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Assessment of gDNA Extracted from *Staphylococcus aureus* Isolate by Polymerase Chain Reaction, Agarose Gel Electrophoresis, and Spectrophotometric Assay

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ABSTRACT

DNA sequencing is crucial in molecular biology techniques to identify *Staphylococcus aureus* isolates' genotype to advance diagnoses, prevent, and control

infections. DNA isolation can be influenced by several factors which can affect the quality of the output. The experiment was conducted to assess the quality and quantity of genomic DNA isolated from *S. aureus*. The gDNA's purity and concentration were determined using the spectrophotometric assay, and the quality was analyzed using PCR and agarose gel electrophoresis. The purity of DNA and RNA was determined using the absorbance ratio at A260 and A280, and the percent coefficient of variation was used for the three replicates. The result showed that the extracted gDNA from *S. aureus* was obtained in large quantities, with good quality, R_1=93.67 µg/ml, R_2=59.85 µg/ml, and R_3=67.50 µg/ml. The concentration was within a 1.8 – 2.0 absorbance ratio and was considered high quantity output. The extracted and amplified pure gDNA fragment from the sample can now be used for other molecular techniques.

***Keywords* —** molecular biology, agarose gel electrophoresis, gDNA, PCR, *S. aureus*, Philippines

INTRODUCTION

About 30% of the world's human population is colonized by *Staphylococcus aureus,* a pathogenic bacteria and a leading cause of benign to life-threatening skin and soft tissue infections, bacteremia, and other device-related infections (Tong et al., 2015). *S. aureus* is a natural microbiota of the human skin and major pathogens that causes a wide variety of infections when it enters the internal tissues, affecting humans, livestock, and wild animals that have hopped across host species genetically epidemiologically (Matuszewska et al., 2020). As one of the frequently isolated pathogens, *S. aureus* infections affect any organ, and strains develop resistance to antibiotics (Gnanamani et al., 2017; Turner et al., 2019). The resistant strains significantly participate in nosocomial infections, damaging the host epithelial cells and neutrophils (Foster & Geoghegan, 2015; Prévost, 2013). *S. aureus* is typically transmitted through direct contact and other transmission methods commonly acquired in the community or hospital settings (Taylor & Unakal, 2019). According to van Hal et al. (2012), *S. aureus* infections are severe and deadly; it accounts for more deaths than other bacterial and viral infections combined.

Deoxyribonucleic acid (DNA) fingerprinting and sequencing is crucial in molecular biology techniques to identify and genotype *S. aureus* isolates to advance diagnoses, prevention, and control of infections. Several approaches

were developed to amplify the nucleic acids as a basis for DNA extraction and isolation of the bacteria. The model method for genomic DNA (gDNA) extraction has to optimize a high quantity of pure yield from the isolated sample with minimal co-extraction of inhibitors during the downstream process or removal of contaminants, minimize DNA degradation, and be cost-efficient (Auricchio et al., 2013; Looi et al., 2012; Miyata et al., 2011). In preparation of bacterial genomic DNA, most common protocols before the precipitation of nucleic acids, comprising of lysis and incubation with a nonspecific protease followed by a series of extractions that effectively remove protein contaminants but not exopolysaccharides which can interfere with the activity of restriction enzymes such as endonucleases and ligases (Andreou, 2013; Canto-Canché et al., 2013; Raven et al., 2021; Vingataramin & Frost, 2018).

Recently, different methods of extracting bacterial DNA have been introduced as an alternative to conventional, time-consuming manipulation and contamination risks. Commercial kits are readily available and offer low-risks, faster than traditional protocols with high-quality DNA recovered (Hassanzadeh et al., 2016). Polymerase Chain Reaction (PCR) is an everyday and indispensable scientific technique use to amplify DNA fragments to generates millions of copies of a particular DNA in a few hours (Garibyan & Avashia, 2013; Kadri, 2019; Kuzdraliński et al., 2017). In PCR, a small amount of DNA is added to a solution containing essential components such as DNA polymerase, primers, and nucleotides and heated in a thermal cycler to separate DNA strands, and once cooled, the DNA polymerase creates the copy of individual DNA strand. The PCR cycle is repeated after five minutes until exponential DNA amplification is produced. After developing the PCR method, several DNA-based technologies were also developed and were applied in the different areas of biomedical research, especially in the investigation of the growing number of diseases, such as coronavirus disease 2019 (COVID-19) (Chung et al., 2021; Han et al., 2021; Udugama et al., 2020; Wang et al., 2020; Zauli, 2019). Agarose gel electrophoresis is another method used for separating DNA fragments by the electric field where negatively-charged molecules migrate toward the anode (positive) pole. The molecular weight of the molecules determines the migration of DNA fragments. In addition, nucleic acid fractionation in gel electrophoresis can be an initial step to purify a band of interest. Agarose gel electrophoresis is most suited for separating DNAs/RNAs in the range of 100bp to about 15kb (Cai, 2020; Kuhn et al., 2018; Motohashi, 2019; Tankeshwar, 2021).

Sufficient quantity and quality of extracted DNA are critical in the amplification-based analysis since DNA amplification is influenced by contaminants' presence from matrix and reagents, which reduce the PCR efficiency. The experiment was conducted to assess the quality and the quantity of the gDNA extracted from the *S. aureus* isolates based on its reaction in agarose gel electrophoresis, PCR, and spectrophotometric analysis, which is essential in identifying *S. aureus* species.

OBJECTIVES OF THE STUDY

This study aimed to determine the quantitative and qualitative aspects of extracted genomic DNA from *S. aureus*. Specifically, the study aimed to (1) determine the quantity of gDNA extracted from *S. aureus* isolates by spectrophotometric method and (2) determine the quality of extracted gDNA from *S. aureus* by a polymerase chain reaction and agarose gel electrophoresis.

MATERIALS AND METHODS

Reagents

All reagents, including agarose, Tris-acetate EDTA (TAE), ethidium bromide (stock concentration of 10 mg/mL), nuclease-free water, Taq DNA polymerase, template DNA, reverse primer, forward primer, deoxynucleotide triphosphates (dNTPs), and standard Taq reaction buffer used in the experiment, were analytical grade and obtained from addgene® (2018). TAE recipe can be found on this site (https://media.addgene.org).

Equipment

The following were the equipment used in the experiment: Eppendorf® Mastercycler® (40) cycler for amplifying the DNA isolate, spectrophotometer for quantifying DNA isolate, and agarose gel electrophoresis: casting tray, well combs, voltage source, gel box, UV source, and microwave.

S. aureus isolate

S. aureus isolates used in the experiment were previously prepared by the laboratory professor, Dr. Sabinay, and obtained from the Biotechnology Research Laboratory at West Visayas State University. The gDNA was isolated from *S. aureus* using the Nucleospin Microbial DNA protocol (Macherey-Nagel, 2018). About 2.5 µL taken from the sample was used PCR amplification.

DNA Extraction

DNA extraction of *S. aureus* isolate was carried out using the protocol described by Macherey-Nagel (Macherey-Nagel, 2018), using the commercial kit Nucleospin® Microbial DNA designed to isolate genomic DNA for microbial samples efficiently.

Sample Preparation. The cells were harvested from the produced isolates by spinning for about 2 minutes at 1000 rpm in a microcentrifuge tube to eliminate the residues. After the supernatant was discarded, resuspend the harvested cells with a 100 µL of Elution Buffer (BE) and centrifuge again at 3000 rpm for 5 minutes to obtain the chloroplast pellets.

Lyse Sample. The cell suspension was then transferred to NucleoSpin® Bead Tube Type B, and 40 µL Buffer MG and 10µL of Liquid Proteinase K were added tube was closed to avoid spilling the mixture during the agitation process. The NucleoSpin® Bead Tube was agitated to a swing mill and centrifuged for about 30s at 10,000rpm to clean the lid.

Adjust DNA Binding Condition. A 600 µL Buffer MG was added to the mixture and centrifuged for about 30s at 10,000rpm to clean the lid, sediment glass beads, and cell debris.

Bind DNA. For DNA binding, an approximately 500–600 µL supernatant was transferred to the NucleoSpin® Microbial DNA column, positioned in a 2mL collection tube, and then centrifuged for about 30s at 10,000rpm. The collection tube was discarded with flowthrough. Put column into a fresh 2 mL Collection Tube.

Wash Silica Membrane. A 500 µL Buffer BW was added and centrifuged for about 30s at 10,000rpm on the first washed. Discard the flowthrough and place the column back into the collection tube. Rewashed and 500 µL Buffer B5 was added to the column and centrifuged again for about 30s at 10,000 rpm; 30s at 10,000rpm.

Dry Silica Membrane. The column was centrifuged for the 30s at 10 000 rpm. In this step, the residual wash buffer was removed.

Elute Highly Pure DNA. The NucleoSpin® Microbial DNA Column was placed into a 1.5 mL nuclease-free tube, and 100 µL BE was added. Then incubated at ambient temperature for a minute and centrifuged for 30 s at 10 000 rpm.

Spectrophotometric Assay of gDNA

The concentration and purity extracted gDNA from *S. aureus* were analyzed using Thermo Scientific™ Multiskan™ GO (4) Spectrophotometer (Thermo Fisher Scientific Corporation, 2010). The analysis was done using the protocol described in the User Manual. The spectrophotometer was used to measure the amount of light a substance can absorb; by combining kinetic measurement and Beer's law principle, analysts could calculate its concentration. It is simple, sensitive, non-destructive, and selective.

First, the instrument was switched on, and it performed a self-diagnostic scan before it could be used. The microplate loaded was fitted well in the microplate carrier and made of quartz or UV compatible microplates to ensure the instrument's output quality. Then, the desired measurement parameters were defined in the plate menu. The device has automatically displayed the data onto its monitor. The selected data were then exported to the attached laptop. After the microplates were removed from the carrier, the instrument was switched off.

DNA Amplification by Polymerase Chain Reaction (PCR)

The extracted gDNA of *S. aureus* was amplified using Eppendorf® Mastercycler® PCR cycler. The instrument can accommodate several standard vessels in 96 well plates.

PCR is a powerful technique for DNA amplification that involves thermal cycling or heating and cooling. There were three stages involved in the amplification of gDNA of *S. aureus*. First, the denaturing process, where the double-stranded DNA was heated to separate into two single-stranded DNA. Next, is annealing where the temperature is cooled to enable the DNA primers to attach to the template DNA. Lastly, extending where the temperature is raised and the Taq polymerase enzymes make the new DNA strand.

In the experiment, the traditional protocol by GenScript ® (2019) was followed. First, all the reagents (nuclease-free water, Taq DNA polymerase, template DNA, reverse primer, forward primer, dNTPs, and standard Taq reaction buffer) was thawed on ice. Assembled reaction mix into 50 µL volume in thin-walled 0.2 mL PCR tubes. The reagents were added: nuclease-free water, standard Taq reaction buffer, dNTPs, $MgCl_2$, template primers, and Taq DNA polymerase. Then, the mixture was gently mixed by tapping the tube and centrifuged to settle the tube contents.

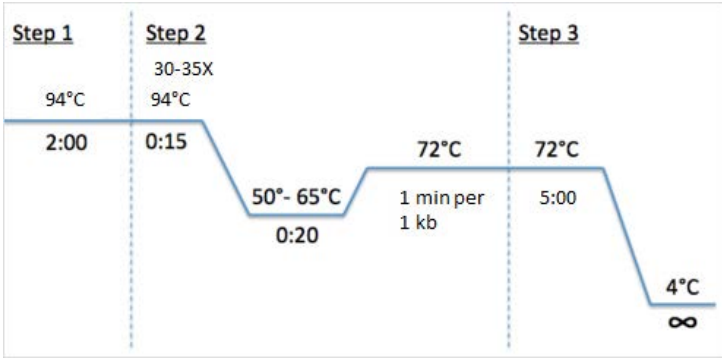

Figure 1. Steps for PCR reaction. Image from GenScript ® (2019).

Agarose Gel Electrophoresis

The gDNA extracted from *S. aureus* was subjected to agarose gel electrophoresis to separate DNA fragments based on size. The method has proven to be an effective and efficient way of separating gDNA.

Agarose gel electrophoresis followed the protocol

Preparation of the Agarose Gel. About 1g of agarose powder was weighed in the analytical balance. The agarose powder was mix with 100 mL 1xTAE in a microwavable Erlenmeyer flask. The mixture was put in the microwave for about 3 min until the powder was dissolved. It cooled down the agarose solution for about 50 °C for 5 mins. Approximately 0.2-0.5 µg/mL of ethidium bromide (EtBr) was added to the final concentration to visualize DNA when viewed under UV light—poured out the agarose solution into a gel tray with the well comb in place. Allow it to cool to ambient temperature until it has solidified completely.

Loading Samples and Running and Agarose Gel. Loading buffer was added to each of the DNA samples. Once solidified, the agarose gel was placed into the gel box (electrophoresis unit). The gel box was then filled with 1xTAE until the gel was covered. A molecular weight ladder was carefully loaded into the first lane of the gel. The gel was run for about 80 – 150 V until the dye line was approximately 75-80% of the way down the gel. The typical runtime is about 1 – 1.5 hr, depending on the gel concentration and voltage. Lastly, the power was turned off, and the gel was carefully removed from the gel box. The DNA fragments were visualized in UV light and analyzed the bands or the fragments of DNA.

RESULTS AND DISCUSSION

The quality of gDNA extracted from *S. aureus* was assessed using the Polymerase Chain Reaction and Agarose Gel Electrophoresis. Spectrophotometric analysis was done to quantify the gDNA of the said bacterium.

DNA extraction has come a long way since its inception in 1869; techniques range from straightforward manual processes to more advanced automated DNA extraction strategies. It would be excellent to identify the solutions that perform best in terms of cost-effectiveness and time efficiency based on the extensive range of possibilities (Chacon-Cortes & Griffiths, 2014). Extraction methods are critical in molecular biology because molecular investigations play a significant role in downstream applications, such as PCR and gel electrophoresis (Boesenberg-Smith et al., 2012). DNA quality indicators, such as DNA purity and integrity, must be verified before employing DNA samples in analytical techniques to ensure their quality and applicability (Lucena-Aguilar et al., 2016).

Amplification of nucleic acids is a powerful molecular biology tool, but its use outside the laboratory is limited. Researchers have successfully created an equipment-free nucleic acid extraction dipstick methodology to obtain amplification-ready DNA and RNA from biological samples. Zou et al. (2017) developed an extraction method that confirmed that untreated cellulose-based paper might fast absorb nucleic acids and retain them after a single washing phase, while contaminants present in complex biological samples are quickly eliminated. On the other hand, Sasagawa (2018), based on the alkaline lysis approach, a novel composition of solution III was devised, allowing for the purification of plasmid DNA without RNase. This method does not require any unique columns or resins, and it is sufficient for the transfection of a cultured cell. It also has a significant benefit in terms of pure plasmid DNA quality.

In the experiment conducted, spectrophotometric analysis was done using the Thermo Scientific™ Multiskan™ GO (4) Spectrophotometer (Thermo Fisher Scientific Corporation, 2010) to determine the quantity, concentration, and purity of the extracted gDNA of *S. aureus*. The most common and simple technique to quantify DNA yield and purity is the measurement of absorbance, which requires only the commonly available equipment in the laboratory.

Table 1 shows the absorbance values of the extracted gDNA at 230 nm, 260 nm, 280 nm, and 320 nm UV wavelengths. UV absorbance is a common way to quantify the DNA since molecules absorb different wavelengths of light to varying degrees, and many molecules have a specific maximum absorption

wavelength (Tsang, 2020). The blank (known as the buffer) was measured first, which served as the background absorbance, followed by the replicates containing the *S. aureus*. The blank absorbance measures gave an idea of the contamination of the DNA preparation.

UV spectrophotometry is a typical yet straightforward approach to estimate the concentration of DNA in a sample. According to Shen (2019), the technique does not require large purified samples; it has the advantage of not requiring any additional reagents or incubation time, and a UV spectrophotometer is widely available. However, it has several drawbacks. It often involves minimum sample volumes of 50–75L to produce an accurate instrument output; it cannot distinguish between signals from DNA and RNA or between double-stranded and single-stranded DNA, and the UV technique is susceptible to biological contamination. Negative values at 320 nm absorbance mean that no particulates are contaminating the solution. According to Olson and Morrow (2012), DNA extract purity is of interest in how contaminants will affect downstream assay performance; UV spectroscopic DNA purity measurements only provide indicators for different types of contaminants and provide no information about the effect of contaminants on downstream applications. However, Kazeminasab et al. (2019) established that sodium dodecyl sulfate (SDS) treatment and incubation at a high temperature significantly impact the extracted DNA from exhaled breath condensate (EBC) samples, but no effect on the quality of the DNA obtained from EBC samples. To Garibyan & Avashia (2013), the future of PCR is promising, combining various assays and approaches to produce greater insight into different gene combinations. As with all methods, the validity of the results should be compared with the specificity associated with the technique.

Table 1. The absorbance of gDNA from S. aureus at different wavelengths

gDNA	Replicates	UV absorption							
		230 nm		260 nm		280 nm		320 nm	
		Tube 1	Tube 2	Tube 1	Tube 2	Tube 1	Tube 2	Tube 1	Tube 2
Blank	Blank	0.003650	-0.003650	0.001200	-0.001200	0.0007000	-0.0007000	0.0004000	-0.0004000
S. aureus	R1	0.06575	0.05425	0.1071	0.06900	0.05310	0.03230	-0.003700	-0.003800
S. aureus	R2	0.05895	0.03315	0.05340	0.05840	0.02560	0.02680	-0.001900	-0.003600
S. aureus	R3	0.002250	0.04365	0.04220	0.07840	0.01840	0.03700	-0.005400	-0.006300

Table 2 shows the concentration of gDNA extracted from *S. aureus*. The concentration of each replicate of *S. aureus* was computed using the formula: concentration (µg/mL) = (A260 measurement – A320 measurement) x nucleic

acid conversion factor x dilution factor. The conversion factor for dsDNA is equal to 50 µg/mL (Barbas et al., 2007; Promega®, 2021). The formula (A260 - A320) * (50/0.049) was used to obtain the concentration. The coefficient of variation (%) or CV% for the three replicates is low, except for $R_{3,}$ which may be attributed to pipetting and contamination errors. Furthermore, the results show that high-molecular-weight DNA was obtained in large quantities and sound quality, R_1=93.67 µg/ml, R_2=59.85 µg/ml, and R_3=67.50 µg/ml.

There are many ways to extract DNA from a sample using various methods, including phenol-chloroform, detergent, and commercial kits. Despite the need for pretreatments to extract DNA from microorganisms other than Gram-negative bacteria, guanidine thiocyanate breaking has been the conventional initial step in genomic DNA (gDNA) extraction of microbial DNA for decades. Vingataramin and Frost (2018) developed a low-cost gDNA extraction procedure, effective for bacteria and yeast over a wide concentration range, known as EtNA, made by lysing ethanol in a hot alkaline solution to break down single-stranded DNA from bacteria and yeast. The EtNa reagent can process a wide range of biological samples for PCR assays in clinical diagnostic and biomedical research. In the study conducted by Hassanzadeh et al. (2016b), the highest concentration of extracted DNA was associated with the TENT method (919 mg/µL), which may be attributed to Triton ×100 along boiling that complete cause lysis of cell wall, which showed satisfactory result for PCR assay. According to Nzilibili et al. (2018), sodium monofluorophosphate (SMFP), a component of toothpaste, can affect the purity and concentration of DNA for forensic analysis by damaging the DNA molecule. Using a spectrophotometer and electrophoresis results, one of the samples examined had an extreme DNA content of 371 g/ml and a minimum purity value of A260/A280 ratio of 1.25. Limited DNA targeted locus for electrophoresis and DNA ionic irresponsive interactions arose as two distinct occurrences.

Table 2. Analysis of the concentration of gDNA extracted from *S. aureus*

gDNA	Replicate	Concentration (µg/ml)		Mean	SD	CV%
		T1	T2			
Blank	Blank	0.8163	-0.8163	-3.497E-15	1.154	-3.301E+16
S. aureus	R_1	113.1	74.29	93.67	27.42	29.27
S. aureus	R_2	56.43	63.27	59.85	4.834	8.078
S. aureus	R_3	48.57	86.43	67.50	26.77	39.66

Figure 2 shows the purity of the extracted gDNA from *S. aureus*. The purity of the extracted gDNA was computed using the formula: (A260 – A320)/ (A280 – A320). The purity of the DNA samples was determined using the 260/280 absorbance ratio with a value of ~1.8 – 2.0; however, if the ratio value is lower (≤1.6), it may specify the presence of contaminants such as proteins phenol, and other contaminants in the sample. Moreover, the 260/230 ratio is an accepted alternative measure for DNA purity with an expected value within the range between 2.0 and 2.2; values lower or higher than this may specify the presence of contaminants such as proteins, carbohydrates, and or salts (DeNovix, 2021). The purity of the extracted DNA can be calculated after correcting for the turbidity absorbance at 320nm.

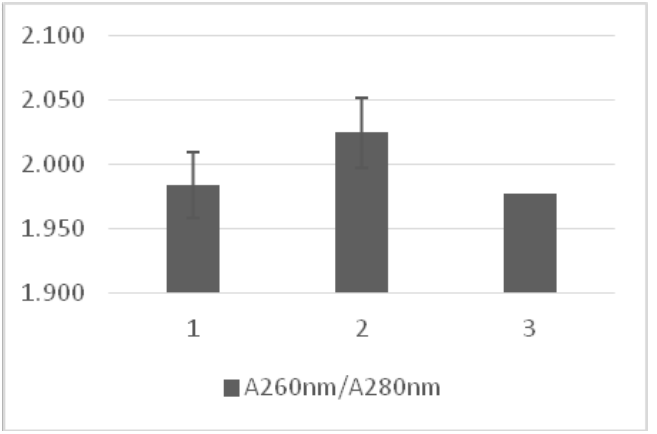

Figure 2. The purity of extracted gDNA at $A_{260/280}$

Table 3 shows the absorbance for determining contaminants present in extracted gDNA from *S. aureus.* The absorbance ratio at 260 nm and 280 nm ($A_{260/280}$) is used to assess the presence of RNA and protein in gDNA, while $A_{260/230}$ absorbance ratio was used to detect the presence of chaotropic salts (Held, 2001). The experimental result showed that the extracted gDNA was contaminated with protein molecules, as demonstrated by the three replicates' absorbance ratio at 260 nm and 280 nm. The values are lower than 1.8. This means that the protein present in the mixture is higher than the amount of DNA molecule. No RNA contamination is observed because the $A_{260/280}$ values are lower than 2.0. Contamination of samples by residual phenol, guanidine, and other chemicals

employed in the isolation technique; samples are very dilute, and concentrations are near the lower detection limit; and an inappropriate solution was used for the blank measurement are the three most common causes of abnormal 260/280 ratios (DeNovix, 2021).

According to Held (2001), several factors may affect A260/A280 ratio, but the most important is the amount of light absorbed by a given sample. Different instruments may produce slightly different ratios due to variability of wavelength accuracy between instruments. The amino acid sequence of proteins has a tremendous influence on the ability of a protein to absorb light at 280 nm.

Table 3. The absorbance of extracted gDNA extracted from *S. aureus* for detection of contaminants

gDNA	Replicate	Chaotropic Salt Contamination		RNA/Protein Contamination	
		A_{260}	A_{230}	A_{260}	A_{280}
Blank	Blank	0.2462	0.2462	2.667	2.667
S. aureus	R1	1.595	1.254	1.951	2.017
S. aureus	R2	0.9088	1.687	2.011	2.039
S. aureus	R3	6.222	1.696	2.000	1.956

Moreover, the extracted gDNA from *S. aureus* was amplified in agarose gel electrophoresis. The agarose gel electrophoresis allows DNA fragments of varying sizes to separate (Lee et al., 2012). Figure 3 shows the extracted gDNA of *S. aureus*, Lanes 2,3,4,5,6, and 7 run-ups to the first linear double-stranded DNA fragment (10,000 bp) of the 1 Kb DNA Ladder (Lane 8) with no DNA fragmentation but do not appear as bright bands. The first well (at Lane 1) does not show any band, although it was loaded with Lambda DNA. Lambda DNA did not appear as a band in the gel because it was still circular, for it was not treated with a restriction enzyme.

The bands of the PCR products in agarose gel electrophoresis appear longer than the gDNA bands. It shows that Lane 2 and 3 run-up to the 9^{th} band (1,500 bp) of the 1 Kb DNA Ladder. Lanes 4,5,6,7,8,9,10,11 run-up to the 8^{th} band (2,000 bp) of the same DNA Ladder. All the bands appear slightly bright except for the band in Lane 4, which appears degraded. This implies that the gDNA extracted from the bacterium is suitable for PCR. PCR amplification also indicates that the DNA was of good quality (Wang et al., 2011).

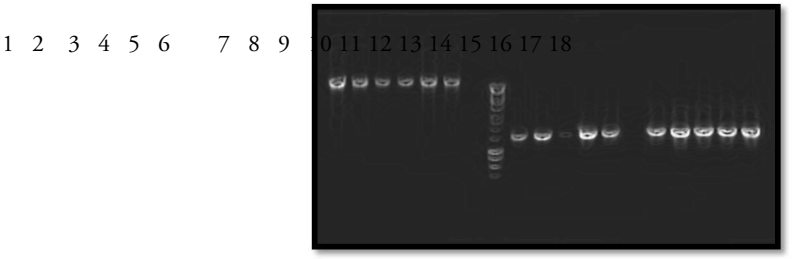

Figure 3. Agarose gel electrophoresis of extracted gDNA of *S. aureus*

CONCLUSION

DNA extraction is one of the most crucial undertakings that can influence all immediate and downstream experiments. DNA purification can extract sufficient amounts of genomic or plasmid DNA from a limited source to meet the researcher's needs, as well as lower the number of contaminants that could jeopardize the study's results and sample shelf life. The value of high-quality, pure DNA cannot be overstated in today's era of multiplex and real-time PCR DNA analysis. Finding a suitable DNA isolation system to meet the downstream application objectives is critical to completing research successfully. The experiment's weakness was the use of only one sample type, the *S. aureus;* more sample types included in the study might have appropriately challenged the result of the experiment. The methods for extraction involved here can be used as a starting point for developing a standard procedure for evaluating gDNA extract quality and quantity for use or downstream microbial research and application. Though the extracted gDNA obtained from the sample was large and sound quality, it may have been contaminated with proteins and chaotropic salts based on its absorbance.

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