Use of Dithiothreitol to Improve the Diagnosis of Prosthetic Joint Infections

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ABSTRACT: Diagnosis of prosthetic joint infections (PJIs) remains a challenge for microbiologists, despite new techniques for bacteria isolation have been developed in recent years. A widely recognized standard method has not yet been indicated mainly because of limitations due difficult procedures and need of dedicated instrumentation. We evaluated the ability of a sulfhydryl compound routinely used in microbiology laboratories, dithiothreitol (DTT), to dislodge bacteria from biofilm, keeping them alive and cultivable for identification and antibiotic susceptibility testing. We compared DTT treatment against sonication of prosthesis and culture of periprosthetic tissues, in order to establish if it could be introduced in routine microbiological diagnosis of PJIs. The study was conducted on 76 patients, 34 with aseptic loosening of their prosthesis and 42 who were diagnosed for PJI. DTT treatment gave results similar to sonication in terms of bacterial yielding. Sonication provided higher sensitivity (71.4%) and specificity (94.1%) respect to periprosthetic tissue culture, while DTT showed the same specificity of sonication but a better sensitivity (85.7%), especially when the causative microorganism was *Staphylococcus epidermidis*. In conclusion, we demonstrated that DTT could be used for PJIs diagnosis, thanks to its ease of use and its high sensitivity and specificity. © 2013 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 31:1694–1699, 2013

Keywords: prosthetic joint infections; dithiothreitol; sonication; bacterial biofilm

Infections of prosthetic joints (PJIs) or non-biological implants are an important part of acute and chronic infections in many hospitals and are related with complication for patients, such as prolonged hospitalization and often additional surgery, associated with an increased risk of complications and long antimicrobial treatment.[1,2] Moreover, treatment of an infected prosthesis requires considerable costs for the healthcare system.[3] For these reasons a prompt diagnosis and the implementation of antibiotic therapy, targeted against the organisms responsible for infection, could improve the patient's prognosis, although surgery is often required to clean up.[4] Unfortunately, PJIs are often difficult to diagnose because a lot of factors such as sampling errors, inappropriate transport of samples, inadequate quantities of vital bacteria retrieved and previous use of antibiotics can affect bacterial isolation, thus causing a high number of negative cultures.[1,5] Another issue regarding conventional culture techniques using swabs or periprosthetic tissue cultures is the high risk of contamination during handling. For this reason, it is generally accepted that two or more positive cultures are needed to define a microorganism as an infecting one.[6,7]

Moreover, a wide range of bacteria responsible for PJIs is able to produce biofilm on prosthetic implants. Biofilm is a complex microbial community protected by self-produced polymeric matrix and adherent to various surfaces.[8] Its formation notably hinders sampling and culturing, because traditional sampling techniques are not able to detach biofilm-embedded bacteria from prosthetic surfaces, thus leading to negative results.[9] In order to improve the diagnosis of PJIs, several techniques for detection of biofilm-related infections have been developed in recent years.[10–13] Nowadays, sonication of explanted prosthetic materials is widely recognized as the most advantageous treatment for detachment and isolation of microorganisms responsible for biofilm-related prosthetic infections.[14] The ultrasonic treatment of the explanted material provides a good recovery of microorganisms with a relatively simple procedure, allowing greater sensitivity when compared to periprosthetic tissue or swabs cultures.[15] However, some limitations of this methodology have been recently highlighted such as the necessity of dedicated laboratory tools and a considerable risk of contamination, due to possible damages or inaccurate sealing of sample's containers, size of explanted prostheses, and bacteria proliferation in sonication water.[7] To avoid contamination, in fact, a continuous intervention for cleaning and disinfecting the sonication bath is needed, as well as an accurate inspection of each sample container.[15] This study evaluated the use of DL–dithiothreitol (DTT), a sulfhydryl compound (empirical formula $C_4H_{10}O_2S_2$, MW 154.2) which is routinely used in clinical microbiology for liquefying specimens from the respiratory tract, for the treatment of explanted orthopedic joint prostheses, in order to assess its sensitivity and specificity in detecting bacteria from biofilm infections, comparing results to those from sonication and periprosthetic tissue cultures. Sulfhydryl compounds are well known for their ability to reduce disulphide bounds between

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polysaccharides and neighboring proteins and to interfere with biofilm formation.[16–18] Therefore, we hypothesized that DTT can alter the extracellular matrix of biofilm and free bacteria from it, permitting their retrieval and cultivation with traditional methods, as previously shown by us in an vitro model of bacterial biofilm on titanium and polyethylene surfaces.[19] Moreover, DTT is characterized by low toxicity (LD_{50} in rat: 400 mg/kg), easy to use, since it does not require special precautions, relatively low costs, also considering that the working solution contains small quantities of reagent (0.1% w/v). For all these reasons we proposed a new, cheap, simple and reliable method for the diagnosis of PJIs.

METHODS

The study was performed on prosthetic joint material explanted from 80 patients undergoing removal of the prosthetic joint or cement spacer at the Unit of Reconstructive Surgery of bone and joint Infections (C.R.I.O. Unit) at Galeazzi Orthopaedic Institute in Milan. Patients, whose characteristics are summarized in Table 1, were divided in two groups: group A ($n = 34$) presenting an aseptic loosening and group B ($n = 42$) with clinical signs of infection of the prosthesis. Patients in group B reported aching joint, swelling, redness and altered biochemical analysis with values of erythrocytes sedimentation rate (ESR) >40 mm/h and C-reactive protein (CRP) >1.00 mg/dl all suggestive for PJIs.[1] Diagnosis of infection was confirmed according to the criteria set forth by Spangehl et al.[20] at least three positive results for ESR, CRP and aspiration, frozen section, or intraoperative cultures.

Periprosthetic tissue samples ($n = 5–8$) and prosthetic implants were aseptically collected in the operating room where removed implants were aseptically divided into two parts and put in two different plastic sterile containers which were sealed and transported to the laboratory. Implants with suspicion to have been contaminated during surgery or received in not-accurately sealed containers or misidentified were excluded from the study.

Prosthetic implant parts were alternatively treated with sonication or DTT; for example, if the femoral portion of a knee prosthesis was sonicated and the tibial portion treated with DTT, the subsequent one was sonicated in the tibial portion and treated with DTT in the femoral portion. The same alternation was applied for titanium and plastic prosthetic components.

One part of the prosthesis was processed by sonication as routinely performed in our laboratory, as previously described.[14] Briefly, the container was filled with sterile saline until complete submersion of the device, carefully sealed and sonicated in an ultrasound bath (VWR, Milan, Italy) for 5 min with a frequency of 30 kHz and a power output of 300 W at room temperature. The other part was immersed in a solution of 0.1% w/v DTT (Sigma-Aldrich, Milan, Italy) in phosphate-buffered saline and mechanically stirred for 15 min at room temperature (Fig. 1). Stirring time and DTT concentration were previously determined from in vitro tests in order to assess the right proportion between time and DTT concentration. At the end of sonication and DTT treatment, the obtained fluids were accurately mixed, collected in sterile 50 ml tubes and centrifuged at 3,000 rpm for 10 min and resuspended in a volume of about 1.5 ml.

Swabs and 150 μl of sonicated and DTT-treated samples were seeded onto Chocolate Agar (CA), Columbia Blood Agar (CBA), Schaedler Blood Agar (SBA), Brain Heart Infusion broth, and Thioglycollate Broth. CA and CBA were incubated at 37°C for 24 h in 10% CO_2 enriched atmosphere, while SBA was incubated in anaerobiosis at 37°C for 48 h. Enrichment broths were incubated for 15 days at 37°C. The remaining fluid from sonicated and DTT-treated samples (about 1 ml) was frozen at −80°C for further analyses.

After incubation, growth and colonies counts were recorded for both aerobes and anaerobes, while at the 15th day each broth was vortexed for 30 s and 150 μl were inoculated on CA and SBA plates.

All the isolates were assessed for biofilm production by means of a spectrophotometric method as previously described.[21]

Sonicated or DTT-treated devices were considered positive if at least five colonies grew on agar plates after 24 h or if a growth was observed during broth enrichment. Periprosthetic tissue cultures were considered as positive if at least three samples yielded microbial growth. Identification was performed at phenotypic (API system and Vitek2 Compact, Biomerieux, Marcy l'Etoile, France) and genotypic level. Phenotypical identification was confirmed by DNA sequencing of about 80 pb of variable regions V1 and V3 of the 16S rRNA gene by Pyrosequencing (PSQ96RA, Diatech, Jesi, Italy).[22] Sequences of the primers used are shown in Table 2. Obtained sequences were inserted in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to perform accurate identification. In addition, a broad-range PCR amplification of bacterial 16S rRNA gene was performed on previously frozen fluids as previously described.[23] Bacterial DNA was extracted using Qiagen DNA mini kit (Qiagen, Inc., Valencia, CA) accordingly to the manufacturer's protocol. PCR was performed on specific regions of 16S rRNA gene, using the RotorGene 3000 (Corbett Research, Cambridgeshire, UK) with the same set of primers used for the genotypic identification described above. After agarose gel electrophoresis, the presence of a band corresponding to the expected molecular weight was considered indicative of presence of bacteria and the amplification products were identified by means of pyrosequencing, as described above. The whole analytical process is shown in Figure 2. Comparison of results obtained from sonication and DTT methods was performed by means of McNemar's test of paired proportions. Differences were considered t-test significant when p-values were equal or less than 0.05.

Table 1. Characteristics of Patients

Patients Characteristics	Group A	Group B
Number of patients	34	42
Sex (female/male)	22/12	25/19
Mean age (range), years	68 (40–83)	71 (50–90)
Mean ESR (range), mm	29 (10–39)	84 (42–120)
Mean CRP (range), mg/dl	0.37 (0.07–0.99)	6.44 (1.22–28.2)

RESULTS

A total of 456 periprosthetic tissue samples and 160 prosthetic devices were received from 80 patients. Samples from four patients were excluded from the study because they were not correctly identified ($n = 1$) or containers were not correctly sealed ($n = 3$).

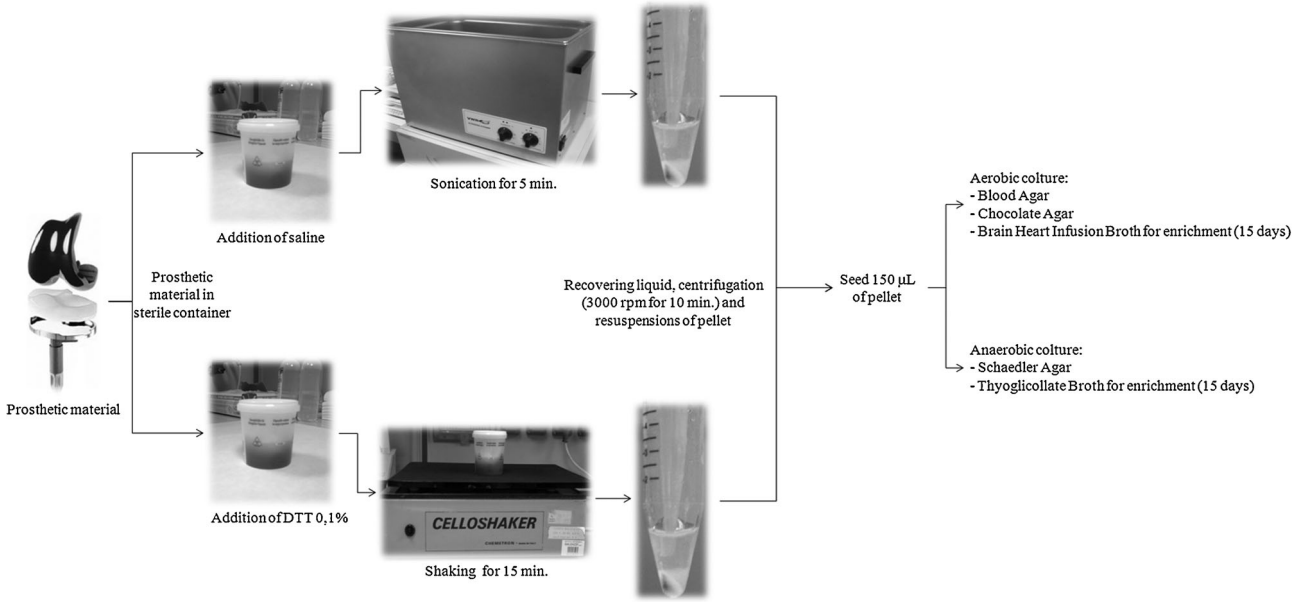

Figure 1. The analytical process.

Therefore, samples from 76 patients were included into the study. Explanted prostheses were equally distributed within the two groups for composition and size.

As shown in Table 3, significant bacterial growth was observed in 32 sonicated prostheses, 38 DTT-treated prostheses, and 38 periprosthetic tissue cultures. Concordance among all methods was observed in 48 out of 76 cases (63.2%). Sonication and DTT treatment yielded concordant results in 92.1% of patients, while the lowest rate of concordance (65.8%) was observed when results obtained after sonication and tissue cultures were compared.

In group B, periprosthetic tissue cultures, sonicated, and DTT-treated devices gave concordant results in 24/42 patients (22 positive and 2 negative). In the same group, microbial growth was obtained from DTT-treated and sonicated devices in eight patients whose periprosthetic tissue cultures resulted negative. For four patients of group B sonicated devices yielded negative cultures while microbial growth was observed for DTT-treated samples and periprosthetic tissues. In two patients, bacterial growth was observed only in DTT-treated devices. In four patients both cultures and PCR of sonicated and DTT-treated samples resulted negative despite cultures of periprosthetic tissue grew *Staphylococcus epidermidis* in three cases and *Pasteurella multocida* in another.

No microbial growth was observed in cultures from 24 patients without clinical signs of infection (group A). One strain of *Pseudomonas aeruginosa* and one of *Pseudomonas putida* were isolated from sonicated and DTT-treated samples while periprosthetic tissue cultures failed to give bacterial growth. Interestingly, coagulase negative staphylococci (five *S. epidermidis* and three *Staphylococcus hominis*) were isolated from less than three samples of periprosthetic tissues after broth enrichment, while no growth was obtained from either the sonicated or the DTT-treated devices.

As reported in Table 4, Gram positive cocci were the most frequently isolated bacteria, followed by Gram negative rods and anaerobes. *Staphylococcus aureus* accounted for 46.8% and 44.7% of bacteria isolated from sonicated and DTT-treated samples, while *S. epidermidis* was found in 12.5% and 21.0% of sonicat-

Table 2. Primers Used for PCR Analysis and Pyrosequencing

Primer Code	Primer Name	Primer Sequence
V1 antisense	bio*-pBR5′.SE	GAAGAGTTTGATCATGGCTCAG
	pBR-V1.AS	TTACTCACCCGTCCGCCACT
V3 antisense	bio*-pJBS-V3.SE	GCAACGCGAAGAACCTTACC
	B-V3.AS	ACGACAGCCATGCAGCACCT
V3 sense	bio*-B-V3.AS	ACGACAGCCATGCAGCACCT
	pJBS-V3.SE	GCAACGCGAAGAACCTTACC

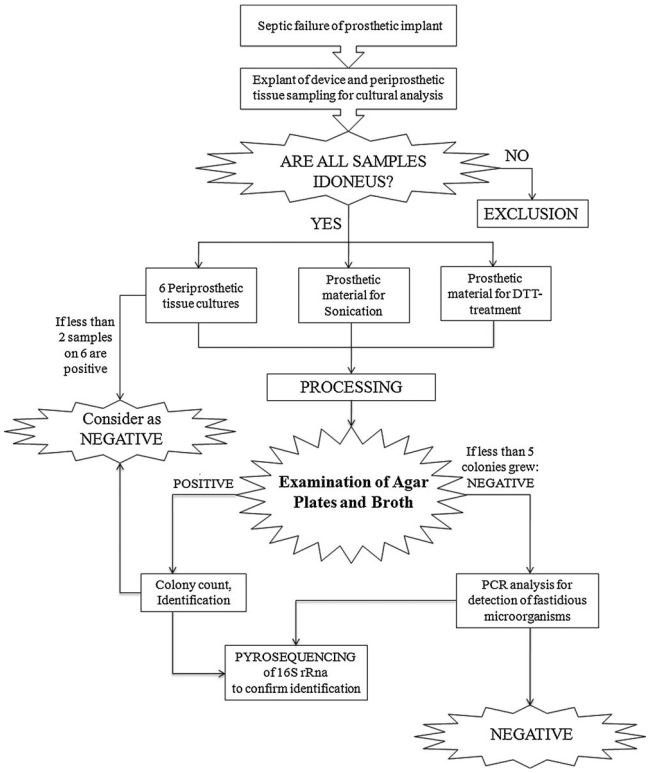

Figure 2. The analytical process flow-chart.

ed and DTT-treated devices, respectively. Gram negative rods were represented by non-fermenting bacteria, such as *Pseudomonas* spp. while, among anaerobes, *Propionibacterium acnes* and *Bacteroides fragilis* were isolated in one patient each. All the isolates were classified as moderate to strong biofilm producers, except for *P. multocida* which was unable to produce biofilm (data not shown).

As expected, DNA was successfully amplified in all culture-positive samples. By contrast, bacterial DNA was found in 6 DTT-treated samples and 10 sonication fluids with no bacterial growth. Of these, bacterial DNA was detected in both DTT-treated and sonicated fluid of six patients of group A, despite they had no clinical signs of infection and negative cultures. Unfortunately, the presence of overlapping sequences hampered pyrosequencing of these samples. Sequencing of DNA from the remaining four sonicated fluids confirmed the presence of *S. epidermidis* as evidenced by cultures of DTT-treated prostheses and periprosthetic tissue.

Sonication procedure presented a sensitivity of 71.4% and a specificity of 94.1%; periprosthetic tissue cultures presented the same value of sensitivity of the sonication procedure but a lower specificity (76.5%). The DTT treatment provided a sensitivity of 85.7% with the same specificity of the sonication procedure. Positive predictive values were 94.7%, 93.7%, and 78.9%, respectively for DTT treatment, sonication, and periprosthetic tissue culture, while negative predictive values resulted 84.2%, 72.7%, and 68.4%, respectively.

Summarizing, treatment with DTT showed the same specificity of sonication but a higher sensitivity and allowed a higher recovery of *S. epidermidis* than sonication.

DISCUSSION

Diagnosis of PJIs remains a challenge for microbiology laboratories because, despite the continuous development of microbiological techniques, a truly valid technique that could serve as a reference for their diagnosis has not been still recognized.[15,24] Periprosthetic tissue culture has been generally considered as the gold standard for identification of pathogens involved in PJIs but the presence of biofilm may

Table 3. Comparison of DTT Treatment, Sonication, and Culture of Periprosthetic Tissue

		Number of Patients With	
Method	Patients' Group	Positive Cultures, *n* (%)	Negative Cultures, *n* (%)
DTT	Group A ($n = 34$)	2 (5.9)	32 (94.1)
	Group B ($n = 42$)	36 (85.7)	6 (14.3)
Sonication	Group A ($n = 34$)	2 (5.9)	32 (94.1)
	Group B ($n = 42$)	30 (71.4)	12 (28.6)
Tissue culture	Group A ($n = 34$)	8 (23.5)	26 (76.5)
	Group B ($n = 42$)	30 (71.4)	12 (28.6)
Concordant results DTT/sonic/tissue	Group A ($n = 34$)	0 (0)	24 (70.6)
	Group B ($n = 42$)	22 (52.4)	2 (4.8)
Concordant results DTT/sonic	Group A ($n = 34$)	2 (5.88)	32 (94.1)
	Group B ($n = 42$)	30 (71.4)	6 (14.3)
Concordant results DTT/tissue	Group A ($n = 34$)	0 (0)	24 (70.6)
	Group B ($n = 42$)	26 (61.9)	2 (4.76)
Concordant results sonic/tissue	Group A ($n = 34$)	0 (0)	24 (70.6)
	Group B ($n = 42$)	22 (52.4)	4 (9.52)

Table 4. Microorganisms Isolated From Biological Specimens

Microorganism	Number of Isolate After		
	DTT Treatment	Sonication	Tissue Cultures
S. aureus	17	15	13
S. epidermidis	8	4	10 (3)
S. hominis	0	0	0 (3)
S. capitis	4	4	4
S. warnerii	4	4	2
Gram negative rods	2 (1)	2 (1)	2
Anaerobes	2	2	1

Numbers in brackets indicates isolated from patients of Group A.

complicate bacterial retrieval. In recent years, molecular biology analysis and prosthetic devices sonication have been developed to improve diagnosis of PJIs.[11] In this study, we assessed the ability of DTT, a sulfhydryl compound routinely used in microbiology laboratories, in detaching bacteria from orthopedic devices, keeping microorganisms alive and cultivable, permitting an easier treatment of explanted devices though maintaining the advantage of sonication to dislodge bacteria from biofilm. Our results show that cultures from DTT-treated devices gave similar or even higher yields when compared to sonication and periprosthetic tissue cultures. When compared with similar studies using sonication,[25,26] the present one shows a lower sensitivity. This discrepancy could be due to the fact that all patients included in group B were under antibiotic therapy in the weeks before surgery. Antibiotic therapy is widely known to dramatically reduce sensitivity of cultural procedures.[1] We could also hypothesized that lower sensitivity might be due to the inclusion of cement samples, which were not included in any previous study, all testing titanium or polyethylene devices. Materials used in orthopedic surgery present different affinity for bacteria,[27] which could be responsible for differences in microbial retrieval from new tested materials.

Anyway, positive and negative predictive values confirmed to be higher in sonication than in periprosthetic tissue cultures, and showed to be even higher in DTT-treated samples.

Sonication demonstrated a high specificity, confirming what have been previously reported,[26] and presented the same value for DTT treatment. The lower specificity of periprosthetic tissue culture probably depends on the high risk of contamination during sampling, which is also confirmed by the eight cases of contaminants bacteria isolated, probably collected from accidental contact with cutaneous regions during sampling.

Finding of *P. multocida* and *S. epidermidis* in periprosthetic tissue cultures with negative results for sonication and DTT treatment, also after bacterial DNA amplification, could be explained by the localization of the infection only in the soft tissues and not on the prosthetic surface, hypothesis supported by the inability of the *P. multocida* isolate to produce biofilm on prosthetic materials. Interestingly, sonication was able to detect only 40% of *S. epidermidis* isolates that were identified after DTT treatment and confirmed by DNA sequencing. As shown by other authors,[28] it is possible that sonication could be less efficient in removing *S. epidermidis* from biofilm in respect to other bacteria. An intensive antibiotic therapy before surgery seems to make bacteria more susceptible to ultrasound treatment and chemical reducing agents and could be responsible for presence of bacterial DNA not confirmed by cultures, as observed in six cases.[29] DNA sequencing was unable to give valid sequences for identification for 12 samples corresponding to six patients. Because it had been demonstrated that commercially available reagents for PCR contains contaminating bacterial DNA that could not be removed for not affecting sensitivity of broad-range PCR assays,[30] it may be hypothesized that, when DNA is present in low amounts, it might compete with the contaminating DNA leading to suppression of amplification of the pathogen DNA or to mixed amplicons, which, in turns, may cause invalid sequencing results.[30] Data on retrieved organisms confirm what previously observed,[31,32] with a predominance of Gram positive cocci, especially *S. aureus* and *S. epidermidis*, on Gram-negative rods. We also observed a low prevalence of anaerobes infection, similar to that observed in other studies.[25]

In conclusion, our data showed that DTT treatment could be used in substitution of sonication for the microbiological diagnosis of PJIs, thanks to its easier use and because this procedure do not require any specific laboratory instrumentation. Predictive values of DTT treatment showed that it could be a useful skill in microbiology laboratory, especially for those in which orthopedic infection are often investigated. We also demonstrate the major sensitivity of this method towards *S. epidermidis*, which is often involved in PJIs in respect to sonication. However, since our study considered a limited number of patients, further larger studies are needed to confirm our findings.

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