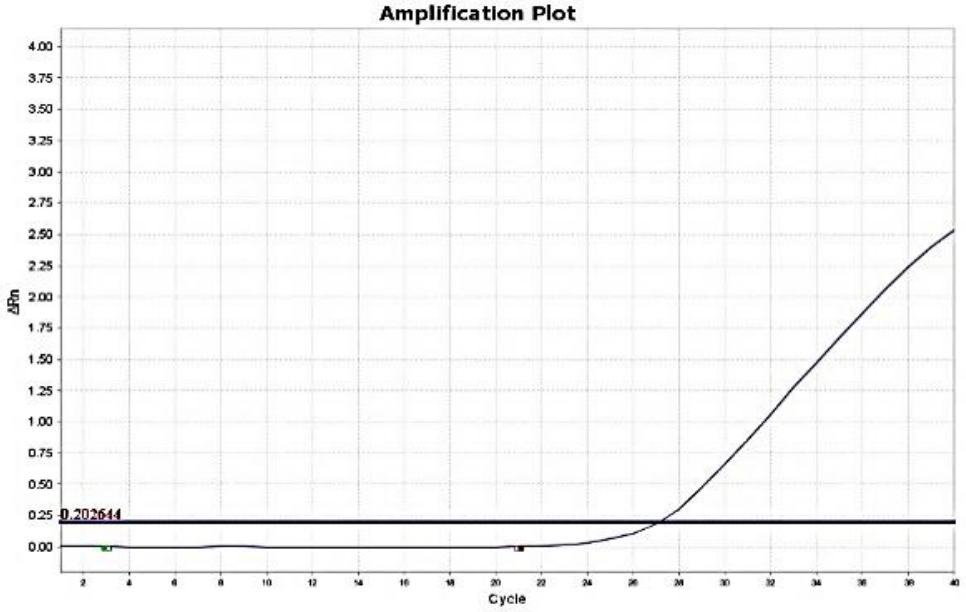

Figure 6.: The amplification plot of the pre-enriched sample (Ct value – 27.119)

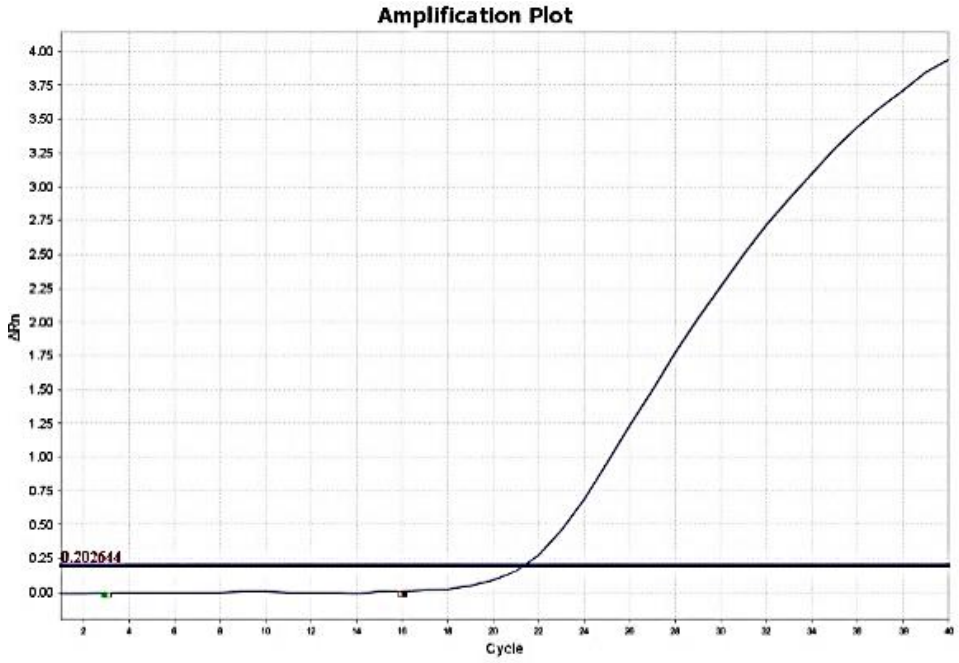

Figure 7. The amplification plot of the enriched sample (Ct value – 21.374)

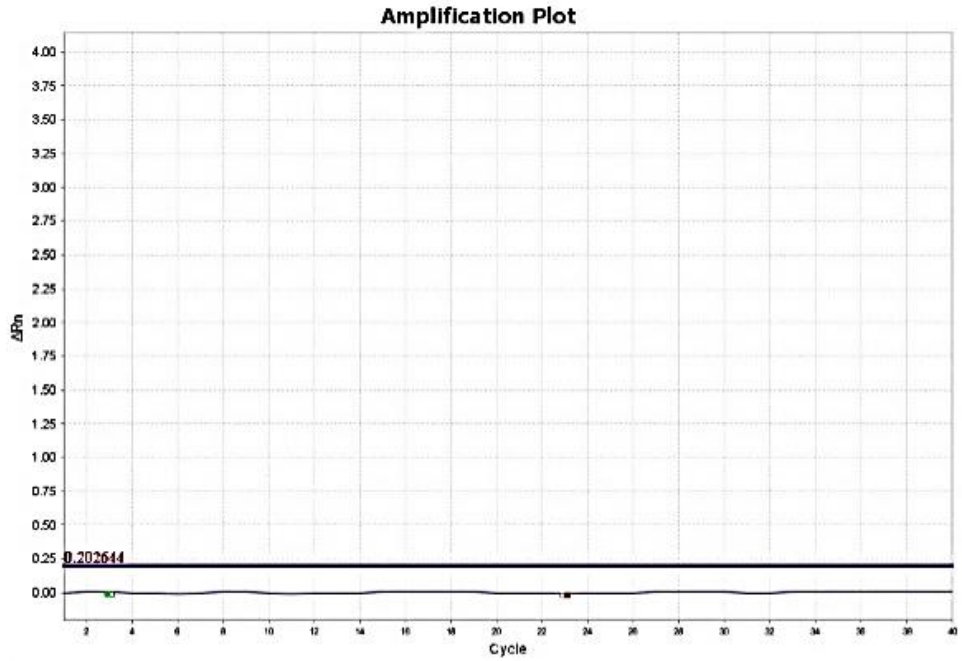

Figure 8. The amplification plot of the negative sample containing *Salmonella* DNA

Table 5: Ct values of the samples

	S. aureus **Positive sample**	***S. aureus*** **Negative sample**	**Pre-enriched sample**	**Enriched sample**	**Negative sample containing *Salmonella* DNA**
Ct value	16.961	Not determined	27.119	21.374	Not determined

Staphylococcus aureus is a gram-positive bacterium. This causes most hospital- acquired infections in the world. Therefore, the detection of *Staphylococcus aureus* is crucial. qPCR presents a fast and reliable method for the detection and quantification of *S. aureus.*[19] This study aimed to develop a qPCR method to detect *S. aureus* species in both non enriched and enriched medium. In the fluorometer results, *S. aureus* has a concentration of 6.6 ng/µL because *S. aureus* is gram- positive, so the breakdown of the cell membrane was difficult; as a result, a low yield of DNA was obtained. Pre-enriched samples have a concentration of 6.8 ng/µL because the sample was obtained before the incubation. The DNA concentration of the enriched sample was higher than the standard due to two factors: first factor is increase of *S. aureus* cells after the incubation period, second factor is homogenized sample includes DNA from meat in addition to *S. aureus*. Due to the higher DNA concentration, the DNA of the enriched sample was diluted five times so the DNA concentration can be measured. NUC is the gene which is detected in the qPCR when detecting *S. aureus* species in food. NUC decodes for thermostable nuclease of *S. aureus* and is a nuclease gene.[21] NUC is a gene marker used to identify the presence of *S. aureus* as the bacteria can function in the presence of heat resistant nuclease gene which is related to the production of enterotoxin.[22,23] The Ct value of *S. aureus* culture was 16.961. The Ct value of the pre-enriched sample was 27.119 and the Ct value of the enriched sample was 21.374. The Ct value of the enriched sample is lower than the pre-enriched sample because the concentration of DNA is more significant in the enriched sample than in the pre-enriched sample. This indicates that the *S. aureus* has grown in the enriched sample after incubation

indicating viable cells of *S. aureus* can be detected by this method.

4. Conclusion

In conclusion, the DNA extraction was successful in both methods; pre- enriched and enriched. However, considering the concentration of the DNA, enriched sample has a higher concentration than the pre-enriched sample. Because the enriched sample contains more DNA than the pre-enriched sample, the amplification plot indicates that the enriched sample has a lower Ct value compared to the pre-enriched sample. This shows that the live *S. aureus* cells could be detected by this method. This suggests that the aim of this study has been achieved, which is the development of a qPCR method to detect *S. aureus* species.

Acknowledgements

The authors would like to acknowledge the Biotechnology section of Industrial Technology Institute (ITI) for the opportunity to conduct the research project.

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