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An improved procedure for the isolation of Ribonucleic acid from methicillin-resistant *Staphylococcus aureus*

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Abstract

Extraction and purification of ribonucleic acid (RNA) from Gram-positive methicillin resistant *Staphylococcus aureus* (MRSA) is problematic, because the MRSA has a rigid cell wall that contains lipoteichoic acid and peptidoglycan, thus causing difficulty when utilizing the standard methods. For this reason, the aim of the current study was to improve and modify the method of extraction of RNA from MRSA, with good integrity, purity, low cost, and with saved time of extraction. A fast and an inexpensive method involving the use of acid phenol: chloroform (5: 1 [v/v]) at low pH (4.5), with lysostaphin and Triton X-100 for effective isolation of RNA from the MRSA is developed. As a result of this study, yields of this method presented high concentration of RNA 1175.26 ng/ µl/ 3 ml of bacterial culture broth, with high RNA integration number (RIN). In similar assays such as using; the RNeasy Mini kit, GeneJET RNA purification kit, TRIzol kit and hot phenol: chloroform (1: 1 [v/v]) extraction method, they yielded low concentrations of RNA (92-700 ng/ µl); with lower purity, quantity, and also little integrity, compared to using the current acid phenol chloroform (5: 1 [v/v]) extraction method. In conclusion, this new method for extraction of RNA from MRSA can be used to save time, cost, and provide high quality of RNA.

Keywords: MRSA, RNA integration number, acid phenol: chloroform, RNA purification

1. Introduction

Staphylococcus aureus (*S. aureus*) is an important human pathogen in the hospitals and in the community. Recent study of Olaniyi *et al*., (2016) highlighted that *S. aureus* is a pathogenic bacterium that can cause a wide range of diseases, ranging from the superficial skin and soft tissue infections (SSTIs),

to the more life threatening diseases such as; necrotizing pneumonia, urinary tract infection, osteomyelitis, endocarditis and septicemia. Methicillin was introduced in 1959 to treat infections caused by penicillin-resistant *S. aureus*. In 1961, several studies from the UK reported that *S. aureus* isolates had acquired resistance to methicillin (Lakshminarayanan *et al*., 2018), and this resistance spread to other European countries, as well as to Japan, Australia, and the United States (Enright *et al*., 2002).

A recent study carried out by Kitagawa *et al*., (2019) reported that methicillin resistant *S. aureus* (MRSA) are a group of *S. aureus* strains that have acquired resistance to a class of β-lactam antibiotics. In addition, these strains are the major cause of many hospital associated infections (HA-MRSA); however in 1990, other strains of MRSA started to emerge as community-associated MRSA (CA-MRSA). A previous study of Brennan and Nikaido, (1995) documented that bacterial RNA isolation has many challenges due to several reasons including; its susceptibility to degradation in the presence of endogenous and exogenous RNase activity, the presence of the bacterial complex cell wall matrix containing complex polysaccharides, covalently bond peptidoglycan, arabino-galactans, mycolic acids, and long-chain multi-methyl-branched fatty acids, as present in *Mycobacterium tuberculosis* (Kassab *et al*., 2019). This cell wall cannot be easily disrupted or lysed, and thus hampers the yield and quality of the RNA. Therefore, analysis of the bacterial transcripts requires novel and very fast techniques for isolation of the RNA, due to its short half-life. According to Dietrich *et al*., (2000); Wang *et al*., (2012), the most important step during the extraction of RNA is the rapid disruption of the bacterial cells, leading to direct release of the RNA into the buffers containing RNase inhibitors such as guanidinium isothiocyanate, to protect the RNA from degradation by the RNase. The objective of this study was to determine the best method for isolating the RNA from MRSA (ATCC 43300); with good integrity, quality, quantity, purity, in addition to being time and cost savings.

2. Material and methods

2.1. Materials and chemicals

In this study, a modified method of RNA extraction from methicillin-resistant *S. aureus* obtained from the American Type Culture Collection (MRSA, ATCC 43300), was described as an example of Gram-positive bacteria. Five protocols were tested for isolation of RNA from this bacterium including three commercials kits such as, RNeasy Mini kit from Qiagen, Germany; GeneJET RNA Purification kit from Thermo Scientific, and USA and TRIzol kit from Ambion®, USA. In addition, two published methods reported by Cox and Arnstein, (1963); Atshan *et al*., (2012) know as hot phenol chloroform (1: 1 [v/v]), and acid phenol chloroform (5: 1 [v/v]) with pH (4.5) were used. Triton X-100, lysozyme, β-mercaptoethanol, proteinase K, acid phenol chloroform, TBE buffer, 1.5 ml Eppendorf tubes and lysostaphin, were purchased from Sigma-Aldrich, Germany. The Trypticase Soy Broth (TSB) was purchased from Difco, USA. The MinElute PCR Purification Column Kit and the bacterial RNA protectant were purchased from Qiagen Germany. Tris-EDTA buffer and TE 10×was purchased from Calbiochem, USA. Guanidinium thiocyanate, NanoDrop 2000 spectrophotometer, 2× RNA loading dye, power generators EC105, DNase I, Nano pure water, 25 mM EDTA, DNase I (1000U) and distilled water were purchased from Thermo Scientific, USA.

A micro-centrifuge (micro22R) was purchased from Nettich, Germany, the gel documentation gel unit from Vilber Lourmat, France. An Agilent 2100 Bio-analyzer with RNA 6000 Nano lab chip from Agilent Technologies, Germany, and an UV trans-illuminator was purchased from Vilber Lourmat, France. The heater from Labtech, Malaysia, a microwave oven was purchased from New Sonic, Malaysia. An electrophoresis tank Mini-Sub Cell GT from Bio-Rad, USA, agarose gel from Vivantis, USA, DNase running buffer from Promega, USA, and Gradient thermal cycler from Eppendorf, Germany.

2.2. Bacterial strain and growth condition

A MRSA (ATCC 43300) strain was grown overnight in 5 ml of TSB medium on an orbital shaker at speed of 200 rpm, at 37^{o}C. These overnight cultures (1: 100 dilution) were used to inoculate about 100 ml of fresh TSB medium in a 250 ml flask. The broth culture flasks was incubated on an orbital shaker at speed of 200 rpm at 37^{o}C, to an early exponential phase (OD_{600}= 0.7). The bacterial cells were pelleted by centrifugation at 10,000×g for 20 min. at 4^{o}C, washed with fresh TSB, and used for isolation of the RNA.

2.3. Isolation and purification of the RNA by the acid phenol: chloroform method

Briefly, 3 ml of MRSA (ATCC 43300) suspension at 2×10^{8} cells/ ml, were used to inoculate TSB broth, followed by incubation at 37^{o}C for 24 h. The bacterial broth culture was then centrifuged twice at 5.000×g for 5 min. at 4^{o}C, in 2 ml RNase-free micro-centrifuge Eppendorf tubes. The supernatant was discarded, and then the cells were re-suspended in 100 µl of RNase-free water. Immediately, 2 volumes (200 µl each) of RNA bacterial protectant solution were added to the bacterial pellets, homogenized by overtaxing for 30 sec, and then incubated at room temperature for 5 min. Cells were then collected by centrifugation at 10.000×g for 10 min. at 4^{o}C. After that, the pellets were re-suspended in 200 µl of TE buffer (pH 8) containing; 50 mg/ ml lysozyme or 200 U of lysostaphin (1mg/ ml), and 1.2% of Triton X-100, then incubated at 37^{o}C for 15 min. After incubation, about 200 µl of lysis buffer guanidinium thiocyanate containing; 2% (v/v) β-mercaptoethanol and 20 µl (200 µg/ ml) of proteinase K were added and then mixed. This solution was re-incubated for 15 min. at 56^{o}C in a shaking incubator at speed of 200 rpm. The mixture was extracted with an equal volume of 200 µl of acid phenol: chloroform (5: 1 [v/v]) with pH (4.5), vortexed immediately for 30 sec, and then incubated for 5 min. at room temperature (RT), with vortexing each 2 min. After that, the cell pellets were collected by centrifugation at 10.000×g for 10 min. at 4^{o}C. Approximately, 200 µl of the supernatant were transferred to new micro-centrifuge tube, 400 µl of isopropanol was added and gently checked for 10 min. Later, cell pellets were collected by centrifugation at 10.000×g for 10 min. at 4^{o}C. The aqueous phase was discarded, whereas the RNA pellets were dried in a clean tissue. About 500 µl of 70% ethanol was added to wash the RNA pellets, and then immediately centrifuged at 10.000×g for 5 min. at 4^{o}C. The tubes were allowed to dry from ethanol under sterile conditions for 5 min. Finally, the RNA pellet was eluted in 30 µl of RNA free H_2O, and then kept at -80^{o}C for long long-term storage. The total RNA was quantified using NanoDrop 2000 spectrophotometer at the wavelength of (A_{260}: A_{280}). The integrity of RNA was checked by mixing 3 µl of RNA with 3 µl of 2× RNA loading dye, mixed well, and then loaded into 1.4% agarose gel in 0.5× TBE buffer, obtained from 10× TBE stock solution.

2.4. The commercials kits for RNA extraction

Three commercials kits were used for RNA extraction from the MRSA (ATCC 43300) including; RNeasy Mini kit from Qiagen, Germany, GeneJET RNA Purification kit from Thermo Scientific, USA, and TRIzol kit from Ambion®, USA. These commercials methods were carried out as indicated in the manufacturer's instructions handbooks.

2.5. The simple hot phenol chloroform extraction method

This method was described by Cox and Arnstein (1963); Atshan *et al*., (2012).

2.6. Determination of the concentration, purity of the RNA, and the RIN

The concentration and purity of all the RNA samples were measured spectrophotometrically by using NanoDrop 2000, to determine the absorbance of the samples at A_{230}, A_{260} and A_{280} nm, by (A_{260}: A_{280}), and (A_{260}: A_{230}) ratios. On the other hand, the integrity of the RNA was evaluated by gel electrophoresis on 1.2 % (w/v) agarose gel containing 6% formaldehyde,

stained with ethidium bromide, and visualized under UV light. Moreover, the integrity of the RNA samples was also analyzed on the bio-analyzer, according to the manufacturer's instructions. This bio-analyzer provided a structure for standardization of the RNA quality control. Thus, the RNA samples were electrophoretically separated on a micro-fabricated chip, and then detected via laser made fluorescence detector.

2.7. RNA used for Real-time PCR (RT-PCR) and DNA microarray

To determine if the extracted RNA from this method is functional, cDNA was synthesized with a starting material of 10 µg of the total RNA isolated by the acid-phenol: chloroform (5:1 [v/v]) method. This cDNA was purified using a MinElute PCR Purification Column Kit, and then quantified by measuring the absorbance at A_{260} nm, using a NanoDrop spectrophotometer.

2.8. Statistical analysis

The data were analyzed using statistical software SPSS by one-way analysis of variance (ANOVA) test. The differences were compared with Post Hoc Test and unpaired t–test, $P < 0.05$ was considered significant. The results were represented as means ± SD (standard deviation).

3. Results

3.1. Concentration and purity of the total RNA

This study was performed to evaluate five protocols for the isolation RNA from MRSA (ATCC 43300). The concentration and purity of the total RNA was spectrometrically determined using a NanoDrop spectrophotometer 2000. Three absorbance values were taken into consideration (A_{230}, A_{260} and A_{280} nm). The ratio between the absorbance at A_{260} /A_{280} nm was used to evaluate the concentration, purity and contamination of the ribonucleic acid. For pure RNA, the ratio around (1.8 - 2.0) is expected for good quality RNA.

According to the quantity of RNA, the highest concentration is recorded from the samples which were extracted using acid phenol: chloroform (5: 1 [v/v]) with a mean value of 1175.26 ng/ µl, then hot phenol chloroform (1: 1 [v/v]) of 699.93 ng/ µl. However, the lowest RNA concentration is from samples extracted using GeneJET kit, RNeasy Mini kit and TRIzol kit, recording mean values of; 92.30, 137.67, and 671.75 ng/ µl, respectively. Significant differences are recorded in RNA concentrations between these methods ($P< 0.01$ unpaired t – test), as shown in Table (1). The method with the highest purity at A_{260}/A_{280} is form RNeasy Mini kit at a value of 1.98, followed by acid phenol chloroform (5: 1 [v/v]) and hot phenol: chloroform (1: 1 [v/v]) at a value of 1.94 for each. On the other hand, the lowest purity is obtained from TRIzol and GeneJET kits recording; 1.93 and 1.75, respectively. No significant differences are found in purity of the RNA between these five methods ($P > 0.05$ unpaired t – test), as clear in Table (1). In addition, the greatest ratio between the level of purity and contamination demonstrated at A_{260}/A_{230} is from RNeasy Mini kit at value of 2.4, then acid phenol: chloroform (5: 1 [v/v]) 2.21, followed by hot phenol: chloroform (1: 1 [v/v]) recording a value of 1.76. Conversely, the lowest ratio is obtained on using GeneJET followed by TRIzol kits, recording 0.68, 1.02, respectively. Significant statistical differences are observed with regard to the RNA purity and contamination at the ratio of A_{260}/A_{230} between the used methods ($P< 0.01$ One-way ANOVA, Post Hoc Test and unpaired t – test).

3.2. Agarose gel electrophoresis

The RNA integrity was assessed using the agarose gel electrophoresis. The expected double banding pattern of 16S/ 23S, and the absence of smear indicated the good integrity of RNA. The best and sharp intact 16S/ 23S RNA bands are arranged respectively from acid phenol: chloroform (5: 1 [v/v]), hot phenol chloroform (1: 1 [v/v]), TRIzol, RNeasy Mini kit, and GeneJET kit, as demonstrated in Fig. (1, 2).

Table 1: Mean of RNA concentration, purity and RIN obtained by the five RNA extraction methods

Extraction Protocol	RNA concentration (ng/µl)	A_{260}/A_{280} Ratio	A_{260}/A_{230} Ratio	RIN± SD
Hot phenol chloroform 1: 1	699.93	1.94	1.76	6.4 ± 0.2*
Acid phenol chloroform 5: 1	1175.26	1.94	2.21	7.2± 0.05*
RNeasy Mini kit (Qiagen)	137.67	1.98	2.4	5.8 ± 0.05*
GeneJET	92.30	1.75	0.68	5.5 ± 0.05*
TRIzol	671.75	1.93	1.02	4.8 ± 0.5*

*Values represent the mean ± (SD) of 35 independent experiments of RNA isolation by each method

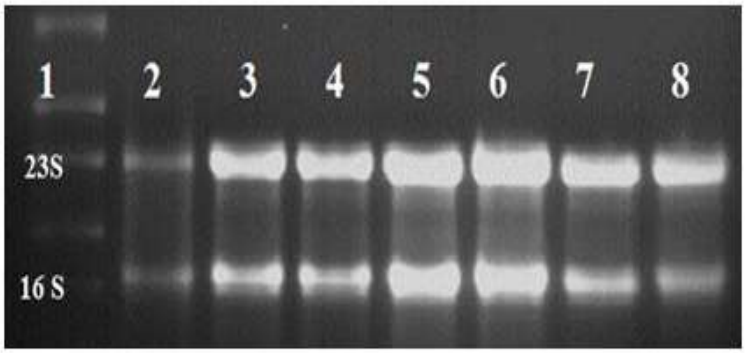

Fig. 1: Agarose gel electrophoresis of the RNA samples using different extraction methods. The amount of RNA loaded in each well was 3 µl. Lane 1: RNA ladder marker; Lane 2: GeneJET kit; Lanes 3 and 4: RNeasy Mini kit; Lanes 5 and 6: Acid phenol: chloroform (5: 1 [v/v]); Lane 7: Hot phenol: chloroform (1: 1 [v/v]); Lane 8: TRIzol kit. The best and sharp intact 16S/23S RNA bands are arranged respectively from acid-phenol: chloroform (5:1 [v/v]), hot-phenol: chloroform (1:1 [v/v]), TRIzol, RNeasy Mini kit, and GeneJET kit.

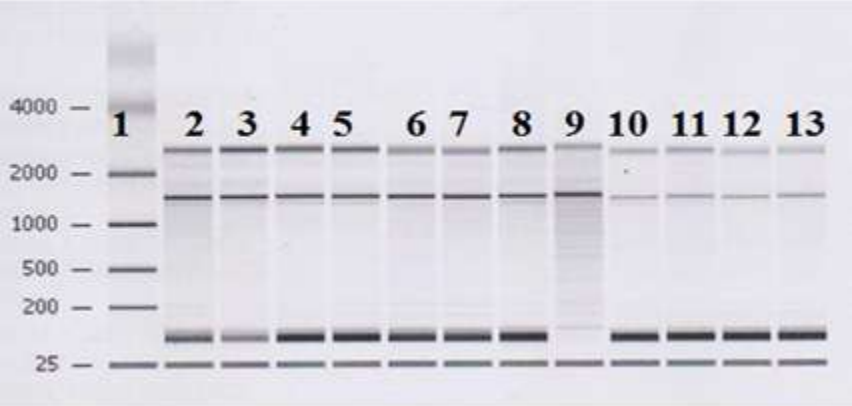

Fig. 2: The RNA Integrity checking: Gel image by the automated electrophoresis system Bio-analyzer 2100. Lane 1: RNA ladder marker (Thermo scientific); Lanes 2 and 3: RNeasy Mini Kit; Lanes 4 and 5: Hot phenol chloroform (1: 1 [v/v]); Lanes 6 and 7: Gene JET; Lanes 8 and 9: TRIzol; and Lanes 10-13: Acid phenol: chloroform (5: 1 [v/v]). The average RIN scores were 6.4 ± 0.2, 5.8 ± 0.05, 7.2± 0.05, 5.5 ± 0.05, and 4.8 ± 0.5, respectively, for each used method. The highest quality of RNA was extracted using the acid-phenol: chloroform (5:1 [v/v]) method, recording the mean values of RIN about 7.2± 0.05.

The presence of DNA in some samples extracted by manual methods is attributed to the absence of DNase treatments between the steps.

3.3. RNA integration number (RIN)

The integrity of the RNA was assessed by using an Agilent 2100 bio-analyzer to calculate the RIN. It required only a very small amount of RNA sample; up to 2 µl of the total RNA, with a concentration up to 500 ng/ µl. The use of RNA ladder as a mass and size standard during electrophoresis allowed the estimation of the RNA band sizes. Integrity of the RNA may be assessed by visualization of the 16S and 23S ribosomal RNA bands, as demonstrated in the electropherogramas (Fig. 2).

From Table (1), the average RIN scores (RIN ± SD) are 6.4 ± 0.2, 5.8 ± 0.05, 7.2± 0.05, 5.5 ± 0.05, and 4.8 ± 0.5, respectively for each used method. The highest quality of RNA was extracted using the acid phenol: chloroform (5: 1 [v/v]) method recording the mean values of RIN of 7.2± 0.05, followed by hot phenol: chloroform (1: 1 [v/v]) protocols recording RIN about 6.4 ± 0.2. The lowest RIN value is obtained from TRIzol kit recording 4.8 ± 0.5.

3.4. RNA used for Real time PCR (RT-PCR) and DNA microarray

The RNA obtained from the acid-phenol: chloroform (5:1 [v/v]), worked effectively in both cDNA synthesis and in downstream applications such as RT-PCR, where the synthesized cDNA was used for the DNA microarray technique. The mean concentration and purity of the cDNA were recorded at absorbance of (A_{260}/A_{280}) nm, were 1200 ng/ µl and 1.87ng/µl; respectively, as demonstrated in Fig. (3). The assay was carried out in triplicates and repeated 3 times.

4. Discussion

Bacterial RNA isolation has many challenges due its susceptibility to degradation in the presence of endogenous and exogenous RNase activity, and presence of complex cell wall matrix. In the present study, the use of acid phenol: chloroform for extraction of the RNA and undergoing enzymatic lysis

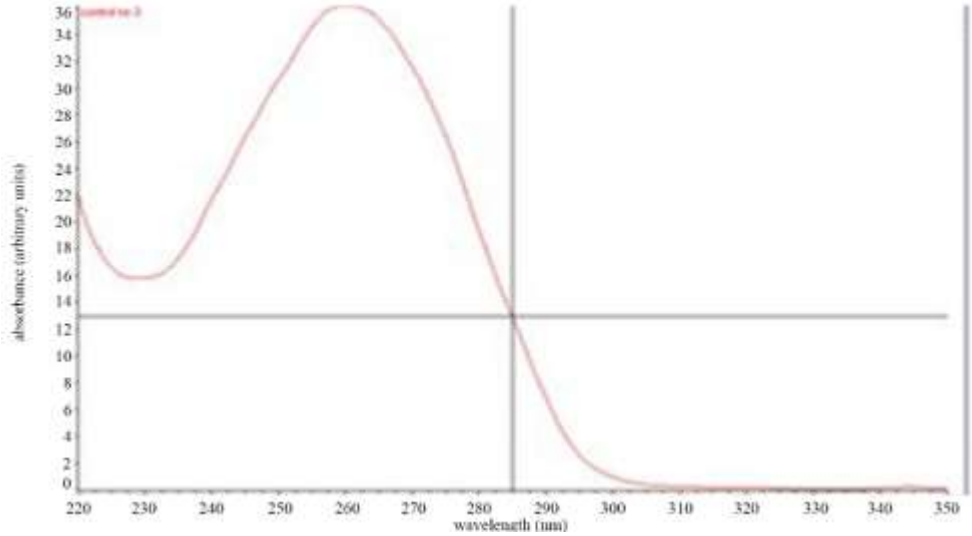

Fig. 3: A quality check of the concentration and purity of cDNA recorded at absorbance of A_{260} /A_{280} nm, with a starting material of 10 µg/µl of the total RNA, extracted by the acid-phenol: chloroform (5:1 [v/v]). The mean recorded concentration and purity values of the cDNA were; 1200 ng/ µl, 1.87 ng/ µl; respectively, for all samples.

procedure at low pH, generated RNA from the bacterial cell within 60 min.

Currently, the isolation of RNA from MRSA (ATCC 43300) required a lysis step using TE buffer with lysostaphin 200 U (1 mg/ ml) or lysozyme (50 mg/ ml), where the lysozyme binds on the bacterial surface and attacks the peptidoglycans, as reported during the early study of Höltje *et al*., (1975). Triton X 100 (1.2 %) was also used in several protocols at the same concentration, to cause autolysis of the bacterial cell wall (Kim *et al*., 2017). Moreover, a commercial lysis buffer was used to protect the RNA from degradation, and it contained guanidinium thiocyanate (GITC), which is a chemical compound commonly used in the extraction of DNA and RNA (Harada, 2016).

According to Das, (2016), the GITC method for isolation of RNA is used frequently when un-degraded mRNA is desired, thus high quantity and quality RNA can be obtained using an acid phenol: chloroform, which is premixed and supplied at pH 4.5 ± 0.2. Previous studies conducted by Asif *et al*., (2000); Sambrook and Russell, (2006) highlighted that this method is used for extraction of RNA from the plant, animal cells, cancer cell lines, yeasts and bacteria. During the RNA extraction processes, the acid phenol: chloroform supports the removal of DNA; in addition, its partitions into the organic phase, helps to stabilize the interface and prevents foaming upon mixing (Wasniewski, 2012). A recent study of Smith *et al*., (2020) added that this method significantly reduces the amount of DNA contamination, which results in fewer DNase treatments and reduced RNA handling. On using this method, the MRSA cell wall was disrupted quickly, efficiently and safely at low temperature of 37^{o}C, which did not affect the RNA integrity; however, some other extraction methods lysis the cell wall at high temperature of 70^{o}C that affect the quality of RNA (Atshan *et al*., 2012).

Wang *et al*., (2019) reported that the principle of the phenol: chloroform method is to increases the efficiency of extraction by causing denaturation of the proteins. In addition, its high density makes separation

of the two phases easier, and also removes any lipids from the sample.

The study of Chomczynski and Sacchi, (2006) showed that the best results of increasing the quantity of RNA were obtained on using acid phenol: chloroform (5: 1 [v/v]), where the mean concentrations recorded was 1175.26 ng/ µl, compared to the other methods of hot phenol: chloroform (1: 1 [v/v]) and TRIzol kit, where the RNA concentration was 699.93 and 671.75 ng/ µl, respectively. However, the lower RNA concentrations recovered were from samples extracted by the GeneJET and RNeasy Mini kits, the mean values recorded were 137.67, 92.30 ng/ µl, respectively. This high concentration of RNA obtained on using this method of extraction might be probably attributed to good lysis of the bacterial cells, whereas the other methods have poor cell lysis due to its short time.

In the current study, the best results in purity of RNA at ratio of A_{260}/A_{280} were from RNeasy Mini kit, followed by acid phenol: chloroform (5: 1 [v/v]) and hot phenol: chloroform (1: 1 [v/v]). On the other hand, the lower purity was from the TRIzol kit and GeneJET kit. Tavares *et al*., (2011); Al Abdulsalam *et al*., (2018) explained that the ratio of absorbance at A_{260}/A_{280} nm and A_{260}/A_{230} nm should be at values of (1.8-2.0); however, any reading that is less than 1.7 suggest that the RNA extraction was contaminated with protein, phenol, or suggestive of contamination with guanidinium thiocyanate. Therefore, all absorbance results obtained at A_{260}/A_{280} nm during the tested extraction method, indicated that there is no significant statistical difference between the used methods of RNA extraction, except on using the GeneJET kit where the ratio is 1.7. However, results of absorbance at A_{260}/A_{230} nm had statistically significant difference between the methods used for RNA extraction ($P < 0.01$ using unpaired t – test).

The agarose gel electrophoresis was used to check the size, integrity of the RNA preparations, and to detect the sharp and intact RNA under UV light. The integrity of the ribosomal RNA sub-units (23S, 16S and 5S for prokaryotes), the presence or absence of low RNA degradation products, and the presence or absence of genomic DNA contamination are commonly visualized using agarose gel electrophoresis (Pinto *et al*., 2009). The 23S rRNA band should have been present at approximately twice the intensity of the 16S rRNA band. However, since the 23S rRNA was more labile than the 16S rRNA, equal intensities of the two bands generally indicated that some degradation had occurred, in reference to Fuchs *et al*., (2001). Current results demonstrated the existence of two well-defined bands in the agarose gel electrophoresis; the best sharp and clear bands of 16S rRNA and 23S rRNA were from acid phenol: chloroform (5: 1 [v/v]). This may be attributed to the low pH and absence of contamination with DNA after some steps of DNA removal.

Similar study of Chung *et al*., (2013) used the same strain of MRSA (ATCC 43300) bacteria, have got good RIN using the RNeasy Mini kit protocol, and disrupted the cell wall of MRSA using the TE buffer that contained 50 mg/ml lysozyme inclosing proteinase K. Moreover, previous study of Jahn *et al*., (2008) used similar standard laboratory kits and methods such as; TRIzol kit, RNeasy Mini kit, and hot SDS/hot phenol alone and in combination, for extraction of RNA from the Gram-negative bacterium *Dickeya dadantii*. They found that high quality RNA was isolated using a hot SDS/hot phenol method, which supports current results that some manual methods can produce high quality and quantity of RNA. The cDNA quality check of the concentration and purity of RNA at A_{260}/A_{280} nm extracted by the acid-phenol: chloroform (5:1 [v/v]) method, represents good indication to use this method for qRT- PCR and transcript profiling of the DNA microarray.

Conclusion

Results of this study indicated that using lysostaphin, lysozyme and acid phenol: chloroform (5: 1 [v/v]) represented a good method to produce high concentration, purity, and good integrity of the RNA from MRSA (ATCC 43300), to be used for qRT- PCR

and transcript profiling of the DNA microarray. This may be attributed to the ability of this method to cause complete lysis of the MRSA cell wall. From the economical point of view, this method saves the cost and time of extraction, compared to the other common methods.

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Conflict of interest

All the authors declare no conflicts of interest.

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Ethical approval

This article does not contain any studies with human participant's and\ or animals performed by the authors.

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