

Short Communication

Comparison of five methods of extraction of *Staphylococcus aureus* DNA for molecular detection by PCR

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Abstract

Introduction: Molecular techniques for the detection of pathogens have been shown to be effective diagnostic tools with high sensitivity and short turnaround times. **Methods:** This study compared five *Staphylococcus aureus* DNA extraction methods for detection by the polymerase chain reaction. **Results:** The concentration and purity of the extracted DNA showed that the methods did not yield DNA of significant quality. However, most protocols yielded 100% positivity, even with low DNA concentrations. **Conclusions:** Although one protocol seemed more efficient than the others, PCR was sensitive enough to allow for detection of *S. aureus* with all the protocols.

Keywords: Polymerase chain reaction. Bacterial DNA. DNA extraction. Nosocomial infection.

Healthcare-associated infections present an overall rate prevalence of 34.5% in patients in intensive care units in the USA¹. This sector experiences a higher rate of healthcare-related infections. Most infections are associated with the use of invasive devices (catheters, bladder catheterization, mechanical ventilation, surgeries, and implants)². Among the most common pathogens that cause these infections within intensive care units, according to the World Health Organization, is *Staphylococcus aureus*, representing more than 20% of isolates².

In most cases, this pathogen is detected using microbiological cultures of samples taken from body fluids or therapeutic devices³. However, this method is not entirely satisfactory, as it is associated with ambiguous results³. This study compared five *S. aureus* deoxyribonucleic acid (DNA) extraction methods for detection by the polymerase chain reaction (PCR).

Strains of *S. aureus* ATCC 25923 were provided by Newprov Ltda (Paraná, Brazil). The strains were cultivated using Brain Heart Infusion medium and kept in a shaker incubator (New Brunswick Scientific, USA) overnight at 37°C. Culture aliquots of 1,000 µL were centrifuged at 10,000rpm for 3 min, and the pellets were frozen at -20°C.

The five conventional DNA extraction protocols⁴⁻⁷ compared here were selected because they are of low cost and easy to use. For each method tested, DNA was extracted in triplicate from independent samples of strands of the strain.

Protocol 1 used a bacterial lysis buffer (Tris-HCl) with Mg²⁺ and Ca²⁺ cations and glucose, and solution II, which contained a mixture of sodium hydroxide and sodium dodecyl sulfate (SDS), followed by purification with phenol:chloroform, as previously described⁴. However, the following adaptation was made for this work: proteinase K was not added, and the sample was freeze-thawed on dry ice and in a 70°C water bath ten times, before purification with phenol and chloroform. The advantages of this method are that it is readily available and its ease of preparation⁴.

Protocol 2 used the GTS™ kit (Phoneutria) in accordance with the manufacturer's instructions and the extraction was subdivided into 2a and 2b. In a, 20µL of Lifton buffer [0.2M sucrose, 0.05M ethylenediaminetetraacetic acid (EDTA), 0.1M Tris, and 0.5% SDS] and 1.2µL of proteinase K (10mg/ml) were added to the pellet. Then, the mixture was incubated at 60°C for at least 90 min (or overnight) to test the effect of the use of these reagents prior to using the kit. While in b, extraction was performed using the GTS kit. This method did not use phenol or chloroform.

Protocol 3 used cetyltrimethylammonium bromide (CTAB), as described in the literature⁵. It used proteinase K (20mg/ml), 10% CTAB, and ribonuclease (RNase) (10mg/ml) to enhance the

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DNA yield, followed by purification with phenol, chloroform, and isoamyl alcohol (25:24:1).

In protocol 4, the pellet was diluted using phosphate-buffered saline (PBS), pH 7.2 (137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, and 1.4mM KH₂PO₄), followed by purification with phenol:chloroform (1:1)⁶.

In protocol 5, the pellet was diluted using NET buffer (50mM NaCl, 125mM EDTA, and 50mM Tris-HCl) and the addition of a denaturing solution, made up of 2.6N NaOH and 24% SDS, in accordance with a protocol described previously⁷.

The DNA concentration was measured using a Qubit® 2.0 Fluorometer (Life Technologies, USA). Using a spectrophotometer (Thermo Scientific NanoDrop™ 2000/2000C, USA), the absorbance at 260nm and 280nm was used to assess the quality and purity of the extracted DNA. High values for both ratios (260/280 > 1.8, 260/230 > 2) are commonly accepted as a good indicator of pure DNA⁸. In contrast, a ratio of less than 1.8 is indicative of protein contamination, while a ratio of greater than 2.0 indicates contamination with ribonucleic acid (RNA)⁶. A low 260/230 ratio may indicate the presence of organic compounds, such as phenolates, thiocyanates, carbohydrates, or salts, in the extract⁸.

Qualitative polymerase chain reaction (PCR) was standardized using a total volume of 25µL, containing 8.5µL of ultrapure water, 12.5µL of pre-mix (buffer IC, Taq DNA, and deoxynucleotides - dNTPs), 1µL of each primer, and 2µL of DNA mold. The forward and reverse sequences of the 16S ribosomal RNA (16S rRNA) gene (5'-GACGGTCTTGCTGTCACCTTA-3' and 5'-AGTTCCAGTGTGGCCGATCA-3', respectively) were used as primers to detect the amplified material (access GenBankPr016589760). The amplified product was approximately 119bp. The reactions were conducted in a thermocycler (Applied Biosystems Veriti® Thermal Cycler, USA), using the following procedure: initial denaturation for 5 min at 94°C, followed by 35 cycles (30 s at 94°C, 30 s at 52°C, and 30 s at 72°C), and final extension (10 min at 72°C). The amplified fragments were then analyzed by electrophoresis using a 1.5% agarose gel, stained with ethidium bromide (0.5µg/mL).

Tests were performed to determine the limit of detection of PCR using serial dilutions of genomic DNA, extracted from 10³

colony-forming units per milliliter (CFU/ml) culture aliquots, plated on brain heart infusion (BHI) agar plates, to reproduce the minimum detectable level of CFUs⁹. In order to determine the sensitivity of the primer to lower DNA concentrations extracted from 10³ CFU/ml, serial dilutions were prepared, and PCR was performed on the titrations using the same bacterial detection parameters.

The initial number of bacterial cells used for extraction with the 100µL culture solution was 4.7 x 10⁷ CFUs for all of the protocols.

The DNA extraction process of conventional protocols can be time consuming, especially given the freeze-thawing cycles and multiple centrifugations, lasting an average of 10 min. The average run-time for each protocol ranged from 2.5h (protocol 2b) to 16.5h (protocol 2a).

The concentration and purity of the extracted DNA reveal that the extraction methods did not obtain significant amounts of DNA. **Table 1** shows the average concentration from three independent extractions with their respective absorbance standard deviations. Only protocol 5 achieved values meeting the ideal purity threshold.

One PCR was performed for each repeating group (A, B, and C for each of the protocols). All protocols yielded 100% positivity rates, except for protocol 4 and protocol 2b, where the rate was 33.3% and 66.6%, respectively (**Figure 1**).

Only protocol 5 was used to extract DNA from culture isolates at a concentration of 10³ CFU/ml, due to the greater DNA concentration and the higher level of purity achieved by this method compared to the other protocols. DNA concentration measurement showed a lower standard deviation than that of the samples at a concentration of 10⁷ CFU/ml, while the purity was below the ideal threshold. The concentrations between 2.1ng/µL and 3.39ng/µL showed purities from 0.85 to 1.67 (absorbance 260/280nm) and 0.18 to 0.44 (absorbance 260/230nm).

Figure 2 shows the PCR results of four extractions of DNA from the culture isolates at a concentration of 10³ CFU/ml using protocol 5. Furthermore, **Figure 2** illustrates the limit of sensitivity of the primer with low concentrations of genetic material.

TABLE 1: Concentration and purity of DNA extracted from *Staphylococcus aureus* (10⁷ CFU/ml).

Extraction protocol	Average concentration of DNA	SD	Purity* 260/280	Purity* 260/230
Protocol 1	3.02ng/µL	2.54	1.34 - 2.09	0.95 - 2.97
Protocol 2a	4.54g/µL	2.14	1.28 - 1.79	0.10 - 0.34
Protocol 2b	2.43 g/µL	0.18	0.80 - 1.71	0.16 - 0.26
Protocol 3	13.10g/µL	9.16	1.42 - 2.14	1.79 - 4.35
Protocol 4	1.36ng/µL	2.19	0.08 - 1.35	0.01 - 1.09
Protocol 5	40.78ng/µL	7.97	1.81 - 1.86	1.74 - 3.80

DNA: deoxyribonucleic acid; **CFU/ml:** colony forming units per milliliter; **SD:** standard deviation. *Minimum and maximum values obtained by the tests.

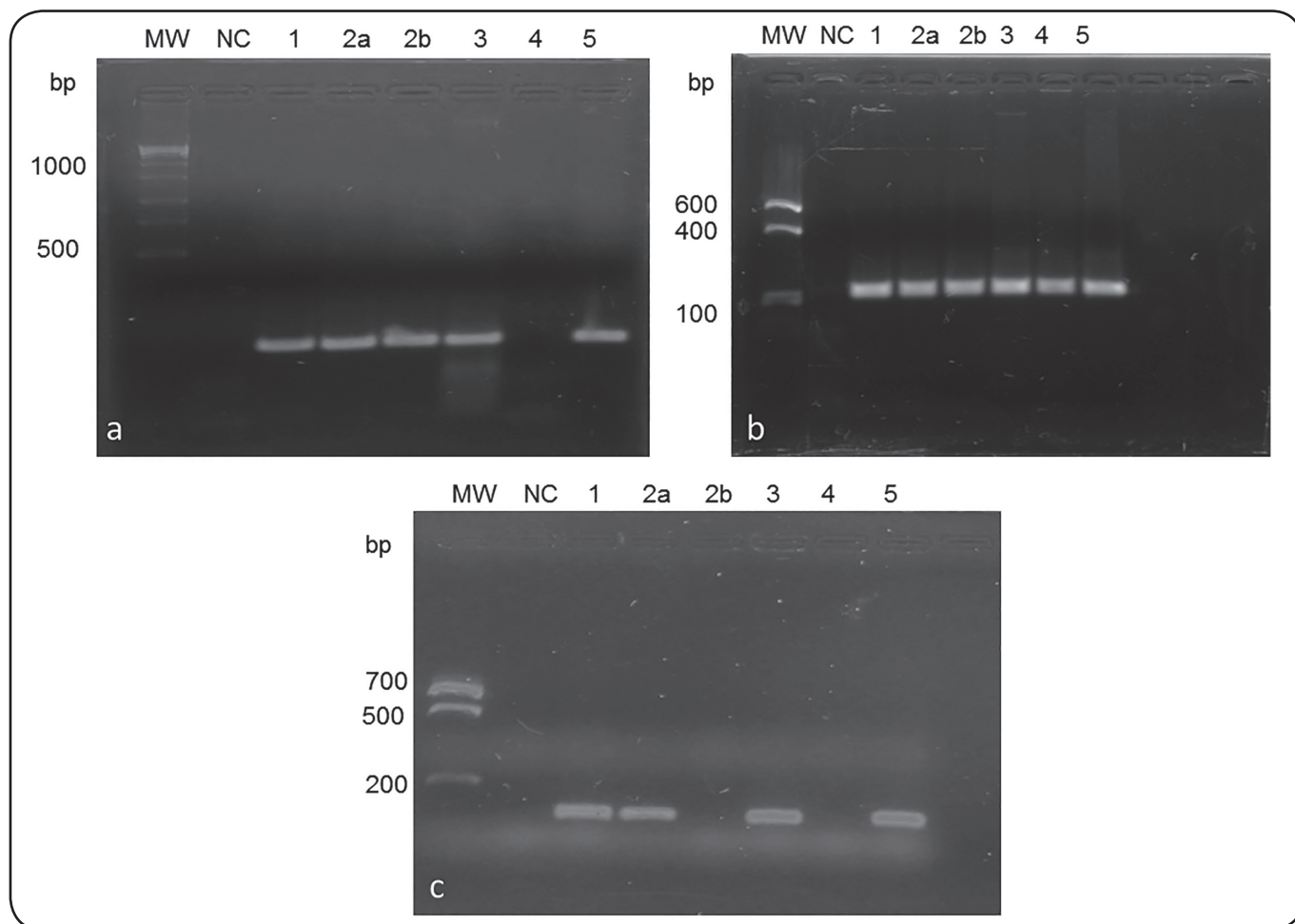


FIGURE 1: Amplification following each of the protocols for the samples at a concentration of 10^7 CFU/ml for each repeating (A): *bp*: base pair; *MW*: molecular weight marker 1,000; *NC*: negative control; 1: protocol 1; 2a: protocol 2a; 2b: protocol 2b; 3: protocol 3; 4: protocol 4; 5: protocol 5. (B): *bp*: base pair; *MW*: molecular weight marker 641; *NC*: negative control; 1: protocol 1; 2a: protocol 2a; 2b: protocol 2b; 3: protocol 3; 4: protocol 4; 5: protocol 5. (C): *bp*: base pair; *MW*: molecular weight marker 752; *NC*: negative control; 1: protocol 1; 2a: protocol 2a; 2b: protocol 2b; 3: protocol 3; 4: protocol 4; 5: protocol 5. *CFU/ml*: colony forming units per milliliter.

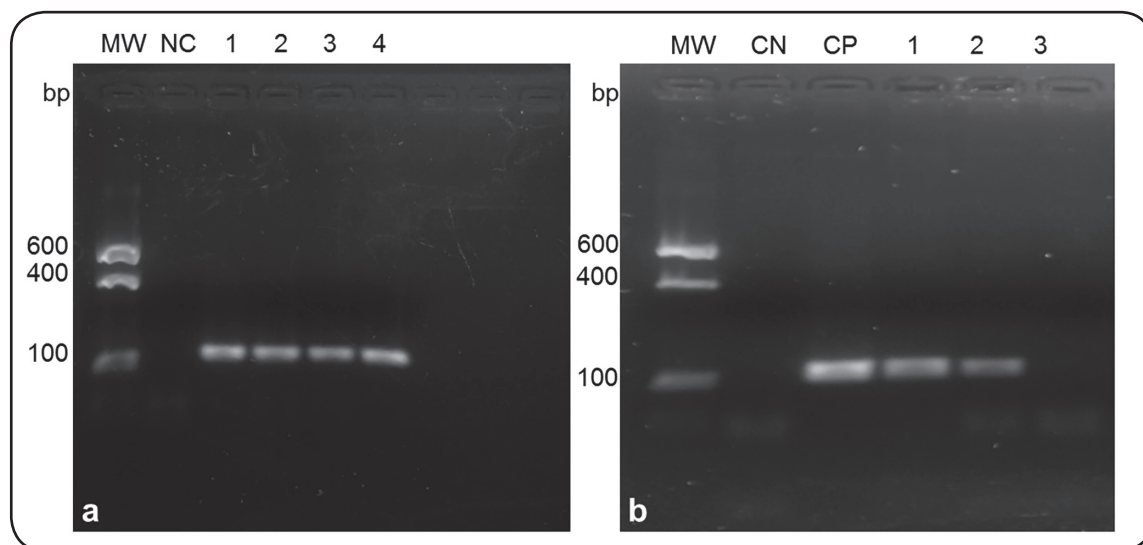


FIGURE 2: Amplification of DNA extracted from the samples at a concentration of 10^3 CFU/ml using protocol 5. A. *bp*: base pair; *MW*: molecular weight marker 641; *NC*: negative control; 1 to 4: four extractions carried out on different days. The limit of sensitivity of the primer with low concentrations of genetic material by serial dilutions. B. : negative control; *PC*: positive control 10^3 ; 1: 10^1 ; 2: 10^2 ; 3: 10^3 . *DNA*: deoxyribonucleic acid; *CFU/ml*: colony forming units per milliliter.

PCR has the potential to become a valuable tool for enhancing the management of patients suspected of having sepsis. Blood culture methods are considered the gold standard for the diagnosis of sepsis. However, they have low sensitivity and their turnaround time for results is between 48 and 72h, while PCR has been shown to provide faster turnaround times and have high specificity¹⁰.

The findings showed that protocol 5 achieved a maximum positivity rate and that the absorbance was within the ideal purity (260 and 280nm). Although the duration of DNA extraction was shorter using protocol 2b, protocol 5 (run-time of 4.5h) was the ideal method for extracting the minimum amount of DNA needed to perform PCR.

Protocol 3 used CTAB, which is commonly used for the extraction of DNA from Gram-positive bacteria^{5,11}. However, despite its low cost, this method is time consuming¹¹. Although this method resulted in a PCR positivity rate of 100% and the second highest concentration of DNA, the purity was lower than expected. As shown by other studies^{5,11} of enzymes, such as proteinase K and RNase, these components does not ensure a high yield of DNA or ideal purity.

The low level of purity of the samples tested using protocol 4 is probably explained by PCR inhibition, due to contaminants, such as proteins and/or residues of the extraction reagents⁸. Another study that used this protocol⁶ with human cell samples observed higher DNA yields and purity.

The cell wall of *S. aureus* consists of a thick peptidoglycan layer that is responsible for its rigidity and for impairing the lysis of the bacterium and the effectiveness of conventional protocols¹¹. Extraction methods should be capable of extracting the highest possible concentration of intact and pure DNA¹². The success of PCR depends largely on ensuring the absence of inhibitory factors¹². Such factors may arise from the sample or could be introduced during one or more of the essential stages of the DNA extraction process: obtaining DNA through cell lysis, during DNA polymerase activity, or during nucleic acid degradation or capture¹². It is important to note that while a given extraction method may work well for one microorganism, it may not be so effective for others¹¹.

Protocol 5 used incubation with NET buffer, a denaturant solution, and heat shock. This procedure may have facilitated cell lysis and DNA purification, thereby leading to a higher DNA concentration and purity⁷.

Although the purity levels of the DNA extracted from the samples at a concentration of 10³ CFU/ml were low, PCR yielded positive results. This is favorable, because the contaminants that were present did not act as PCR inhibitors. A possible explanation for the large difference between the rates observed by this test and those produced by the other protocols is the low number of cells.

The use of proteinase K, Lifton buffer, and the incubation for protocol 2a led to an increase in the DNA concentration, purity, and PCR positivity, showing that these reagents play an important role in cell lysis.

PCR had greater clinical diagnostic accuracy compared to standard blood culture methods for the detection of associated

bacteremia¹³. The present study sought to assess DNA extraction using a concentration of 10³ CFU/ml, with currently accepted diagnostic thresholds for conventional blood culture methods, in order to validate the protocol, based on its capacity to extract DNA at these concentrations and the PCR sensitivity.

Studies using real-time PCR to analyze biofilms formed in central venous catheter removed from intensive care unit patients found that *S. aureus* was the most prevalent bacteria in the catheter tip^{14,15}. Another important point is the contribution that PCR can make to the management of antibiotic therapy.

Its turnaround time enables the rapid start of therapy with the correct medication, thereby positively influencing clinical outcomes and mortality, the incidence of sepsis, and the control of antimicrobial resistance³. Although molecular techniques may be more expensive, they can contribute to reducing mortality and the length of the hospital stay³.

In summary, the protocol developed for the present study is reproducible, and the method uses reagents, inputs, and equipment that are readily available in routine diagnostic laboratories. However, important questions should be explored further, such as the yield and DNA quality.

The main limitation of this study is that only isolated strains were used, and the protocols were not tested on samples taken from catheters from patients. Therefore, it is not possible to confirm whether the extraction method that showed the best performance would suffer from interference of blood cells and other blood products.

Although one protocol seemed more efficient than the others, PCR was sensitive enough to allow for the detection of *S. aureus* in all of the protocols. Furthermore, the primers used were shown to be sensitive for the detection of *S. aureus*, even with low DNA concentrations.

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Conflicts of interest

The authors declare no conflict of interest. The funding sponsors had no role in the design of this study; the analyses, or the interpretation of data.

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