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OPTIMIZATION OF GRAM POSITIVE BACTERIA DNA EXTRACTION PROTOCOLS AND PURITY AND CONCENTRATION ANALYSIS BY GEL ELECTROPHORESIS

OTIMIZAÇÃO E ANÁLISE DE CONCENTRAÇÃO DE PROTOCOLOS DE EXTRAÇÃO DE DNA EM Staphylococcus aureus

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RESUMO

This work aims to optimize protocols for extraction of bacterial Deoxyribonucleic Acid (DNA) in order to develop more practical, fast and less costly methodologies, therefore bringing improvements to the development of research and applications in laboratory routine based on the difficulty of establishing an ideal method that can extract Gram-positive bacterial DNA with excellent quality. The material and the bacterial strain *Staphylococcus aureus* 15 were provided by the Leão Sampaio University Center, where tests for DNA extraction were also carried out. The tests used were Sodium Dodecyl Sulfate (SDS); *salting-out*; PROMEGA's Kit and CTAB / Phenol / Chloroform and were analyzed using agarose gel electrophoresis. The best results were obtained through the use of PROMEGA's commercial kit, salting-out without the use of Proteinase K and CTAB / Phenol / Chloroform, with salting-out having greater advantages in relation to other methods because it brings speed, low cost, good concentration of extracted material and not be toxic. The molecular tests for the extraction of *Staphyloccocus aureus* DNA were successful in general, when comparing issues such as low cost, extracted concentration, sharpness of the bands in electrophoresis and practicality of the performance. The best method for a small laboratory routine or for future research would be the *salting-out* without proteinase K, thus concluding that it is possible to obtain an excellent DNA extraction with less complexity and low cost methods.

Keyword: Electrophoresis; Molecular biology; Molecular tests.

RESUMO

Este trabalho tem como objetivo otimizar protocolos de extração de Ácido Desoxirribonucleico (DNA) bacteriano no intuito de desenvolver metodologias mais práticas, rápidas e com menor custo, assim trará melhorias para o desenvolvimento de pesquisas e aplicações em rotina laboratorial tendo como base a dificuldade de estabelecer um método ideal que consiga extrair com ótima qualidade o DNA bacteriano em Gram-positivas. O material e a cepa bacteriana *Staphylococcus aureus* foram cedidos pelo Centro Universitário Leão Sampaio, onde também foram realizados os testes para extração de DNA. Os testes utilizados foram Dodecil Sulfato de Sódio (SDS); *salting-out*; Kit PROMEGA e CTAB/Fenol/Clorofórmio e foram analisados por meio de eletroforese em gel de agarose. Os melhores resultados foram mediante uso do kit comercial da PROMEGA, o *salting-out* sem o uso da Proteinase K e o CTAB/Fenol/Clorofórmio, sendo que o *salting-out* apresentou maiores vantagens em relação aos outros métodos por trazer rapidez, baixo custo, boa concentração de material extraído e não ser tóxico. Os testes moleculares para a extração do DNA de *Staphyloccocus aureus* obtiveram êxito no geral, quando comparados questões como baixo custo, concentração extraída, nitidez das bandas na eletroforese e praticidade da realização. O melhor método para uma rotina laboratorial pequena ou para pesquisas futuras seria o *salting-out* sem a proteinase K, assim concluí-se que é possível obter uma ótima extração de DNA com métodos de menor complexidade e baixo custo.

Palavras-chave: Eletroforese; Biologia Molecular; Testes Molecular.

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INTRODUÇÃO

There are countless tests to identify the genus and bacterial species, such as biochemical tests carried out in microbiology laboratories, however the analysis after a molecular method groups more information than just the identification, being able to classify them in subtypes and also map specific characteristics with precision (ROSSEN; FRIEDRICH; MORAN-GILAD, 2018. REBIÈRE, 2015). In molecular tests, the choice of the ideal method is essential for the extraction of Deoxyribonucleic Acid (DNA), which in turn will be the basis for the tests to be carried out with excellence, providing greater chances of obtaining good quality and quantity of DNA (ZHOU et al., 2016).There is a difficulty in establishing manual ideal methods for each sample, so a lot of times the commercial kits are chosen, because they guarantee a greater confiability of good quality and purity for analysis, however, the bigger disadvantage is the high cost of each kit (LIPAY; BIANCO, 2015).

Another difficulty is the purification of the bacterian DNA, in this phase the cellular structure of the organism must be considered, as seen that a Gram-positive, has a thicker cellular wall than a Gram-negative, which brings prejudice to its disruption in the procedures perfomed in some tests, therefore, it becomes necessary the addition of Proteinase K in used methods for a succesful extraction of DNA in a satisfatory quantity (ANGTHONG et al., 2020. TORTORA.; FUNKE; CASO, 2017).

After extraction, the quality and quantity of DNA are evaluated, which can be determined by spectrophotometry or by electrophoresis on an agarose gel followed by reading the intensity of ethidium bromide and / or other commercially available DNA intercalators (REBIÈRE, 2015. MAYJONADE et al. 2016).

The interest of researchers in optmization of methods for DNA extraction keeps growing, searching for practicality, low cost, extractions free of residue such as proteins and without toxicity (ABDEL-LATIF; OSMAN, 2017). Based on the exposed, this scientific work has as goal to optimize bacterian DNA extraction protocols with the intention to obtain practical and quicker methods with lower cost, bringing improvements to the development of researches and applications in laboratory routine, based on the difficulty of establishing an ideal method able to extract the bacterian DNA in Gram-positive with great quality.

MATERIALS AND METHODS

For being practical and efficient, the chosen methods were Sodium Dodecyl Sulfate (SDS), CTAB/Phenol-Chloroform, *salting-out* with and without Proteinase K, this one being double time of incubation and different temperature and also with the Kit Wizard® Genomic DNA Purification from PROMEGA for Gram-positive.

Sample preparation

It has been used bacterias from the *Staphylococcus aureus* 15 standard lineage, sown in rich liquid environment, Brain Heart Infusion (BHI), each tube containing 4ml of environment, which were transferred after a 24 hours period of incubation for eppendorfs. After the transfer, the properly identified samples were centrifuged at 10.000 rpm for 2 minutes, and discarding the supernatant after this phase, bacterias were conditioned in temperature of -20ºc to proceed for extractions.

Salting-out methods

Salting-out without use of Proteinase K for 1h at 60ºC

200µL of TE (Tris HCl 10mM pH 7,6; EDTA 1mM; SDS 0,6%) were added to polypropylene tubes of 1,5 mL containing the *Staphylococcus aureus*15 bacteria and were incubated for 1 hour at 60ºC. After incubation, it were added 35µL of saturared NaCl (5M), manually shaken with vigor. It were centrifuged for 2 minutes at 13.000 rpm. Transferring the supernatant for a new tube previously identified and it was added 400 µL of absolut ethanol, after that the tube was shaken and centrifuged for 10 minutes at 13.000 rpm, the ethanol was carefully discarded and added 1mL of ethanol 70%, reversing the tubes a lot of times to clean the pellet, centrifuged for 10 minutes and despising the supernatant. The washing must be repeated with ethanol 70% one more time and the supernatant despised. The tube dried in ambient temperature for 30 minutes for evaporation of residual ethanol; After this period the DNA was resuspended in 50µL autoclaved Milli-Q water and DNA was stocked at -20 ºC (ABRÃO et al., 2005).

***Salting-out* with Proteinase K for 1 hour at 60ºC**

200µL of TE (Tris HCl 10mM pH 7,6; EDTA 1mM; SDS 0,6%) were added to polypropylene tubes of 1,5 mL containing the *Staphylococcus aureus*15 and 5 µL de proteinase K (10mg/mL) bacteria and were incubated for 1 hour at 60ºC. After incubation, it were added 35µL of saturated NaCl (5M), manually shaken with vigor. It were centrifuged for 2 minutes at 13.000 rpm. Transferring the supernatant for a new tube previously identified and it was added 400 µL of absolut etanol, after that the tube was shaken and centrifuged for 10 minutes at 13.000 rpm, the ethanol was carefully discarded and added 1mL of etanol 70%, reversing the tubes a lot of times to clean the pellet, centrifuged for 10 minutes and despising the supernatant. The washing must be repeated with etanol 70% one more time and the supernatant despised. The tube dried in ambient temperature for 30 minutes for evaporation of residual etanol; After this period the DNA was resuspended in 50µL autoclaved Milli-Q water and DNA was stocked at -20 ºC (ABRÃO et al., 2005).

***Salting-out* with Proteinase K for 2h at 42ºC**

200µL of TE (Tris HCl 10mM pH 7,6; EDTA 1mM; SDS 0,6%) were added to polypropylene tubes of 1,5 mL containing the *Staphylococcus aureus*15 and 5 µL de proteinase K (10mg/mL) bacteria and were incubated for 2 hours at 45ºC. After incubation, it were added 35µL of saturated NaCl (5M), manually shaken with vigor. It were centrifuged for 2 minutes at 13.000 rpm. Transferring the supernatant for a new tube previously identified and it was added 400 µL of absolut etanol, after that the tube was shaken and centrifuged for 10 minutes at 13.000 rpm, the ethanol was carefully discarded and added 1mL of etanol 70%, reversing the tubes a lot of times to clean the pellet, centrifuged for 10 minutes and despising the supernatant. The washing must be repeated with etanol 70% one more time and the supernatant despised. The tube dried in ambient temperature for 30 minutes for evaporation of residual etanol; After this period the DNA was resuspended in 50µL autoclaved Milli-Q water and DNA was stocked at -20 ºC (ABRÃO et al., 2005).

Method – Dodecyl Sodium Sulfate (SDS)

It were added 300 µL of TE (Tris HCl 10mM pH 7,6; EDTA 1mM) to the polypropylene tube containing the Staphylococcus aureus15 bacteria, and then left to rest for 20 minutes. After that, it was centrifuged for 2 minutes at 10.000 rpm and disposing the supernatant. The precipitaded lysis buffer (Tris-HCl 400mM e EDTA ,50 mM, NaCl 500mM, SDS 1%) was resuspended. After that it was vigorously shaken in a vortex for 15 seconds and centrifuged for 5 minutes at 10.000 rpm. Transferring the supernatant (watery phase) to a new identified tube and disposing the tube with the precipitate. 1 mL of concentrate cold Ethanol was added, shaken slowly by inversion. Centrifuged for 5 minutes at 10.000 rpm again and disposing the supernatant by inversion. 700 µL of cold Ethanol 70%, centrifuged for 5 minutes at 10.000 rpm. Disposing the supernatant by inversion, the inversed tube was placed over absorbing paper to dry the pellet for about 30 minutes. 50µL of ultrapure H20 was added to the pellet-m then the sample was stocked at -20Cº (LIMA JÚNIOR et al., 2017).

The WIZARD® Genomic DNA Purification kit from the PROMEGA for Positive Gram methods

1 ml of bacterial culture was centrifuged for 2 minutes at 13.000 rpm, disposing the supernatant and the tube with *Staphylococcus aureus* 15 in 480µL of EDTA was resuspended. Adding 600µL of Nuclei Lysis Solution and 10 µL of Proteinase K. It was resuspended pipetting it gently. Incubated at 80Cº for 5 minutes to smooth the bacterial wall. Incubates. After the ambient temperature went cold, 3µL of RNAse solution was added to the cell's lysate. Inversing the tubes from 2 to 5 times to mix and then incubated at 37ºC for 15-60 minutes. The sample was colded to ambient temperature, 200µL of protein precipitation solution was added to the cell's lysate. Shaken vigorously in a vortex for 20 seconds to mix the solution with the cell's lysate. Incubating the sample on ice for 5 minutes and then centrifugating it for 3 minutes at 13.000 rpm. Transferring the supernatant containing the DNA to new microtube containing 600µL of isopropanol to ambient temperature. It will be kindly mixed by inversion until a thread similar to DNA is observed. It was centrifuged for 2 minutes at 13.000 rpm. Disposing the supernatant and adding 600µL of Ethanol to ambient temperature, kindly inversing the tube a lot of times to clean the pellet. It was centrifuged for 2 minutes at 13.000 rpm. Carefully disposing the ethanol; The tube must be drained in clean absorbent paper and leave the inversed tube to rest for 10-15 minutes for the pellet to dry. It was added to the pellet 50µL of ultrapure H20 and the sample stocked at -20ºC (PROMEGA, 2019).

CTAB/Phenol-Chloroform methods

In a polypropylen microtube 250µL of TE were added, after the artificial contamination, it was added 10µL of sodium chloride (NaCl) and 100µL of Cetyltrimethylammonium Bromide (CTAB) preheated at 65ºC. Agitating the suspension for ten seconds until milky aspect is obtained, with posterior incubation at 65 ºC for ten minutes. After this period it were added 750µL phenol and 750µL chloroform-isoamylic-alcohol (24:1), agitating for ten seconds with soft movements and centrifuged for six minutes at 15.000 rpm; the supernatant was transferred to another polypropylen tube and added 200µL of chloroform-isoamylic-alcohol 24:1, centrifuged for 6 minutes at 15.000 rpm; The supernatant was transferred to a new tube and 1\10 of sodium acetate 3M and 3X the volume of ETOH 100% were added, slowly homogenizing and then put in overnight at -20 ºC; After those phases the supernatant was transferred to a new tube and 450µL of absolut cold ethanol was added to it, it was incubated during 10 minutes at -20 ºC. After that, centrifuged for twenty minutes at 15.000 rpm, the alcohol was disposed and 500µL of etanol 70% in ambient temperature was added, following the same centrifugation procedure previously quoted. Disposing the supernatant and getting the pellet dry at 56 ºC in dry bath. After the pellet was completely dry, it was added 50µL of dilution buffer (10 mM Tris-HCL, 1 mM EDTA) pH 8,0 or ultrapure water. The DNA must be kept in ambient temperature for twenty minutes and posteriorly stocked at -20 ºC (OLIVEIRA et al., 2006).

ELECTROPHORESIS ANALYSIS IN AGAROSE GEL

For analysis of the genetic material extracted during the previously performed methods, electrophoresis in agarose gel was performed, so the differences can be visually observed for each extraction.

Gel preparation

0,3g of agarose was weighted; transferring the agarose to a recipient containing 30ml of the TAE plug and then diluted; it was placed in a heating plate and heated until the gel was clear, then it was left in ambient temperature and transferred to the electrophoresis chamber, the clip was added to form places for DNA placement and then waited for solidification.

Electrophoresis procedure

It was mixed 8 µL from sample with 5 µL from the racing plug (Blues Green Dye I (Ludwig Biotechnology) + intercalators of nucleic acids) and softly homogenizing with the pipette; The plug TAE 1X was placed in the vat until it covered the parse's vat; it was placed on the vat containing the gel and the clip removed; it was carefully applied to the sample so it wouldnt leak out from the formed well; With the vat closed the position of the electrodes identified by color was verified; the power supply was turned on and the current and voltage was adjusted. After the race, the power supply was shut down, the electrodes were removed and the gel carefully taken out, which was placed in the UV light transilluminator and the results were observed and compared with Ladder DNA 1Kb (Ludwig Biotecnology). This method was performed using the EMBRAPA protocols accordingly (EMBRAPA, 1998. EMBRAPA, 2007).

RESULTS

As seen in Figure 1, the genomic material extracted from the *Staphylococcus aureus* bacteria lineage 15, resulted in crisp and good quality bands in general, although some methods stood out in terms of concentration of DNA extracted with PROMEGA Kit, *salting-out* without PK 1hour at 60°C e Cetyltrimethylammonium Bromide (CTAB) with Phenol-Chloroform, between the ones that stand out the most in their concentration, the *salting-out* method is the one that is not as expensive as the PROMEGA Kit and also it does not have as much toxicity as CTAB/Phenol-Chloroform, being the better method choice when it comes to low cost and non-toxicity.

The Table 1, shows quantifications of extracted material, comparisons of DNA ladder which is a genomic sample, were made through electrophoresis, which were calculated according to the quantity of sample introduced in gel.

Figure 1. Gutters M= DNA Ladder 1 Kb; 1= method SDS; 2= method Salting out without PK 1h a 60°C; 3= method Salting out with PK 1h at 60°C; 4= method Salting out with PK 2h a 42°C; 5= Kit Promega; 6= method CTAB/Phenol-Chloroform

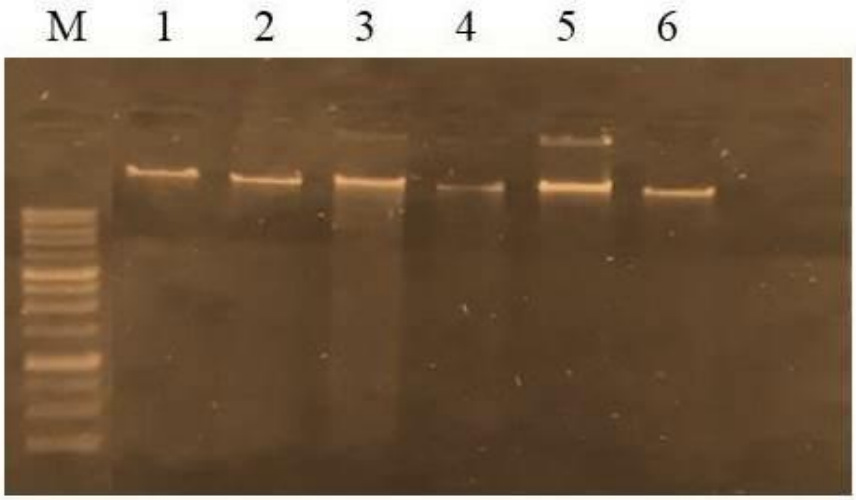

Table 1. Concentrations obtained through comparative analysis with DNA ladder.

Methods	ng/8µL	ng/µL
SDS	44,8	5,6
Salting-out with PK 1h à 60ºC	44,8	5,6
Salting-out without PK 1h à 60ºC	72,0	9,0
Salting-out with PK 2h à 42ºC	28,8	3,6
Kit PROMEGA	147,2	18,4
CTAB/Phenol-Chloroform	72,0	9,0

DISCUSSION

The best test with relation to accuracy extraction as expected was the PROMEGA Kit, because this commercial test has shown great results related to genetic material extraction, still brings the disadvantage of being amongst all the one with higher cost. According to Lipay and Bianco (2015), commercial kits for DNA extraction show with higher frequency better quality and purity, than the manual methods, but the bigger disadvantage with wearing kits, it's the cost of the test.The Dodecyl Sodium Sulfate method (SDS) was the most practical and fastest method performed, it was shown that is possible to have a good result even using a simple method with few constituents in its step by step, for not using enzymes or another high cost reagents.

According to Baratto and Megiolaro (2012), even though the SDS method had better quantitative performance to positive Gram, it was not efficient in qualitatives terms, so it was made necessary to add Proteinase K to the SDS method, the analysis confirmed great purity and great quantity of genetic material extracted. Also was noticed that for the ideal DNA extraction in Gram-positive it would be necessary to add

proteinase K, for cell lysis once that Gram-positive bacteria have a thick wall cell, which makes the extraction harder.

Salting-out without Proteinase K for 1 hour at 60°C was the best method of *salting-out*, because this test had a short time of realization and without the use of proteinase K, which left it cheaper, and besides that, in electrophoresis the band showed itself without residues and traces. The *salting-out* method with the use of proteinase K with temperature of 1 hour at 60ºC was quick like the period of time from the equivalent test to SDS, with low increasement in cost due to enzymes use, in the bands this test has shown itself with traces of DNA, with relation to its quality, this extraction showed traces meaning residues. The last test realized with *salting-out*, this time with change of temperature to 42ºC for 2 hours still using proteinase K, between the three methods using *salting-out*, it was the one who had a lower concentration of extracted DNA, it was concluded that this test besides being extended for one hour was not effective.

Currently, researchers are looking for DNA extraction methods, which must be fast, economical, free from residual contamination and toxicity. It was possible to observe the efficiency of saline solution (NaCl) as a precipitant, which had the same results as the DNA samples extracted with Phenol / Chloroform, with no differences in the result. The use of NaCl allows the DNA to be free of toxic reagents, such as Phenol and Chloroform, also because it is cheaper and more compatible with the laboratory reality (RIVERO et al., 2006). The results of this research corroborate with Shokrzadeh and Mohammadpour (2018), who previously showed that saline can be an alternative method for replacing Phenol and Chloroform.

CTBA / Phenol-Chloroform was the test that resulted in the same concentration of the *salting-out* without proteinase K for an hour at 60º C, it is a slightly more expensive method than salting, as it has two more substances to be purchased, Phenol and Chloroform. This method does have a good extraction, but it has a significant disadvantage because it carries toxicity risks, it is often not used. According to Monteiro, Montanhini and Bittencourt (2014) & Shetty, Ghosh and Paul (2017) between the two extraction methods used in their research, CTAB and thermal lysis, it was proven that the best method of extraction was by thermal lysis, this method proved to be effective for certain Gram-positive bacteria, which has a thick layer of glycan peptide resulting in better extraction in terms of quantity and purity. However, Andreatti Filho et al. (2011) also used the same methods to extract the DNA of a certain Gram-negative bacterium in their research and analyzed that between two tests, both extractions had satisfactory quantity, quality and purity of genetic material, CTAB was the one that showed high sensitivity and quality with good clarity in the bands visualized.

The tests for extracting DNA from Staphyloccocus aureus were succesfull, being possible to identify three methods as the best ones when it comes to concentrated extraction, they are the PROMEGA Kit's method, *salting-out* without Proteinase K and CTAB/Phenol/Chloroform. When compared to matters of lower cost, good extracted concentration, sharpness in electrophoresis bands, practicity in realization and lower toxicity, the best method for a small laboratory routine or for future researches would be *salting-out* without proteinase K.

CONCLUSION

So its concluded that it is possible to obtain a great DNA extraction with methods of lower complexity. In future researches it will be tested or should be tested in new temperatures and different times for phases of resting in the water, because it might lead to variations in results and generate a new procedure that might improve the quality and quantity of extracted DNA.

Conflict of interest declaration

The authors declare that there was no conflict of interest in this study. This study contains no research involving animals or humans.

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