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Original article

Impact of *Staphylococci* DNA extraction methodology on microsatellite-based PCR banding profile

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ABSTRACT

Background: Microsatellite-based polymerase chain reaction (PCR) has great utility in microbial typing by analysis of band profiles of PCR amplicons. Bacterial DNA extraction methods affect yield and quality of DNA extract, and consequently subsequent molecular-based studies. The study aimed to evaluate impact of bacterial DNA extraction methods on band profiles of microsatellite-based PCR. **Methods:** Genomic DNA was extracted from *Staphylococcus epidermidis* ATCC 12228 using QIAamp kit, tween and SDS methods, and submitted to microsatellite-based PCR using $(GACA)_4$ primer. For each extraction method, 4 concentrations were tested: the first crude extracted DNA and other 3 concentrations obtained by 1:1, 1:3, and 1:5 dilutions of the crude samples. **Results**: Yield and purity of DNA extracted by QIAamp kit were higher than those of other two methods. The PCR amplified all tested DNAs with generation of band profiles ranged from 2 to 11 bands in number, and from 200 bp to 1500 bp in size. Marked inter-method differences were found due to variation in band numbers and sizes. Band patterns obtained for QIAamp kit and tween methods were more robust than for SDS method. Variations of PCR patterns of different DNA concentrations within each method were minimal. **Conclusions:** Microsatellite-based PCR band profiles vary with different genomic DNA extraction methods for the same bacterial strain. Quality of extracted DNA is more influencing than its concentration. Therefore, it is recommended to unify the extraction method of bacterial DNA for all tested bacterial strains that are submitted to such type of PCR.

Introduction

Polymerase chain reaction (PCR) is a sensitive and specific molecular-based technique used in identification of causative pathogens in infectious diseases. There are numerous modalities of PCR including, for example, simple conventional PCR, nested PCR, multiplex PCR, random amplified polymorphic DNA (RAPD), and real-time PCR. Microsatellite-based PCR utilizes short nucleotide repeats as primer(s) targeting microsatellites which comprise DNA tandem repeats in the target genome [1]. It is considered as a simple, rapid, single-step PCR, and proved to have utility in detection, species differentiation, strain identification and genotyping of several bacteria and fungi [2-4]. The key factor used in the interpretation of the results of this PCR variety is the band pattern of the amplified products electrophoresed in agarose gel. This pattern depends mainly on polymorphisms in lengths of the target repetitive DNA sequences in tested nucleic acid. Many simple repetitive primers

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were previously utilized in microsatellite-based molecular techniques, one of them is $(GACA)_4$ primer [1,3,5,6].

One of the basic primary steps preceding PCR and other molecular-based typing techniques is the DNA extraction from the tested specimens. There are numerous approaches to extract DNA, either physical, chemical, or kit-based. The quantity and quality of the extracted DNA vary markedly according to the used extraction method, so that the extraction method has remarkable effects on the results of the subsequent PCR test [7-9].

This study aimed to investigate the impact of staphylococci DNA extraction methods on the results of microsatellite-based single-step PCR to test if change in DNA extraction methodology for the same bacterial strain can affect the reproducibility of this PCR variety when performed on DNAs extracted by different methods for the same bacterial strain. Three different DNA extraction methods including QIAamp DNA kit, tween method, and SDS method were used to extract DNA from *Staphylococcus epidermidis* ATCC 12228 and the extracted DNAs were submitted to microsatellite-based PCR using $(GACA)_4$ primer, then, the profiles of the amplified products were compared. Additionally, the study tested the effect of changing DNA concentration within each extraction method on the yield of PCR.

Materials and Methods

Tested bacterial strain

Staphylococcus epidermidis ATCC 12228 was used in the study. It was subcultured in 50 ml tryptone soya broth (Oxoid) in 250 ml sterile flaks, incubated at 37°C using shaker incubator for 18 hours, then mixed thoroughly and distributed into equal 1 ml aliquots in sterile tubes. The densities of bacterial suspensions were adjusted to 1 McFarland standard.

DNA extraction

The bacterial suspensions were centrifuged at 13000 rpm for 5 minutes followed by discarding the supernatants, and washing the bacterial pellets four times using phosphate buffered saline (PBS). Then, genomic DNA was extracted from all bacterial pellets using the following three methods (triplicate for each method): A) Kit-based method using QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany); the extraction steps were followed according to the manufacturer's instructions. B) Tween-based method using the lysis buffer (10 mM Tis-HCl,1 mM EDTA (pH 8), 0.5% tween, 200 µg/ml proteinase K) according to **Lazarevic et al.** [10] with modifying the incubation with the lysis buffer to 3 hours, followed by heating at 100°C for 10 min. C) SDS method as mentioned by **Goldenberger et al.** [11] using the lysis buffer (10 mM Tis-HCl, 1 mM EDTA (pH 8), 0.5% SDS, 200 µg/ml proteinase K). For purification, the DNAs extracted from tween method were treated by 3 M sodium acetate, precipitated using absolute ethanol, then washed by 70% ethanol, while the DNA extracts from SDS method were washed using 2 M sodium chloride, precipitated by absolute ethanol, and then washed by 70% ethanol. In both methods, the DNA pellets were let to air dry, and then finally eluted in 200 µl of TE buffer.

Assessment of yield and quality of extracted DNA

The yield (concentration in ng/µl), OD_{260}, OD_{280} of all extracted DNAs were measured using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The purity of extracted DNA was determined by using OD_{260}/OD_{280} ratio.

Microsatellite-based PCR analysis

The global effect of the DNA extraction methodology on the yield of PCR was tested by mixing together the 3 samples of DNA extracted by each extraction method to obtain one collective sample representative for each extraction method, and then these samples were subjected to PCR analysis. The effect of the concentration of DNA on the yield of PCR was tested by diluting the collective representative samples from each method using sterile RNase and DNase free water to get different concentrations of DNA by performing dilutions (1:1, 1:3, 1:5) and all diluted samples were also subjected to PCR. In this microsatellite-based PCR, the repetitive primer $(GACA)_4$ (TIB MOLBIOL, Germany) was used. The PCR was performed according to **Faggi et al.** [5] with some modifications. The reactions were performed in 50 µl reaction volume: 25 µl of 2× Go *Taq* Green Master Mix (Promega, Madison, USA), 160 ng $(GACA)_4$ primer, 12 µl DNA, made up to a total volume of 50 µl with sterile RNase and DNase free water. Negative control reactions were included with each PCR run with replacement of genomic DNA with sterile RNase and DNase free water. PCR was performed in 39 cycles of denaturation at 93°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, followed by a final extension step at 72°C for 7 min.

Polymerase chain reaction products were separated in 1.5% agarose gels. The ExactMark 100 bp DNA

ladder (1st Base, Singapore) was used as a molecular marker. The gels were stained with ethidium bromide, and then images were captured using the Uvitec gel documentation system (Cambridge, UK).

Inter-method and intra-method variations were studied. Inter-method means comparing the PCR amplified products patterns for DNA obtained by the three different tested DNA extraction methods. Intra-method means comparing PCR patterns of the original crude sample (1st concentration) and the three diluted samples (2nd, 3rd and 4th concentrations) of DNA extracted by each tested extraction method.

Results

DNA yield and purity

The spectrophotometric assessment of the concentration and purity of the extracted DNAs is shown in **table (1)**. QIAamp kit method gave the highest DNA yield (81.89 ng/µl), followed by tween method (69.76 ng/µl) and SDS (34.96 ng/µl) method. The OD_{260}/OD_{280} ratios were 1.82, 1.54 and 1.21 for QIAamp kit, tween and SDS methods, respectively.

Microsatellite-based PCR analysis

The used PCR succeeded in amplification of all tested DNA samples. The amplified products appeared in multiple bands rather than one band. Different band patterns were obtained with the different tested extraction methods, with band numbers ranged from 2 to 11 bands, and sizes ranged from 200 bp to 1500 bp. There was variation in the strength of the bands from faint to bright.

The results showed obvious inter-method variation (in band number and sizes) and little intra-method variation (in number of bands, while the sizes were the same).

The PCR of DNAs extracted by QIAamp kit method are shown in **figure (1), panel (A)**. The number of bands ranged from 3 to 6 bands and sizes ranged from 400 bp to 1200 bp approximately. Some intra-method variation was noted; as the tested first and third concentrations gave nearly same pattern of 6 bands with little difference in the size of the smallest band (500 bp and 400 bp, respectively), the tested second concentration gave pattern of five bands (with sizes ranging from 650 bp to 1200 bp approximately), while the fourth concentration gave a pattern of three bands (of sizes: 700, 900, and 1200 bp).

Amplification of DNAs extracted from tween method gave one band pattern, which was the most complexed pattern among the tested methods, and formed of 11 bands ranged from 200 bp to 1200 bp in size, with most of the bands (8 bands) in the size range of 200 bp to 900 bp. The band of the size 700 bp was the most prominent (brightest) one in all patterns of tested 4 concentrations. No obvious variation was found between different concentrations tested, except the brightness of the resulted bands; as the larger 3 bands (900 bp, 1000 bp, 1200 bp) were fainter in the first three concentrations than in the fourth concentration, while the smaller bands (200 bp, 230 bp, and 300 bp) of the pattern of fourth concentration were fainter than those in the first tested three concentrations **(Figure 1, Panel B)**.

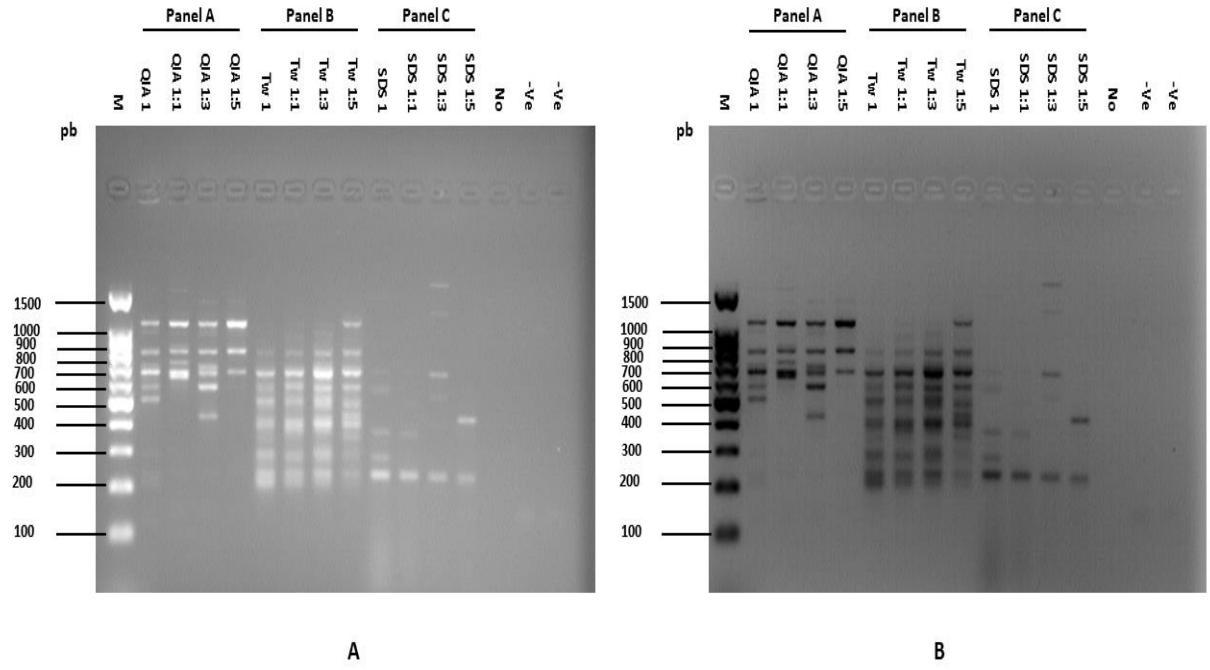
The PCR of the DNAs extracted from SDS method produced patterns of 2 to 6 bands with sizes ranged from of 200 bp to 1500 bp. The 200 bp band was the brightest one in all tested concentrations, while the rest of the bands were faint. The patterns of the tested 4 concentrations varied slightly in band number as the first concentration had 5 bands, the second and fourth concentrations had 2 bands, and the third concentration had 6 bands **(Figure 1, Panel C)**.

Table 1. Spectrophotometric assessment of yield and purity of the extracted DNA.

	QIAamp kit M±SD*	**Tween method M±SD***	**SDS method M±SD***
Yield (ng/µl)	81.89±13.06	69.76±11.88	34.96±5.04
Purity (OD_{260}/OD_{280})	1.82±0.20	1.54±0.34	1.21±0.02

* Mean ±Standard deviation

Figure 1. Agarose gel electrophoresis of products of microsatellite-based PCR using $(GACA)_4$ primer for DNA extracted by three different methods. A) Positive photo. B) Negative photo. Lanes: M, Molecular weight marker (1st Base, Singapore); Panel A, for DNA extracted by QIAamp kit; Panel B, for DNA extracted by tween method; Panel C, for DNA extracted by SDS method. Each panel contains 4 concentration named by initials of each method; Number 1 denotes the crude (1st) concentration; 1:1, 1:3, 1:5 denote different dilutions of the crude concentration to obtain the other three concentrations (2nd, 3rd, 4th, respectively). No, no samples; -ve, negative control (no template DNA).



Discussion

The study evaluated the impact of methods of bacterial genomic DNA extraction on amplification products of a single-step microsatellite-based PCR using $(GACA)_4$ primer. In addition to testing the effect of changing the concentration of template DNA on the pattern of amplification.

This PCR modality uses repetitive primers that target microsatellites repeat regions in template DNA, resulting in amplification of the regions of DNA flanked by these repeats, consequently, the number of these amplification bands varies according to the number of repeats of these microsatellites.

This variety of PCR was selected to perform the current study as it is sensitive to detect differences in quality and yield of DNA obtained from different extraction methods because it depends on characters of the microsatellites sequences repeats regarding their number, length, and pattern of repeating within the template DNA. All of these characters can be influenced by the state of the template DNA, which is remarkably affected by the extraction method. Indeed, the protocol of DNA extraction affects quantity and/or quality of DNA and results of subsequent molecular-based procedures carried out on it [12,13]. Moreover, certain protocols of DNA extraction were found to be superior in extraction of genomic DNA appropriate for subsequent bacterial typing, profiling, fingerprinting and diversity discrimination PCR studies [8,14].

In the current study, the PCR analysis of all tested DNA samples generated amplified products in form of multiple bands. These bands could be either true amplification products resulted from specific annealing of $(GACA)_4$ primer to its complementary sequences in target microsatellites regions dispersed in the tested DNA template, or false (spurious) bands resulted from primer-template mismatches as a result of non-specific binding of the primer with the template DNA. These mismatches occur frequently in microsatellite-primed PCR carried out on prokaryotic genomes [15].

Overall, the study results revealed marked diversity in the band patterns of the PCR amplicons of DNAs obtained from the three tested DNA extraction methods for the same bacterial strain. This inter-method variation resulted from obvious differences in number, sizes and density of the bands. QIAmp kit and tween methods showed patterns of more diversity than SDS method. This inter-method variation could originate from several factors related to extraction protocols, as the DNA extraction methodologies were found to vary in purity, yield, shearing of resultant DNA and representation of microbial diversity [14].

Purity of genomic DNA, is among the most crucial factors that are influenced by the DNA extraction approach and has paramount effect on the yield of PCR [16]. In the current study, DNA extracted by QIAamp kit method was purer than that extracted by tween and SDS methods. This agrees with **Dilhari et al.** [14] who found that DNeasy kit approach was better than other 5 conventional physical and chemical methods regarding purity and revealing diversity in bacterial profiling using PCR-denaturation gradient gel electrophoresis. Other studies [17,18] revealed high quality of DNA extracted by QIAamp kit-based methods, in addition to accuracy and more efficiency of subsequent molecular-based analyses carried out on it. The high purity and quality could result from the efficient spin column procedure, applied in these methods, which ensures specific adsorption of DNA to silica-gel membrane and elimination of contaminants, in addition to the two washing steps using different washing buffers for removal of any residual contaminants.

The impurities found in the DNA extract include proteins, polysaccharides, salts, traces of chemicals used in extraction, and RNA. These impurities can interfere with PCR and affect the quality and band profiles of its amplification products. SDS inhibits *Taq* polymerase and can interfere with PCR amplification even at very low concentrations [19,20]. Also, salts such as sodium chloride have inhibitory effect on PCR. This could explain, in part, the poor and less diverse band profiles obtained from PCR for DNA extracted by SDS method in the current study, as the DNA extract may contain traces of SDS and/or NaCl used in removal of SDS in post extraction purification as mentioned in methods section. On the other hand, although non-ionic detergents such as tween could inhibit PCR, this occurs with relatively high concentrations [19], so the PCR products for DNA of tween method in the current study are less likely to be affected by this inhibitory effect as most of the tween is removed by the post extraction treatment step using Na acetate, which reduces the final concentration of the tween in DNA extract, so its band profiles are more robust than those of SDS method.

The yield of genomic DNA differs according to the extraction methodology. Indeed, the yield can influence the PCR amplification products, especially in microsatellite-based PCR, in which the amplicons are usually appear as profiles of multiple bands. This effect can include number of bands and/or their densities. In the current study, QIAamp kit and tween methods gave higher DNA yields than SDS method, and produced more robust banding profiles consisting of more bands. This concurs with **Carrigg et al.** who found that DNA extraction method significantly affected bacterial profiles regarding number of bands and ribotypes of PCR amplicons for soil bacterial communities [21].

Shearing of extracted genomic DNA occurs with many extraction protocols and affects the PCR amplification. It is a degradation process that results from exposing DNA to heat, physical shearing such as sonication, bead beating, repeated freezing and thawing, insufficient purification resulting in residual nucleases, and improper preservation of extracted DNA [22,23].

Shearing affects the integrity of genomic DNA and consequently can influence the geometry of the microsatellite sequence repeats and other tandem repeats within the genome. It can disrupt the normal distribution, sizes, and repeating pattern of these repeats, resulting in production of different banding profiles of microsatellite-based PCR performed on this DNA. In addition, high degrees of shearing decrease the gene copies in template DNA rendering them insufficient for PCR detection or quantitation [24,25]. The shearing occurs with varying degrees with different extraction methods, so it can lead to different band patterns of PCR amplicons. In all three tested extraction methods of the study, some degradation of DNA can occur due to exposure to high temperatures applied during processing. Moreover, shearing results in DNA fragments of low molecular weight that when amplified by PCR can lead to formation of chimeric structures or products that are considered as PCR artefacts [25-27]. These products may give extra bands when electrophoresed in agarose gel.

Replication slippage (or slipped-strand mispairing) could be a cause of diversity (either inter-method or intra-method) of the results of the current study. It is a replication error that may occur during *in vitro* amplification of DNA by PCR and results from misalignment of DNA strands [28,29]. It is common in microsatellite PCR [30,31] and results in formation of minor amplification products, in addition to the main products, which give additional bands called stutter or shadow bands. These extra bands can contribute to variation and diversity of banding profiles of PCR amplicons [32-34].

Moreover, the PCR amplicons banding profiles vary with PCR conditions such as annealing temperatures and extension times [35,36], however, this is excluded in the current study because the PCR conditions were the same as one PCR protocol was applied for all DNA samples tested in the study. Primer dimerization can also be responsible for diversity of band profiles of PCR products. It occurs when there is complementarity of sequences within the same primer or between different primers, as presence of more than two overlapping nucleotides at 3' ends of primers was confirmed to have a considerable effect in formation of primer-dimers [37]. The primer-dimers are considered as primer secondary structures (hairpins, self-dimers and cross-dimers) and result in formation of nonspecific amplicons, that may appear as extra band (s) along with the main PCR amplicon. The formation of such extra amplicons (extra bands) is excluded in the current study, as one primer type $(GACA)_4$ was used with no self-complementation.

Regarding testing the impact of changing the concentration of tested DNA within each extraction method on the yield of PCR, the current results found that decreasing the concentration has a minimal effect on banding profiles (as evidenced by minimal intra-method variation), even some lower concentrations gave patterns of increased band numbers than higher concentrations as found with QIAmp method and SDS method, in which the third tested concentrations patterns were more rich than patterns of the second concentrations, which indicates that the integrity and purity of the genomic DNA extract are more influencing factors than its yield in determining band patterns of the PCR amplicons.

As regards the interpretation that the obtained band profiles of the current study have originated from non-specific priming that resulted in primer-template mismatches, the generation of PCR products can be explained as follows. Some types of primer-template mismatches can be amplified with the same efficiency as the fully matched primer-template duplexes [38]. So that, in the first PCR cycles, if there is perfect matching between the template and just few bases of the primer at its 3' end, the *Taq* polymerase can overcome the mismatched priming and the extension occurs. The success of first extension allows extensions of the subsequent cycles with production of amplified products that fully match with the primer [15]. According to this explanation, the presence of multiple microsatellite sequence units in template DNA that are partially complementary with $(GACA)_4$ primer can enhance multiple mismatched annealing with this primer resulting in production of multiple amplification bands.

There are many factors underlying the mismatched priming, including increased primer concentration, low annealing temperatures, long annealing and extension times, improper concentration of Mg^{2+} ions and increased number of cycles. Reviewing these factors in relation to the current study showed that the concentration of the primer that was used (160 ng) and the number of cycles that were applied (39 cycles) are relatively increased and could enhance mismatching between the primer and template DNA, but annealing temperature (50 °C) is proper and even higher than the calculated one that should be 3-5 °C less than the melting temperature (*Tm*) of the primer (48 °C, calculated according to GC/AT content rule and provided by the manufacturer). The occurrence of such mismatches and their affection by many PCR conditions interferes with the reproducibility of the microsatellite-primed PCR.

Although mismatches have drastic effects on detection and quantitation of target nucleic acids by PCR [39], they have discriminative value in genotyping PCR studies which depend on polymorphisms of nucleotides between tested nucleic acids, as the patterns of these mismatches differ according to nucleotides sequences in target genome [40]. Indeed, DNA extraction methods can affect the pattern of the primer-template mismatches due to their original effects on the quality and integrity of the extracted DNA.

The limitations of the study included restriction on Gram positive bacteria, testing one strain of staphylococcus species, and evaluation of three DNA extraction methodologies only, so the

results may not come concordant with other bacterial species and other DNA extraction methods. However, the current study highlighted the impact of changing DNA extraction methodology on variation of microsatellite-PCR patterns for the same bacterial strain, and can be taken as a guide for more sophisticated studies needed to investigate factors affecting reproducibility of this PCR modality.

Conclusions

The generated band profiles could result from either specific amplification of microsatellites repeats or mismatched annealing of $(GACA)_4$ primer. Extraction methods of bacterial genomic DNA affect band profiling of the microsatellite-based PCR as each method gives unique band pattern. Integrity and purity of extracted DNA have more influencing effects than its concentration. Therefore, DNA extraction methodology affects the reproducibility of microsatellite-based PCR, so it is important to apply the same extraction method of bacterial DNA from all tested bacterial strains when planned to be submitted to such type of PCR to obtain reproducible results. More additional studies are needed to investigate the behavior of the microsatellite-based PCR carried out on bacteria by testing more different bacterial species, additional DNA extraction methods, and varying PCR conditions.

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