Detection of Microorganisms in Clinical Sonicated Orthopedic Devices Using Conventional Culture and qPCR

Detecção de microrganismos em dispositivos ortopédicos sonicados clínicos usando cultura convencional e qPCR

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Rev Bras Ortop 2022;57(4):689–696.

Abstract

Objective To evaluate the sensitivity and specificity of the quantitative real-time polymerase chain reaction (qPCR) for *16S rDNA* gene screening using sonicated fluid from orthopedic implants.

Methods A retrospective study was conducted on 73 sonicated fluids obtained from patients with infection associated with orthopedic implants. The samples were subjected to conventional culture and molecular testing using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and qPCR for *16S rDNA*. The cycle threshold values were used to define a cut-off of the qPCR of the *16S rDNA* for negative and positive cultures.

Results No statistical differences were observed between the positive and negative culture groups based on the time from the first surgery to infection ($p = 0.958$), age ($p = 0.269$), or general comorbidities. Nevertheless, a statistical difference was found between the mean duration of antibiotic use before device removal (3.41 versus 0.94; $p = 0.016$). Bacterial DNA was identified in every sample from the sonicated fluids. The median cycle thresholds of the positive and negative cultures were of 25.6 and 27.3 respectively ($p < 0.001$). As a diagnostic tool, a cycle threshold cut-off of 26.89 demonstrated an area under the curve of the receiver operating characteristic of 0.877 ($p \leq 0.001$).

Keywords
- sonication
- infections
- qPCR
- spectrometry, mass, matrix-assisted laser desorption-ionization
- prostheses and implants

received
September 11, 2020
accepted
February 19, 2021
published online
October 1, 2021

DOI https://doi.org/10.1055/s-0041-1732386.
ISSN 0102-3616.

Thieme Revinter Publicações Ltda., Rua do Matoso 170, Rio de Janeiro, RJ, CEP 20270-135, Brazil

Conclusion The presence of antimicrobial agents for more than 72 hours decreased culture positivity, but did not influence the qPCR results. Despite this, amplification of the *16S rDNA* may overestimate infection diagnosis.

Resumo

Objetivo Avaliar a sensibilidade e a especificidade da reação em cadeia de polimerase em tempo real quantitativa (*quantitative real-time polymerase chain reaction*, qPCR, em inglês) para a triagem do gene *rDNA 16S*, com a utilização do fluido sonicado de implantes ortopédicos.

Métodos Um estudo retrospectivo foi realizado em 73 fluidos sonicados obtidos de pacientes com infecção associada aos implantes ortopédicos. As amostras foram submetidas a cultura convencional e a teste molecular utilizando ionização e dessorção a laser assistida por matriz com espectrometria de massa por tempo de voo (*matrix-assisted laser desorption/ionization time-of-flight mass spectrometry*, MALDI-TOF MS, em inglês) e qPCR para o *gene rDNA 16S*. Os valores limiares do ciclo foram usados para definir um ponto de corte para a qPCR do gene *rDNA 16S* para culturas negativas e positivas.

Resultados Não foram observadas diferenças estatísticas entre os grupos de cultura positiva e negativa com base no tempo desde a primeira cirurgia até a infecção ($p = 0{,}958$), na idade ($p = 0{,}269$), ou nas comorbidades em geral. No entanto, uma diferença estatística foi encontrada entre a duração média do uso de antibióticos antes da remoção do dispositivo (3,41 *versus* 0,94; $p = 0{,}016$). O DNA bacteriano foi identificado em todas as amostras dos fluidos sonicados. Os limiares do ciclo médio de culturas positivas e negativas foram de 25,6 e 27,3, respectivamente ($p < 0{,}001$). Como uma ferramenta de diagnóstico, um corte do limite do ciclo de 26,89 demonstrou uma área sob a curva da característica de operação do receptor de 0,877 ($p \leq 0{,}001$).

Conclusão A presença de agentes antimicrobianos por mais de 72 horas diminuiu a positividade da cultura, mas não influenciou os resultados da qPCR. Apesar disso, a amplificação do *rDNA 16S* pode sobrestimar o diagnóstico de infecção.

Palavras-chave
- sonicação
- infecções
- qPCR
- espectrometria de massas por ionização e dessorção a laser assistida por matriz
- próteses e implantes

Introduction

Orthopedic implant-associated infections (OIAIs) and periprosthetic joint infections (PJIs) are associated with high morbidity, mortality, and costs.[1] Biofilm-associated microorganisms are the main etiological agents of OIAIs and PJIs, including *Staphylococcus* spp., *Pseudomonas aeruginosa*, and some species of *Enterobacterales*.[2,3] The structure of biofilms develops after an initial attachment of microorganisms to a substratum, wherein the microorganisms adhere irreversibly to the surface and produce extracellular polymers, forming a structural matrix that plays an essential role in the pathogenesis of OIAIs and PJIs.[4] Biofilm formation is not only prevalent in prosthetic devices; it also occurs in bone and/or bone cement, synovial fluid, and fibrous tissue.[5]

The accurate diagnosis and early identification of infectious agents are vital for a successful treatment. Multiple cultures of the periimplant tissue are the gold standard for microbial detection in OIAI and PJI.[6,7] However, this method has low sensitivity, with only 62% of detection of the infectious bacteria,[6,8] and requires at least 24 hours until the microbial growth can be assessed.[9] Additionally, conventional culture is associated with false-negative results in low-grade infections or in patients undergoing antimicrobial treatment.[10] However, implant sonication, which dislodges the biofilm from the device, increases the culture sensitivity when compared with periimplant tissue biopsy or culture.[1]

Modern techniques, such as molecular testing, have redefined the methods of microbiological investigation. Several techniques have been described for the molecular examination of sonicated fluids, with the aim of improving the diagnostic sensitivity or detection of periprosthetic infection.[11–15] For instance, the polymerase chain reaction (PCR), broad-range 16S ribosomal DNA (rDNA) PCR, or multiplex PCR, offer significant advantages in the detection of active as well as non-viable microorganisms (even in cases in which antibiotics were administered before

sampling).[16] However, the results can be controversial due to DNA contamination (while detecting mixed infections) when using broad-range PCR, but they are less controversial with multiplex PCR.[17] In addition, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been used in various settings for the direct detection of biological samples for the early and reliable identification of the microorganisms, as an alternative to culture.[18–20]

The aim of the present study was to evaluate the sensitivity and specificity of the quantitative real-time polymerase chain reaction (qPCR) to screen the *16S rDNA* gene from the sonicated fluid samples obtained from orthopedic implants.

Methods

Setting

The present was a single-center, retrospective study following the implementation of a sonication method after orthopedic surgery. The study evaluated a period of 12 months (from December 2018 to December 2019) in a tertiary care, medical, surgical, and academic teaching hospital with a capacity of 206 beds. The hospital is a referral center for trauma patients, admitting approximately 1,100 inpatients, with 4,800 patient-days per month.

Patients and Devices

Different types of orthopedic devices were obtained under surgical conditions after medical recommendation (suspicion of infection). Infection criteria met the definitions of the International Consensus Group on Periprosthetic Joint and of the International Consensus Meeting on musculoskeletal infection.[21,22] Patients with external fixation devices were excluded from the study. Clinical data were evaluated for a group analysis.

Sonication of the Orthopedic Devices

All explanted devices were placed into sterile and nuclease-free polyethylene sampling bags with a removable seal and a wire closure system (Labplas, Sainte-Julie, Quebec, Canada), and immediately sent for sonication. The sonication was performed in a 0.9% NaCl solution in an amount sufficient to cover the device; the solution was sonicated for 5 min in an ultrasonic bath using a Soniclean 15 (Sanders Medical, Santa Rita da Sapucaí, MG, Brazil) at a frequency of approximately 40 kHz and 35°C.[1] One aliquot was used for microbiological tests, and 50-mL aliquots were stored at -20°C for molecular tests. In total, 39 samples with bacterial growth (detected by conventional culture) and 34 samples without bacterial growth were used.

Laboratory Tests

For the conventional culture, 100 µL of the sonicated fluid were spread onto tryptic soy agar plates supplemented with 5% sheep blood and MacConkey agar (Laborclin, Pinhais, PR, Brazil), and incubated for 5 days at 35°C. The anaerobic culture was performed for 14 days in a standard anaerobic medium (Bactec, BD, Franklin Lakes, NJ, US). Colony growth was evaluated using a direct detection protocol with MALDI-TOF MS.

Direct detection of microorganisms using MALDI-TOF MS was performed on the Vitek MS equipment (bioMérièux, Durham, NC, US). The sample-extraction process was adapted from a previously-described protocol.[23] Briefly, 4 mL of the sonication fluid were centrifuged at 367 × g for 5 minutes, and the pellet obtained was washed with deionized water. The pellet was resuspended in 50 µL of deionized water, followed by the addition of 900 µL of absolute alcohol. After vortexing, the tube was centrifuged at 18,000 × g for 2 minutes, and the supernatant was discarded. A total of 50 µL of formic acid (70% v/v) and 50 µL of acetonitrile were added to the pellet. After vortexing, the tube was centrifuged at 18,000 × g for 2 minutes. Then, 1 µL of the supernatant was spotted directly onto the target plate. After drying, each inoculum was covered with 1 µL of the alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (bioMérièux). After drying, the samples were analyzed on the VITEK MS system. Quality control was performed using a reference strain of *Escherichia coli ATCC 8739*. All procedures were performed in duplicate.

Microbial genomic DNA (gDNA) was detected by performing qPCR for the *16S rDNA* gene screening (broad-range qPCR). Microbial DNA was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, US) according to the manufacturer's instructions, using 1 mL of the sonication fluid, and 50 µL of DNA were extracted. For the molecular detection of *16S rDNA*, the TaqMan Universal PCR Master Mix (Applied Biosystems, Inc., Foster City, CA, US) was used; the detection was adapted from a previously-described protocol,[24] using forward and reverse primers, and a probe with the following sequences: *5'-TGGAGCATGTGGTTTAATTCGA-3'*, *5'-TGCGGGACTTAACCCAACA-3'*, and *(CY5)-5'-CACGAGCTGACGACARCCATGCA-3'-(BHQ_2)*.[25] The reaction was performed in triplicate for each sample, using 12.5 µL of the TaqMan Universal PCR Master Mix, 8.7 µL of ultrapure water, 0.6 µL of each primer (forward and reverse; 20 mM), 0.6 µL of probe (10 mM), and 2 µL of DNA, with a total volume of 25 µL *per* well. Furthermore, no template controls (NTC, using water instead of DNA) and positive controls were included, and the reactions were run on the ABI-7500 Fast real-time PCR instrument (Applied Biosystems, Inc.) using the following steps: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute.

Standard curves using Gram-negative bacterium, *Pseudomonas aeruginosa ATCC 27853* (Laborclin), and Gram-positive bacterium, *Staphylococcus aureus ATCC 25923* (Laborclin) were generated for *16S rDNA* to determine the efficiency and analytical sensitivity of the assay. Briefly, the saline solution was inoculated with *S. aureus* and *P.*

aeruginosa at progressive dilutions, from 10^8 to 10^2 CFU/mL (performed in triplicate), to determine the standard curve. The cycle threshold (Ct) values were used to calculate the performance of *16S rDNA* qPCR. The last three concentrations detected in the standard curve ($10^2 \pm 1$) were amplified with 30 repetitions to define the limit of detection (LOD), which must amplify 100% of molecular targets to ensure minimal detection with a 95% confidence interval (95%CI). Dilutions were made before each experiment. The standard curve was plotted from the $Cq \times \log_{10}$ plot of target gene concentration/reaction based on the determination of the copy number of the *16S* gene in *S. aureus* and *P. aeruginosa* strains. After the linear regression of the obtained points, R^2 and the equation of a straight line ($y = mx + n$) were determined using the following equation:

$$Cq = \text{slope x log (n)} + \text{y-intercept},$$

in which: **Cq** = cycle of quantification;
slope = angular coefficient of the line;
log (n) = logarithmic base 10 of the gene copies per reaction; and
y-intercept = linear coefficient.

Statistical Analysis

The continuous variables were expressed as means with standard deviations (SDs), and they were analyzed using the Student *t*-test. The categorical variables were expressed as absolute frequencies and proportions, and they were analyzed using the Chi-squared or Fisher tests. The sensitivity, specificity, and positive and negative predictive values were calculated using the culture as a reference (culture-positive infection versus Culture-negative infection). The Ct was determined to improve the accuracy of the PCR using the cultures as the gold standard. The area under the receiver operating characteristic (ROC) curve was calculated to quantify the discriminative ability of the qPCR. The statistical significance was set at $p < 0.05$. The Statistical Package for the Social Sciences (SPSS, IBM Corp., Armonk, NY, US) software was used for the statistical analysis.

Results

General Characteristics

In total, 148 sonicated fluids were collected. The clinical characteristics were present in 132 samples (52 positive cultures; 80 negative cultures). However, 59 stored samples could not be recovered for the qPCR. Thus, 73 sonicated fluids were included in the final analysis.

The median age was 54 years (range: 39 to 64 years) and 68.4% of patients (n = 50) were male. The median time between the first surgical procedure and infection was of 220 days (range: 30.5 to 962 days). The sonicated implants consisted of parts of the prosthesis (hip and knee) and fixation devices (screws, plates, wires, and pins). The main comorbidities were arterial hypertension (n=30; 41%), trauma (n=27; 36.9%), and diabetes mellitus (n=11; 15%). Before samples were collected, 30 patients (41%) received antimicrobial therapy. The main clinical characteristics of the patients are listed in ►**Table 1**.

Cultures and Etiologies

Using the conventional culture method, 39 samples (53.5%) were found positive. Infections due to *Staphylococcus* spp. were found in 64% (n = 25) samples. Of these, 68% (n = 17) were due to methicillin-susceptible *S. aureus* (MSSA), and 28% (n = 7) were due to methicillin-resistant *S. aureus* (MRSA). Gram-negative bacilli (GNBs) were present in 25.6% (n = 10) of the samples, mainly nonfermenting GNBs, such as *P. aeruginosa* and *Acinetobacter baumannii* (n = 4). Mixed infections (polymicrobial) were found in 10% (n = 4) of the cases. The major etiologies are listed in ►**Table 2**.

There were no statistical differences between the positive and negative groups based on the time from the first surgery to infection ($p = 0.958$), age ($p = 0.269$), or general comorbidities. Nevertheless, a statistical difference was found between the mean duration of the antibiotic intake before device removal (3.41 versus 0.94; $p = 0.016$) (►**Table 1**).

16S rDNA qPCR

Bacterial DNA was identified in all samples from sonicated fluids, regardless of the culture results. The median Ct values of the positive and negative cultures was of 25.6 and 27.3 respectively ($p < 0.001$), and those of *S. aureus* and GNBs were of 25.07 ± 2.97 and 23.53 ± 3.31 respectively ($p = 0.123$). As a diagnostic tool, a Ct cut-off of 26.89 demonstrated an area under the curve (AUC) of the ROC of 0.877 ($p \leq 0.001$) (►**Fig. 1**). The Ct cut-off values are listed in ►**Table 3**.

In general, we observed that samples from patients who received antimicrobial therapy for more than 3 days before the surgical procedure were more likely to result in negative cultures ($p = 0.016$). However, the *16S rDNA* qPCR was positive in all samples, even in patients with negative culture results. Further, the use of the Ct cut-off of 26.89 as a diagnostic tool demonstrated an AUC of 0.877 ($p \leq 0.001$).

Discussion

Despite the progress made in the diagnosis of infections associated with orthopedic implants, tissue culture remains the gold-standard tool. Therefore, the standard criteria to diagnose PJI are closely related to the type and number of samples collected. Although cultures from pus may present higher sensitivity than that of other samples, no single tissue sample is reliable regarding the PJI criteria.[26] Thus, multiple cultures are traditionally needed to achieve a higher sensitivity. Additionally, culture positivity is directly influenced by the growth medium used. Samples inoculated directly in blood culture during the surgical procedure yielded results

Table 1 Clinical and laboratory characteristics of patients with infection and negative or positive cultures of sonicated orthopedic devices

	Negative culture			Positive culture			
	n = 34			n = 39			
	Mean or N	Standard deviation or %	Median	Mean or N	Standard deviation or %	Median	*p*-value
Cycle threshold	27.22	0.89	27.35	24.51	3.14	25.64	< 0.001
Time from surgery to infection (days)	1,649	7,355	126	2,715	9,158	240	0.958
Age (years)	55.82	18.58	57.50	51.12	17.37	48.00	0.269
Duration of the antibiotic therapy before implant removal (days)	3.41	5.96	1.00	0.94	1.65	1.00	0.016
Sonicated device							
Hip prosthesis	15	44%		9	23%		
Knee prosthesis	3	9%		5	13%		
Shoulder prosthesis	0	0%		1	3%		
Plate and screws	4	12%		9	23%		
Only screws	8	24%		9	23%		
Only plate	3	9%		6	15%		
Wires	1	3%		0	0%		
Male gender	23	68%		27	69%		0.542
Trauma	13	38%		14	36%		0.514
Smoking	4	12%		6	15%		0.460
HIV	0	0%		0	0%		–
Diabetes mellitus	5	15%		6	15%		0.274
Chronic renal failure	1	3%		0	0%		–
Hearth failure	2	6%		0	0%		–
Peripheral vascular disease	1	3%		3	8%		0.361
Previous stroke	2	6%		3	8%		0.566
Chronic pulmonary disease	1	3%		0	0%		–
Arterial hypertension	15	44%		13	33%		0.241
Neoplasm	0	0%		1	3%		–
Liver diseases	1	3%		0	0%		–
Antibiotic therapy prior material sampling	16	47%		14	36%		0.233

within 48 to 72 hours, with a sensitivity comparable to that of the conventional methods.[27,28] However, even with improved sample collection, inoculation, and processing methods such as sonication, the presence of antimicrobial agents at the surgical site for more than three days before the procedure reduces culture positivity.

Given the difficulties associated with the clinical management of PJI, cultures may not always be obtained in the absence of antibiotics. Thus, molecular tools, such as the PCR, may be advantageous compared to traditional culture techniques, once DNA can be amplified during the early clinical treatment phase.[16] However, depending on the molecular technique, specificity may decrease due to contamination.[17] In the present study, all samples were positive for *16S rDNA*. Once this technique amplifies any bacterial gene, it is probable that it will present low specificity when used indiscriminately and broadly used. However, when advanced molecular tests, such as 16S rRNA or next-generation sequencing, are used according to clinical and laboratory criteria, they may present benefits.[29,30] Interestingly, compared to traditional methods, 16S rRNA failed to identify polymicrobial infection.[30] Therefore, given the complexities

Table 2 Etiology of positive cultures from sonicated orthopedic devices from patients with infection

Microorganism		N
Staphylococcus spp.		25
	Methicillin-susceptible *S. aureus*	17
	Methicillin-resistant *S. aureus*	7
	S. epidermidis	1
Gram-negative bacilli		10
	Enterobacter spp.	2
	Morganella morgannii	1
	Proteus spp.	2
	Pseudomonas aeruginosa	2
	Serratia marcescens	1
	Acinetobacter baumannii	2
Mixed		4
	Enterobacter spp. + *S. aureus*	1
	Enterococcus spp. + *S. aureus*	1
	Klebsiella spp. + *S. pyogenes*	1
	Serratia spp. + *P. aeruginosa*	1

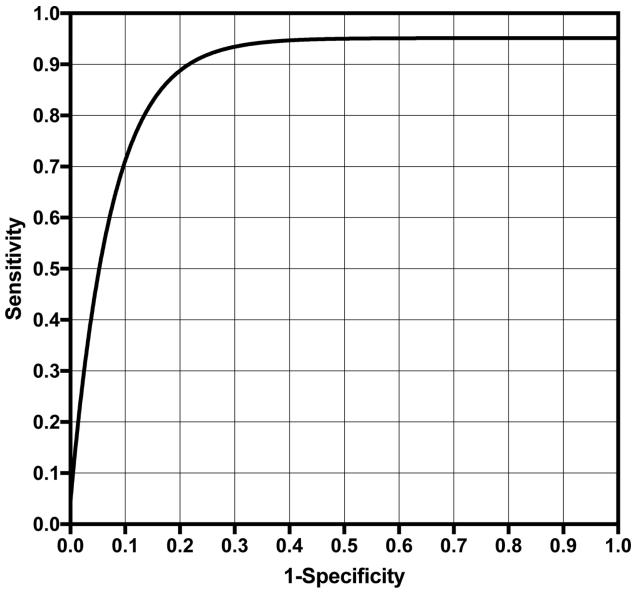

Fig. 1 Receiver operating characteristic curve of the cycle threshold of 26.9 to separate positive and negative cultures from the sonicated fluid of orthopedic devices.

that arise during the evaluation of orthopedic infections (such as misdiagnosed infections and contaminations), a combination of techniques is important to reach a final diagnosis.[31]

Table 3 Positive predictive value (PPV), negative predictive value (NPV), sensitivity, specificity, and accuracy of different cycle threshold (Ct) values, considering culture positive infections versus culture negative infections

Ct value	26.25	26.89	27.17	27.45
Sensitivity	62%	79%	90%	97%
Specificity	94%	85%	68%	47%
PPV	92%	86%	76%	68%
NPV	68%	78%	85%	94%
Accuracy	77%	82%	79%	74%

Tunney et al.[11] stated that the incidence of prosthetic joint infection is grossly underestimated by current culture-detection methods, and that molecular tests should be included in the routine. Despite the recommendation, Ryu et al.[15] confirmed that PCR presents a low sensitivity, but high specificity. Thus, for the etiological diagnosis, we can conclude that PCR may not be the ideal test, but a negative test excludes the presence of infection. In contrast, Gomez et al.[13] reported that PCR is equivalent to culture. We believe that these inconsistent results may be associated with the in-house method used for the qPCR. Unfortunately, the methods used in each study cannot be compared directly.

Improving diagnostic tools is necessary to establish the correct treatment and decrease therapy failure. Once *S. aureus*, mainly MRSA, has been associated with poor prognosis in PJI,[32] choosing the correct antimicrobial therapy (such as those with anti-biofilm properties) may be considered a treatment cornerstone. Future studies should explore the applicability of molecular tools in patients at a higher risk of treatment failure (such as those with immunosuppression) with a negative culture, despite clinical and laboratory results suggesting the presence of an infection.

The present study has some limitations. First, given the retrospective design, the "suspicion of infection" may have been overestimated. Second, after two to three years, the clinical outcomes were not evaluated to establish the significance of *16S rDNA* positivity in patients with negative cultures. Still, the present study highlights the importance of adequate sample collection and the role of specialized laboratory techniques performed by an infectious disease expert. The *16S rDNA* qPCR cannot identify the species; it is only used to identify the presence or absence of bacterial DNA. The test should be complemented with gene sequencing to identify the species.

Conclusion

In conclusion, the presence of antimicrobial agents for more than 72 hours decreased culture positivity without influencing qPCR results. Despite this, *16S rDNA* amplification may overestimate

the diagnosis infection. Further studies are warranted to evaluate the role of *16S rDNA* qPCR in the diagnosis of PJI.

Financial Support
This study was supported by a grant of the MCTIC/CNPq, number 28/2018, level B.

Conflict of Interests
Felipe Tuon is a CNPq researcher.

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