Rev Soc Bras Med Trop 51(4):528-532, July-August, 2018
doi: 10.1590/0037-8682-0352-2017

Short Communication

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

***Maristela Oliveira Lara*[1], *Thabata Coaglio Lucas*[1], *Evanguedes kalapothakis*[2], *Ronaldo Luis Thomasini*[3] *and Carla Jorge Machado*[4]**

[1]. Departamento de Enfermagem, Universidade Federal dos Vales do Jequitinhonha e Mucuri, Diamantina, Minas Gerais, Brasil.
[2]. Laboratório de Biotecnologia e Marcadores Moleculares, Departamento de Biologia Geral, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brasil.
[3]. Faculdade de Medicina, Universidade Federal dos Vales do Jequitinhonha e Mucuri, Diamantina, Minas Gerais, Brasil.
[4]. Programa de Pós-Graduação *Stricto Sensu* em Saúde Pública, Departamento de Medicina Social e Preventiva, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brasil.

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 USA[1]. 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 isolates[2].

In most cases, this pathogen is detected using microbiological cultures of samples taken from body fluids or therapeutic devices[3]. However, this method is not entirely satisfactory, as it is associated with ambiguous results[3]. 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.

***Corresponding author*:** Dra. Maristela Oliveira Lara.
e-mail: maryslara@hotmail.com
Received 3 September 2017
Accepted 1 March 2018

The five conventional DNA extraction protocols[4-7] 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 Mg^{2+} and Ca^{2+} 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 described[4]. 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 70ºC 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 preparation[4].

Protocol 2 used the GTS™ kit (Phoneutria) in accordance with the manufacturer's instructions and the extraction was subdivided into 2*a* and 2*b*. 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 literature[5]. It used proteinase K (20mg/ml), 10% CTAB, and ribonuclease (RNase) (10mg/ml) to enhance the

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 Na_2HPO_4, and 1.4mM KH_2PO_4), 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 previously[7].

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 DNA[8]. 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 extract[8].

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'-GACGGTCTTGCTGTCACTTA-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 10^3 colony-forming units per milliliter (CFU/ml) culture aliquots, plated on brain heart infusion (BHI) agar plates, to reproduce the minimum detectable level of CFUs[9]. In order to determine the sensitivity of the primer to lower DNA concentrations extracted from 10^3 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 10^7 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 10^3 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 10^7 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 10^3 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* (10^7 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.

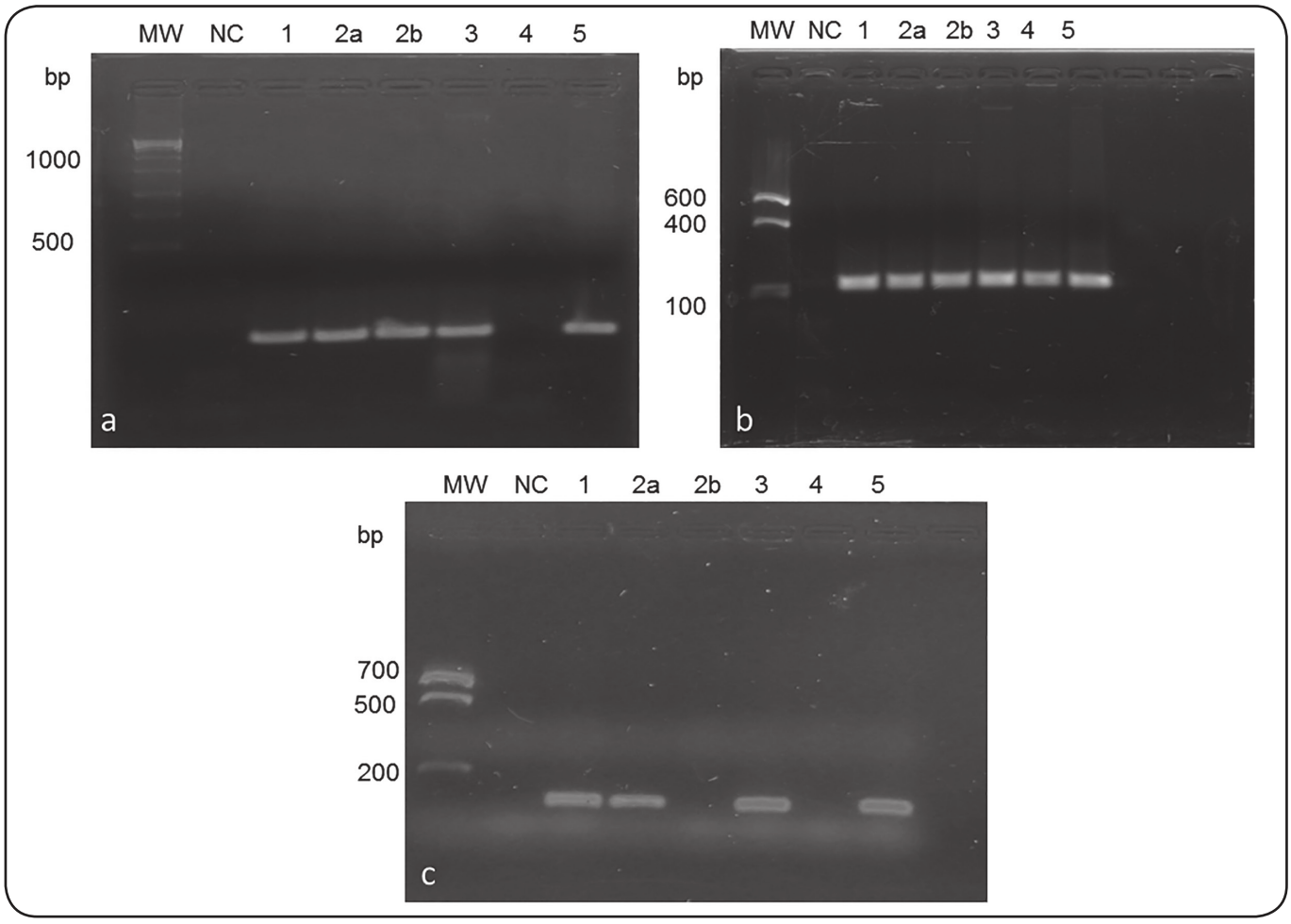

FIGURE 1: Amplification following each of the protocols for the samples at a concentration of 10^7 CFU/ml for each repeating **(A): *bp****: base pair;* **MW**: molecular weight marker 1,000; **NC**: negative control; **1**: protocol 1; **2a**: protocol 2a; **2b:** protocol 2b; **3:** protocol 3; **4:**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

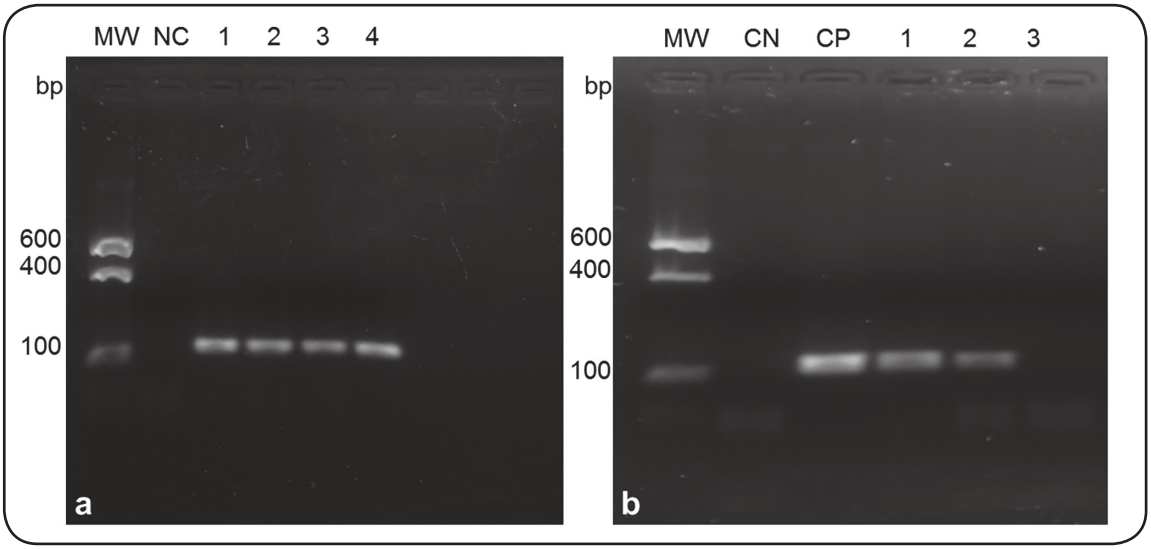

FIGURE 2: Amplification of DNA extracted from the samples at a concentration of 10^3 CFU/ml using protocol 5. **A. *bp****: base pair;* **MW:** molecular weight marker 641; **NC:** negative control; **1** to **4**: four extractions carried out on different days. The limit of sensitivity of the primer 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 specificity[10].

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 consuming[11]. 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 reagents[8]. Another study that used this protocol[6] 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 protocols[11]. Extraction methods should be capable of extracting the highest possible concentration of intact and pure DNA[12]. The success of PCR depends largely on ensuring the absence of inhibitory factors[12]. 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 capture[12]. It is important to note that while a given extraction method may work well for one microorganism, it may not be so effective for others[11].

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 purity[7].

Although the purity levels of the DNA extracted from the samples at a concentration of 10^3 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 bacteremia[13]. The present study sought to assess DNA extraction using a concentration of 10^3 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 resistance[3]. Although molecular techniques may be more expensive, they can contribute to reducing mortality and the length of the hospital stay[3].

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.

Acknowledgements

We are grateful for the Biotechnology and Molecular Markers Laboratory of the Institute of Biological Sciences at the Federal University of Minas Gerais for support and collaboration.

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.

Financial support

This study was supported by *Fundação de Amparo à Pesquisa de Minas Gerais* (FAPEMIG), Project Number APQ-01862-15.

REFERENCES

1. Magill SS, Edwards JR, Bamberg W, Beldavs ZG, Dumyati G, Kainer MA, et al. Multistate point-prevalence survey of health care–associated infections. N Engl J Med. 2014;370(13):1198-208.
2. World Health Organization (WHO). Report on the burden of endemic health care-associated infection worldwide. Switzerland: WHO; 2011. 40p.

3. Riedel S, Carroll KC. Early identification and treatment of pathogens in sepsis: molecular diagnostics and antibiotic choice. Clin Chest Med. 2016;37(2):191-207.

4. Herrmann BG, Frischauf AM. Isolation of genomic DNA. Methods Enzymol. 1987;152:180-3.

5. Minas K, McEwan NR, Newbold CJ, Scott KP. Optimization of a high-throughput CTAB-based protocol for the extraction of qPCR-grade DNA from rumen fluid, plant and bacterial pure cultures. FEMS Microbiology Lett. 2011;325(2):162-9.

6. Ghatak S, Muthukumaran RB, Nachimuthu SK. A simple method of genomic DNA extraction from human samples for PCR-RFLP analysis. J Biomol Tech. 2013;24(4):224-31.

7. Romero C, Lopez-Goñi I. Improved Method for Purification of Bacterial DNA from Bovine Milk for Detection of Brucella spp. by PCR. Appl Environ Microbiol. 1999;65(8):3735-7.

8. Morin N, Vallaeys T, Hendrickx L, Natalie L, Wilmotte A. An efficient DNA isolation protocol for filamentous cyanobacteria of the genus Arthrospira. J Microbiol Methods. 2010;80(2):148-54.

9. Brito CS, Ribas RM, Resende DS, Brito DV, Abdallah VO, Santos KR, et al. Genotypic study documents divergence in the pathogenesis of bloodstream infection related central venous catheters in neonates. Braz J Infect Dis. 2014;18(4):387-93.

10. Furtado I, Xavier PCN, Tavares LVM, Alves F, Martins SF, Martins AS, et al. *Enterococcus faecium* and *Enterococcus faecalis* in blood of newborns with suspected nosocomial infection. Rev Inst Med Trop São Paulo. 2014;56(1):77-80.

11. Willner D, Daly J, Whiley D, Grimwood K, Wainwright CE, Hugenholtz P. Comparison of DNA Extraction methods for microbial community profiling with an application to pediatric bronchoalveolar lavage samples. PLoS One. 2012;7(4):e-34605.

12. Schrader C, Schielke A, Ellerbroek L, Johne R. PCR inhibitors – occurrence, properties and removal. J Appl Microbiol. 2012;113(5):1014-26.

13. Al Wohoush I, Rivera J, Cairo J, Hachem R, Raad I. Comparing clinical and microbiological methods for the diagnosis of true bacteraemia among patients with multiple blood cultures positive for coagulase-negative staphylococci. Clin Microbiol Infect. 2011;17(4):569-71.

14. Pozzi C, Waters EM, Rudkin JK, Schaeffer CR, Lohan AJ, Tong P, et al. Methicillin resistance alters the biofilm phenotype and attenuates virulence in *Staphylococcus aureus* device-associated infections. PLoS Pathog. 2012;8(4):1-15.

15. Guembe M, Marín M, Martín-Rabadán P, Echenagusia A, Camúñez F, Rodríguez-Rosales G, et al. Use of universal 16S rRNA gene PCR as a diagnostic tool for venous access port- related bloodstream infections. J Clin Microbiol. 2013;51(3):799-804.