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BRIEF REPORT

Optimized DNA extraction protocol for *Staphylococcus aureus* strains utilizing liquid nitrogen

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DNA extraction;
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Abstract DNA extraction is crucial for conducting procedures, such as whole-genome sequencing, which demand methods that are reproducible and cost-effective. Lysing *Staphylococcus aureus* cells is particularly challenging due to their peptidoglycan layer that is resistant to common treatments. Traditional methods involve costly enzymatic lysis using lysostaphin. Here, we developed a novel approach for lysis utilizing liquid nitrogen and mechanical disruption in a mortar. DNA from *S. aureus* USA300 and related clinical isolates were purified using phenol–chloroform extraction followed by precipitation. The integrity and purity of DNA were confirmed, obtaining suitable concentration and purity for various molecular biology techniques. The quality of the employed DNA was validated by amplifying fragments of different genes using PCR. This method circumvents lysostaphin, yielding DNA that is suitable for use in other techniques.

PALABRAS CLAVE
Extracción de DNA;
Staphylococcus aureus;
Lisis de nitrógeno líquido

Protocolo de extracción de ADN optimizado para cepas de *Staphylococcus aureus* utilizando nitrógeno líquido

Resumen La extracción de ADN es crucial para llevar adelante procedimientos, como la secuenciación de genomas completos, que requieren el empleo de métodos reproducibles y de bajo costo. La lisis de las células de *Staphylococcus aureus* presenta desafíos debido a su capa de peptidoglicano resistente a los tratamientos más comunes. Los métodos tradicionales

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implican una costosa lisis enzimática con lisostafina. Aquí desarrollamos un enfoque novedoso utilizando nitrógeno líquido y disrupción mecánica en mortero para la lisis. El ADN de *S. aureus* USA300 y de aislamientos clínicos de dicha especie se purificó utilizando el método de extracción con fenol-cloroformo seguida de una precipitación. Se confirmaron la integridad y la pureza del ADN, cuya concentración también fue la adecuada para ser empleado en diferentes técnicas de biología molecular. La calidad del ADN se corroboró amplificando fragmentos de diferentes genes mediante la técnica de PCR. Este método evita el uso de lisostafina y permite obtener un ADN de calidad, adecuado para su empleo en diversas técnicas.

DNA extraction is pivotal in high-throughput procedures, such as whole-genome sequencing, to determine not only the complete sequence of a particular organism, but also to investigate point mutations, insertions, or genomic rearrangements[6]. To ensure adequate sensitivity of the molecular biology assay, it is essential that DNA extraction is performed efficiently with optimal reproducibility and, ideally, at a low cost. Furthermore, the DNA samples to be used for sequencing libraries must be pure and unfragmented. The most critical step in a DNA extraction protocol is the lysis stage that depends on the bacterial envelope characteristics. *Staphylococcus aureus* is a Gram positive bacterium with clinical relevance, with a thick, complex and highly cross-linked peptidoglycan layer that is very difficult to disrupt. Different methods have been employed to extract DNA from clinical samples, including enzymatic, chemical, or thermal lysis, mechanical disruption of the cell wall using beads or sonication, or a combination of these approaches[4,5]. Nucleic acid extraction from *S. aureus* is particularly challenging since this bacterium exhibits resistance to commonly used enzymes in enzymatic lysis, such as lysozyme and mutanolysin, arising from an elevated level of O-acetylation in peptidoglycan[1].

The most widespread approach to disrupt *S. aureus* cells involves an enzymatic lysis step using lysostaphin, an enzyme from *Staphylococcus simulans* biovar *staphylolyticus* that cleaves the pentaglycine cross-bridges within the peptidoglycan of *S. aureus*, which is very effective, but highly expensive[2]. In addition, the gold standard method based on lysostaphin may be ineffective against clinical isolates of *S. aureus* that exhibit resistance due to alterations in cell wall cross-bridges, such as truncations of pentaglycine[3]. Here, we present an innovative method using liquid nitrogen to disrupt *S. aureus* strains, the reference strain USA300 FPR3757 and five clinical isolates. After this lysis step, genomic DNA was purified using similar procedures as those used for other Gram positive bacteria based on the phenol–chloroform DNA extraction protocol. With this method, summarized in Figure 1, we obtained substantial amounts of pure DNA at an average concentration of 1413 μg/ml for USA300, which met all purity and integrity criteria to successfully tackle molecular biology assays.

Strains and culture conditions: The reference strain *Staphylococcus aureus* USA300 FPR3757 and clinical isolates were used for this study. A total of 5 clinical isolates employed in this study were obtained from samples of chronic infections in children with a cystic fibrosis diagnosis (Dr. Pedro Elizalde General Children Hospital from Ciudad de Buenos Aires, Argentina, under the ethical committee PRIISA BA: 4466). Initially, a single colony from each strain was subcultured from tryptone soy agar plates into 4 ml tryptone soy broth (Oxoid). The tubes were incubated for 17 h at 37 °C at 200 rpm and were subsequently used for DNA extraction.

Cold mortarization: *S. aureus* cultures were pelleted by centrifugation at 12 000 rpm for 5 min in an Eppendorf tube and the supernatant was discarded. The supernatant must be completely removed without any remaining liquid to ensure efficient cellular disruption. Eppendorf tubes containing the pellet were submerged in 100 ml liquid nitrogen and immediately removed (supplementary material S1). The frozen pellet was subsequently placed in a ceramic mortar, and an additional 10 ml of liquid nitrogen was added. The pellet was carefully crushed by incrementally adding small volumes of liquid nitrogen until achieving a liquid and homogeneous suspension without any traces of aggregates.

Resuspension and incubation: The resulting bacterial lysate was resuspended in 500 μl of buffer (25 mM Tris–HCl pH 7.5, 10 mM EDTA) plus 0.05 mg/ml RNAase (SIGMA) and 0.2 mg/ml proteinase K (SIGMA) and incubated for 30 min at 37 °C.

Addition of sarkosyl: Sodium louroyl sarcosinate (sarkosyl) was added to the mixture to achieve a final concentration of 1% (v/v). The mixture was then incubated overnight at 42 °C with rotation in a tube rotor for protein denaturation and membrane disruption.

DNA extraction with phenol–chloroform: Purification with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) was performed. After vigorous shaking with a vortex and centrifugation at 4 °C at 14 000 × *g* for 15 min, the upper aqueous phase was carefully recovered. Two consecutive extractions with chloroform: isoamyl alcohol (24:1) were performed saving the upper aqueous phase.

Precipitation of nucleic acids: For nucleic acid precipitation, 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol were added to the solution.

DNA recovery: The solution was centrifuged at 14 000 × *g* for 20 min. Subsequently, the resulting pellet was allowed to dry to facilitate ethanol evaporation.

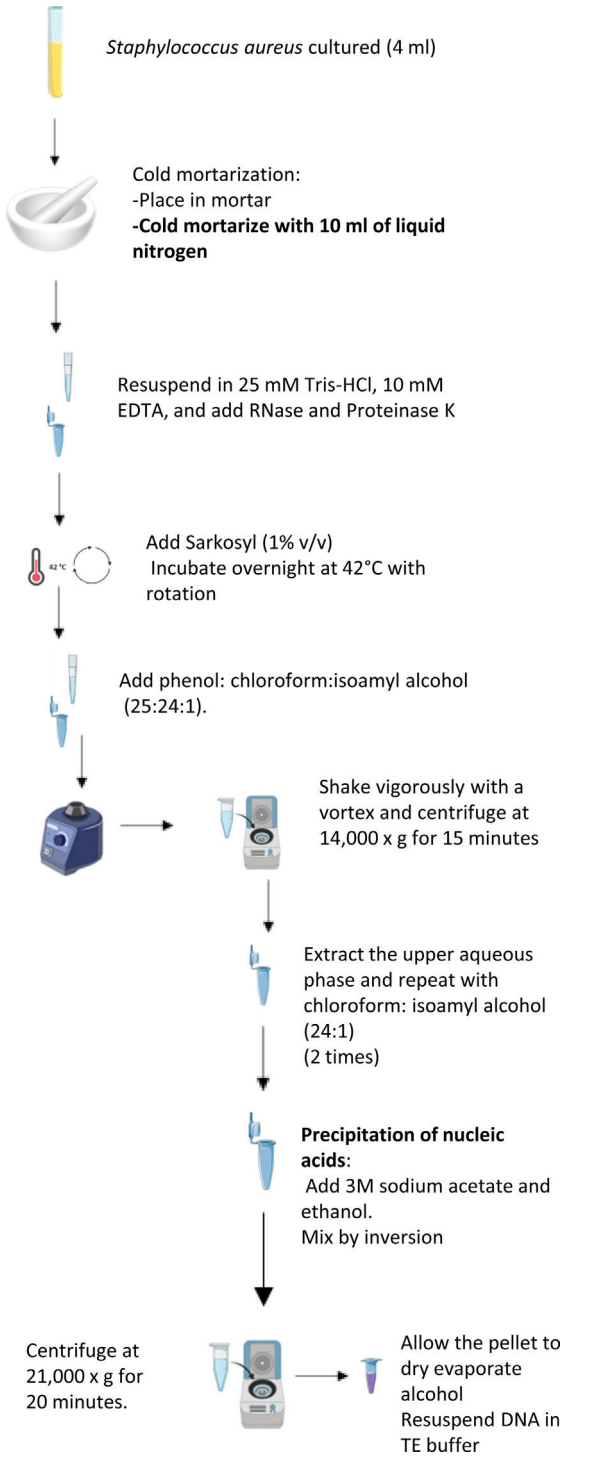

Figure 1 Protocol scheme for *S. aureus* DNA extraction using cold mortarization to disrupt cell wall.

Resuspension of DNA: The DNA pellet was resuspended in 50 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) or an appropriate buffer suitable for sequencing services.

Integrity and purity assessment: DNA integrity was verified by agarose electrophoresis and DNA concentration was assessed using a Nanodrop spectrophotometer (Thermo Scientific NanoDropTM 2000/2000C, USA) and a Qubit Fluorometer T (Invitrogen). Additionally, DNA purity

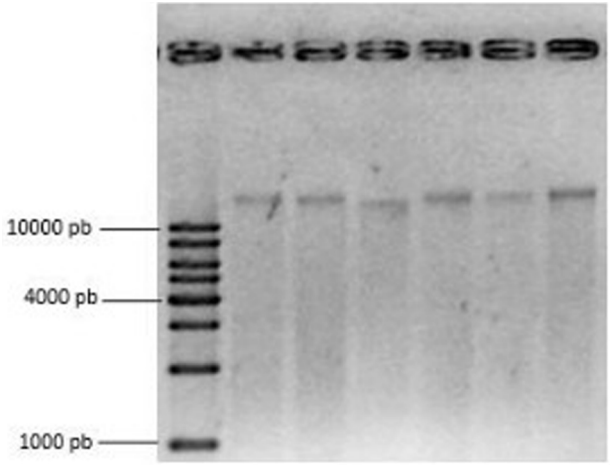

Figure 2 Electrophoretic run of the purified DNA in 1% agarose gel. Gel was loaded with 100 ng of DNA per well. 1: molecular marker (1 kb DNA ladder, TransGen Biotech); 2: USA300; 3: OK; 4: JC; 5: IW; 6: HZ; 7: HL.

was analyzed by using Nandrop to determine the 260/280 and 260/230 absorbance ratios. Values for both ratios near the value of 2 are indicative of high-quality DNA. Conversely, a ratio below 1.8 suggests protein contamination, whereas a ratio exceeding 2.0 indicates the presence of ribonucleic acid (RNA). A low 260/230 value suggests the presence of unwanted organic compounds such as phenolates, carbohydrates, or salts in the DNA extract.

The results of DNA concentration and quality factors 260/280 and 260/230 were as follows: For *S. aureus* USA300: 1413.2 ± 553.8 ng/μl, 1.83 ± 0.07 and 1.98 ± 0.30 and for hospital isolates OK: 4484.7 ± 238.4 ng/μl, 1.85 ± 0.08 and 1.97 ± 0.24; JC: 788.5 ± 253.8 ng/μl, 1.86 ± 0.08 and 1.89 ± 0.04; IW: 289.5 ± 47.2 ng/μl, 1.93 ± 0.09 and 2.26 ± 0.33; HZ: 1141.5 ± 108.2 ng/μl, 2.01 ± 0.03 and 2.07 ± 0.20 and finally HL: 2909.5 ± 154.1 ng/μl, 1.88 ± 0.03 and 2.25 ± 0.16. We analyzed the integrity of the extracted genomic samples by agarose electrophoresis on a 1% agarose gel (Fig. 2). The samples were diluted to a final concentration of 20 ng/μl and a total of 100 ng was loaded into each well. The extracted DNA migrated as a single, high molecular weight band, demonstrating the integrity required for downstream applications.

To verify the quality of the obtained DNA, PCR reactions were performed using Taq Platinum Polymerase to amplify two housekeeping genes, *aroE* and *yqiL*. The following primers were used for the amplification of *aroE* 5′ ATCGAAATCCTATTTCACATTC 3′ and 5′ GGTGTTGTATTAATAACGATATATC 3′ and *yqiL*: 5′ CAGCATACAGGACAGGACACCTATTGGC 3′ and 5′ CGTTGAGGAATCGATACTGGAAC 3′ (Figs. 3A and B). The amplification product of both genes has a molecular weight of 600 bp.

In summary, the protocol proposed in this work allowed us to obtain *Staphylococcus aureus* DNA without using the standard lysostaphin enzyme, leading to cost reduction through the use of liquid nitrogen during the lysis step. In addition to cost savings, this approach also results in high DNA purity and concentration, suitable for performing molecular biology assays effectively.

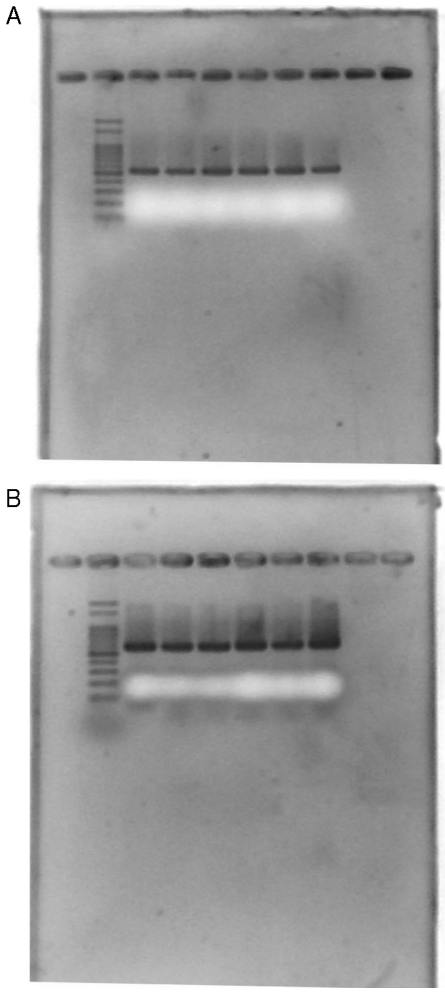

Figure 3 Agarose gel 1%. (A) PCR products of the housekeeping gene *aroE*. (B) PCR products of the housekeeping gene *yqiL*. 1: molecular marker (100 pb ladder, Productos Bio-Lógicos); 2: USA300; 3: OK; 4: JC; 5: IW; 6: HZ; 7: HL.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version available at https://doi.org/10.1016/j.ram.2024.12.006.

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