Detection of bacteria with molecular methods in prosthetic joint infection: sonication fluid better than periprosthetic tissue

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Background and purpose — The correct diagnosis of prosthetic joint infection (PJI) can be difficult because bacteria form a biofilm on the surface of the implant. The sensitivity of culture from sonication fluid is better than that from periprosthetic tissue, but no comparison studies using molecular methods on a large scale have been performed. We assessed whether periprosthetic tissue or sonication fluid should be used for molecular analysis.

Patients and methods — Implant and tissue samples were retrieved from 87 patients who underwent revision operation of total knee or total hip arthroplasty. Both sample types were analyzed using broad-range (BR-) PCR targeting the 16S rRNA gene. The results were evaluated based on the definition of periprosthetic joint infection from the Workgroup of the Musculoskeletal Infection Society.

Results — PJI was diagnosed in 29 patients, whereas aseptic failure was diagnosed in 58 patients. Analysis of sonication fluid using BR-PCR detected bacteria in 27 patients, whereas analysis of periprosthetic tissue by BR-PCR detected bacteria in 22 patients. In 6 of 7 patients in whom BR-PCR analysis of periprosthetic tissue was negative, low-virulence bacteria were present. The sensitivity and specificity values for periprosthetic tissue were 76% and 93%, respectively, and the sensitivity and specificity values for sonication fluid were 95% and 97%.

Interpretation — Our results suggest that sonication fluid may be a more appropriate sample than periprosthetic tissue for BR-PCR analysis in patients with PJI. However, further investigation is required to improve detection of bacteria in patients with so-called aseptic failure.

Distinguishing between aseptic failure (AF) of joint prosthesis and prosthetic joint infection (PJI) can be challenging when there are subtle signs of infection. Despite the now well-established definition of PJI (Parvizi et al. 2011), culture of periprosthetic tissue still remains the gold standard for confirmation of PJI. However, culture can be negative in 7% of PJIs despite obvious clinical signs of infection, mainly because of previous antimicrobial therapy (Berbari et al. 2007). Furthermore, broad-range PCR (BR-PCR) has shown the presence of bacterial DNA in culture-negative samples from patients with PJI (Tunney et al. 1999, Panousis et al. 2005, Fihman et al. 2007). Over and above the introduction of molecular methods in PJI diagnostics, high microbiological yields from sonication of retrieved implants have shown that one reason for culture-negative results from periprosthetic tissue is the presence of biofilm on the implant surface.

There have been several studies in which culture was used for comparison of analysis of sonication fluid and periprosthetic tissue (Trampuz et al. 2006, 2007, Dora et al. 2008, Esteban et al. 2008, Piper et al. 2009, Holinka et al. 2011, Bjerkan et al. 2012, Gomez et al. 2012, Portillo et al. 2012). However, only a few of these studies also involved molecular methods for analysis of sonication fluid (Tunney et al. 1999, Dora et al. 2008, Esteban et al. 2008, Achermann et al. 2010, Gomez et al. 2012, Portillo et al. 2012), and even fewer involved molecular methods for analysis of both periprosthetic tissue and sonication fluid (Bjerkan et al. 2012, Ryu et al. 2014). Since the literature does not provide strong evidence of what type of sample should be used for molecular analysis, we compared detection of bacteria in periprosthetic tissue and in sonication fluid using BR-PCR and sequencing.

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Patients and methods

In this prospective study, we included 87 patients who underwent revision operation of total knee or total hip arthroplasty. Criteria for patient selection were inexplicable pain, repeated dislocation, stiffness, and no obvious explanation for premature failure of the joint (Trebse 2012). Furthermore, we only included those patients for whom all the diagnostic tests that were part of the criteria in the definition of PJI used in this study had been performed. Implants and samples of periprosthetic tissue were retrieved during revision operation. Upon retrieval, prostheses and samples of periprosthetic tissue were placed in sterile plastic containers and transferred to the laboratory, where they were analyzed. Sonication fluids from explanted prostheses and periprosthetic tissue samples were analyzed with culture techniques and broad-range 16S rRNA real-time PCR (BR-PCR). Previous antimicrobial therapy was defined as any antibiotic given within 14 days before the revision operation.

Analysis of periprosthetic tissue

From each patient, 3–6 samples of periprosthetic tissue were retrieved at the revision operation. The volume of liquid thioglycollate medium (TYO) that was added aseptically to the samples varied according to the size of the sample (samples had to be completely submerged in the TYO). Submerged tissue samples were disrupted using a homogenizer for 90 s (Masticator Digital; IUL Instruments GmbH, Königswinter, Germany). Aliquots of 1 mL were prepared for molecular methods, but the rest of the sample was used for routine culture (Rak et al. 2013, 2015).

Molecular analyses of periprosthetic tissue samples were done as previously described (Rak et al. 2013, 2015). Briefly, total DNA was extracted with a PureLink Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's instructions. Additional care was taken to prevent contamination during sample handling. The work flow was unidirectional, with separate designated work benches and pipetting devices. A negative control was included during isolation of DNA. The presence of bacteria was confirmed by detection of the 16S rRNA gene with broad-range primers. Amplification of the human glyceraldehyde-3-phosphatedehydrogenase (GAPDH) gene served as a control for DNA isolation and possible inhibition of PCR. PCR products from the 16S rRNA gene were sequenced in both directions. Sequences were analyzed with the BLAST algorithm at the website of the NCBI, and with software application BIBI V5 (Devulder et al. 2003). Bacteria identified were considered significant if the same species was present in at least 2 samples of periprosthetic tissue.

The detection limit of our BR-PCR was determined based on serial 10-fold dilutions of *S. aureus*, *S. epidermidis*, *P. aeruginosa*, and *S. agalactiae* being used to inoculate negative periprosthetic tissue, and isolation of total DNA was based on the same protocol as for periprosthetic tissue. Viable bacterial counts were used for enumeration of bacteria in samples.

Analysis of sonication fluid

An appropriate volume of Ringer solution (250–800 mL) was added to prostheses after their arrival in the laboratory. Submerged prostheses were sonicated in an ultrasonic bath (BactoSonic 14.2; Bandelin, Berlin, Germany). Containers with prostheses were first vortexed for 30 s, followed by sonication at 100% power (40 kHz) for 5 min and vortexing for 30 s (Trampuz et al. 2007). Sonication fluid was centrifuged (1,490 g for 10 min) and sediment was used for isolation of total DNA using the same protocol as for periprosthetic tissue.

The results of molecular analysis of periprosthetic tissue and sonication fluid did not change the plan for treatment of patients. BR-PCR was only used as an additional method without affecting the routine diagnostic protocol.

Definition of PJI

A patient was considered to have an infection if one of the following criteria were fulfilled: (1) sinus tract communicating with the prosthesis; (2) identification of bacteria with culture methods from at least 2 samples of periprosthetic tissue; or (3) at least 4 of the following 6 additional minor criteria were fulfilled: elevated serum erythrocyte sedimentation rate (ESR) (> 30 mm/h) and elevated serum C-reactive protein (CRP) concentration (> 10 mg/L); elevated synovial leukocyte count (> 1.7×10^9/L); elevated synovial neutrophil percentage (> 65%); presence of purulence in the affected joint; isolation of a microorganism in 1 culture of periprosthetic tissue; > 5 neutrophils per high-power field (HPF) in 5 HPFs observed from histological analysis of periprosthetic tissue (Parvizi et al. 2011).

Statistics

Sensitivity, specificity, and positive and negative predictive value were calculated for the molecular methods based on the definition of PJI. McNemar test was used for comparisons between diagnostic methods. SigmaPlot 11.0 was used for calculation of the McNemar test result and Clinical Calculator 1 (Lowry 2001) was used for the remaining statistical calculations. In all statistical calculations, confidence levels (CIs) were set to 95%.

Ethics

All patients gave informed consent to participate in the study. The Slovenian National Medical Ethics Committee approved the study protocol (approval number 40/06/11).

Results

Based on the definition of PJI, AF was diagnosed in 58 cases and PJI in 29 cases (Table 1). Molecular analysis of periprosthetic tissue showed the presence of bacteria in 22 of 29 patients with PJI (Table 2). Analysis of sonication fluid with molecular methods was positive in 21 of 22 patients with posi-

Table 1. Demographic and clinical data

	Prosthetic joint infection	Aseptic failure
No. of patients	29	58
Age (range)	70 (54–88)	70 (29–86)
No. of females	12	44
No. of males	17	14
Joint		
Knee	12	29
Hip	17	29
First revision	20	39
Repeated revision	9	19
Median no. of samples retrieved (range)	6 (5–8)	6 (5–10)
Time of implant failure		
Early (0–3 months]	6	0
Delayed (3–24 months)	6	19
Late (< 24 months)	17	39
Median time of prosthesis in vivo, months (range)	36 (0–213)	45 (5–270)
Median time of transport, h (range)	1:30 (0:32–18:40)	1:49 (0:40–3:29)
Median ESR (range), mm/h	80 (0–81)	3 (0–30)
Median CRP (range), mg/L	62 (15–170)	9 (0–165)
Histology: no. of patients with > 5 PMN/HPF	16	4
Antimicrobial therapy before operation	8	3

ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; PMN: polymorphonuclear neutrophils; HPF: high-power field.

tive periprosthetic tissue. However, sonication fluid was BR-PCR-positive in 6 additional patients with PJI, while periprosthetic tissue was negative. In 1 patient, periprosthetic tissue was BR-PCR-positive whereas no bacteria were detected in sonication fluid.

In 17 of 21 patients who were PCR-positive in tissue and sonication fluid samples, the same bacterial species were identified in both types of samples by molecular methods. In 2 of the 4 remaining patients, analysis of sonicate enabled identification of additional species by molecular methods. In the third patient, sonicate was BR-PCR-positive but identification was not possible due to multiple patterns on electropherograms, indicating polymicrobial infection. In the fourth patient, additional species were identified in periprosthetic tissue by molecular methods.

In 6 of 7 patients where tissue was negative and sonication fluid was positive by molecular methods, low-virulence bacteria were identified as the causative agent of infection. Bacteria from all 7 patients were considered significant, as they were also isolated in sonication fluid by routine culture.

In all but 1 patient with PJI, where molecular analysis of periprosthetic tissue or sonication fluid identified bacteria, at least 1 major criterion from the definition of PJI was fulfilled, with positive culture of the periprosthetic tissue being the predominant one (Table 2). In the remaining patient, no major criterion was fulfilled, but more than 4 minor criteria were recorded.

Of the 58 patients with AF, molecular methods detected the presence of bacteria in 13 (Table 3). In 9 patients, only periprosthetic tissue was positive; in 3 patients, only sonication fluid was positive; and in the remaining patient, both types of samples showed the presence of bacteria. In 7 of 13 patients with AF in which molecular methods detected the presence of bacteria in periprosthetic tissue, 1 additional minor criterion was fulfilled: positive culture from periprosthetic tissue in 1 sample (4 cases), positive histology (2 cases), and positive white blood cell count (WBC) in synovial fluid (1 case) (Table 3). In 6 of 9 patients with AF where only periprosthetic tissue was BR-PCR-positive (patients 10, 14, 31, 36, 39, and 79), the bacteria identified were considered insignificant based on criteria for interpretation of BR-PCR results of periprosthetic tissue analysis (Rak et al. 2015). The bacteria identified in periprosthetic tissue of the remaining 3 patients (patients 16, 17, and 63) were considered significant based on the criteria for interpretation of BR-PCR results. Bacteria that were identified with molecular methods in sonication fluid or periprosthetic tissue—or both—of the remaining 4 patients with AF (patients 8, 9, 75, and 87) were also considered significant. Interpretation was based on culture results (patients 8 and 87) or on the fact that patients were receiving antibiotic before the revision operation (patients 8, 9, and 75).

The sensitivity of molecular methods with periprosthetic tissue and sonication fluid was 76% and 93%, respectively ($p = 0.06$). The specificity was the same for both types of samples: 93% (Table 4). Concordance of molecular methods with culture results was higher for analysis of sonication fluid (93%) than for analysis of periprosthetic tissue (89%).

Discussion

We found that using molecular methods, bacterial DNA was detected more often in sonication fluid than in periprosthetic tissue. To our knowledge, this is the largest study comparing analysis of periprosthetic tissue and sonication fluid from the same patients with molecular methods.

Molecular analysis of periprosthetic tissue and sonication fluid showed that the sensitivity with sonication fluid is higher than the sensitivity with periprosthetic tissue. Despite the fact that the difference was not statistically significant ($p = 0.06$), our results confirm observations of Bjerkan et al. (2012) and Ryu et al. (2014), who tested periprosthetic tissue and sonication fluid from the same patients by molecular methods and compared the results. In comparison to these 2 studies, the sensitivities of detection of bacteria in tissue and sonication fluid in our study were higher (tissue: 76% as compared to 72% and 16%; sonication fluid: 93% as compared to 82% and 78%).

Overall, molecular methods detected the presence of bacteria (based on either periprosthetic tissue or sonication fluid, or both) in all but 2 of the 29 patients who were considered to

Table 2. Identified bacteria with molecular methods in periprosthetic tissue and sonicate fluid among the patients with PJI

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	86	25	M	K	+	-	+	+	+	+	-	*S. aureus*	2	*S. aureus*
2	79	39	M	H	-	-	+	-	-	+	+	*S. aureus*	6	*S. aureus*
3	60	11	F	H	+	-	-	-	+	-	-	/	0	*P. avidum*
												S. aureus	5	*S. aureus*
												S. epidermidis	5	*S. epidermidis*
4	83	66	M	H	+	-	-	-	-	-	+	*Streptococcus* sp	4	*Streptococcus* sp
7	87	89	F	K	+	-	-	-	+	+	+	*Staphylococcus* sp	6	*Staphylococcus* sp
12	73	29	M	H	+	-	-	-	+	+	-	*S. mitis/oralis*	2	*S. mitis/oralis*
13	65	1	M	K	-	-	-	-	-	+	+	*S. aureus*	5	*S. aureus*
15	78	2	M	H	+	+	-	-	-	-	+	/	0	*S. epidermidis*
												E. faecalis	6	
18	70	64	F	H	+	-	-	-	+	+	+	*S. epidermidis*	1	*E. faecalis*
21	70	1	M	H	+	-	+	+	-	-	-	/	0	*S. epidermidis*
22	70	100	M	H	+	-	+	+	+	-	-	*S. epidermidis*	3	*S. epidermidis*
23	59	80	M	H	+	-	-	-	+	-	-	/	0	*Parvimonas micra/ Micromonas micros*
												Staphylococcus sp	4	
												S. aureus	2	
24	66	12	F	K	+	+	+	+	+	-	-	*S. epidermidis*	2	*S. epidermidis*
29	73	189	F	H	-	-	+	+	+	+	-	*S. aureus*	6	*S. aureus*
35	66	152	F	H	+	-	+	+	+	+	-	*E. coli*	6	*E. coli*
37	61	0	M	H	+	-	-	-	-	-	-	/	0	*S. aureus*
40	74	46	M	K	+	-	-	-	-	+	-	*S. epidermidis*	2	*S. epidermidis*
44	83	36	M	H	+	-	-	-	-	+	-	*S. aureus*	3	*S. aureus*
47 [a]	54	60	F	H	-	-	-	-	-	-	-	/		/
50	56	6	F	K	+	-	+	+	+	-	+	*S. sanguinis*	2	*S. sanguinis*
51	69	1	M	K	+	-	-	-	+	-	-	*Staphlyococcus* sp	3	/
52	69	118	F	K	+	-	-	-	+	-	+	*E. faecalis*	4	*Polymicrobialy electroferogram*
53	78	51	M	H	-	-	+	-	-	+	-	*S. aureus*	3	*S. aureus*
64	74	104	M	K	+	-	-	-	+	+	+	*E. cloacae*	4	*E. cloacae*
67	71	8	M	H	+	-	-	-	-	-	-	*E. faecium*	3	*E. faecium*
68	88	213	F	K	+	-	-	-	-	+	-	*S. pasteuri*	3	*S. pasteuri*
73	63	23	F	K	+	-	-	-	+	-	-	/	0	*S. epidermidis*
77	74	2	F	K	+	-	-	-	-	-	-	*K. oxytoca/ K. michiganensis*	3	*K. oxytoca/ K. michiganensis*
82	65	12	M	H	-	-	+	+	+	-	-	*K. oxytoca/ K. michiganensis*	3	*K. oxytoca/ K. michiganensis*

[a] The patient was diagnosed with PJI based on culture results of periprosthetic tissue.
A Patient number
B Age of patient
C Time of implant in vivo, months.
D Sex
E Joint: H – hip, K – knee.
F C-reactive protein > 10 mg/L.
G Erythrocyte sedimentation rate > 30 mm/h.
H White blood cell count in synovial fluid > 1.7×10^9/L.
I Polymorphonuclear neutrophil (PMN) count in synovial fluid > 65%.
J Histology: > 5 polymorphonuclear neutrophils/high-power field.
K Purulence
L Fistula/sinus
M Identified bacteria in periprosthetic tissue.
N Number of positive samples with the same bacterial species identified with molecular methods.
O Identified bacteria by sonication.

have PJI. Overall concordance between the patients with PJI and those with AF was 86%.

Despite the good concordance between the results from sonication fluid and periprosthetic tissue, there were 6 patients with PJI where molecular analysis of periprosthetic tissue was negative and analysis of sonication fluid was positive. This result is in agreement with the common belief that bacteria are present on the surface of the prosthesis in the form of a biofilm, and not in the surrounding tissue (Trampuz et al. 2006, 2007). However, the results of culture from periprosthetic tissue showed bacteria in 2 or more specimens in all 5 cases (data not shown). Because we used aliquots of the same tissue sample for culture and molecular analysis, we conclude that bacteria were present in the sample but that

Table 3. Identified bacteria with molecular and culture methods in periprosthetic tissue and sonicate fluid 2 among the patients with AF

A	B	C	D	E	F	G	H	I	J	K	L	M	L	N
Identified bacteria with molecular methods were considered insignificant due to criteria [a]														
10	66	13	F	K	-	-	+	-	< 5	/	0	*Enterobacter* sp.	1	/
14	62	16	M	K	-	-	-	-	< 5	*S. epidermidis*	1			
										S. haemolyticus	1	*S. epidermdis*	1	/
										S. lentus	1	*Staphylococcus* sp.	1	
										P. acnes	1			
31	82	116	F	H	-	-	-	-	0	/	0	*S. epidermidis*	1	
36	81	60	F	K	-	-	-	-	< 5	/	0	*A. calcoaceticus*	1	/
39	83	58	F	H	-	-	-	-	> 25	/	0	*H. parahaemolyticus*	1	/
79	74	9	M	H	-	-	-	-	0	*S. cohnii*	1	*S. nepalensis*	1	/
Bacteria identified with molecular methods, considered significant due to criteria [a]														
16	75	17	F	K	+	-	-	-	< 5	/	0	*S. aureus*	2	/
17	43	30	M	H	-	-	-	-	< 5	/	0	*S. aureus*	5	/
63	54	21	F	K	-	-	-	-	0	*S. warneri*	1			
										S. epidermidis	1	*Staphylococcus* sp.	2	/
										P. granulosum	1			
Identified bacteria with molecular methods were considered significant due to patient history and culture results														
8 [b]	62	59	F	K	+	-	-	-	> 25	/	0	/	0	*S. aureus*
9 [b]	73	28	M	K	-	-	-	-	< 5	/	0	/	0	*Streptococcus* sp.
75 [b]	75	20	F	K	+	-	-	-	0	/	0	/	0	*S. dysgalactiae*
87	55	17	F	K	+	-	-	-	< 5	*S. capitis*	1	*Staphylococcus* sp.	3	*Staphylococcus* sp.

A Patient number
B Age of patient
C Time of implant in vivo, months.
D Sex
E Joint: H – hip, K – knee.
F C-reactive protein > 10 mg/L.
G Erythrocyte sedimentation rate > 30 mm/h.
H White blood cell count in synovial fluid > 1.7×10^9/L.
I Polymorphonuclear neutrophil (PMN) count in synovial fluid > 65%.
J Polymorphonuclear neutrophils/high-power field.
K Identified bacteria by culture from periprosthetic tissue
L Number of positive samples with the same bacterial species
M Identified bacteria by molecular methods from periprosthetic tissue
N Identified bacteria by sonication
[a] Criteria for interpretation of BR-PCR results. Bacterium was considered significant if it was identified in at least 2 samples of periprosthetic tissue.
[b] Patient was on antibiotic therapy prior to revision operation. In Case 8 *S. aureus* was isolated from synovial fluid.

Table 4. Statistical parameters for analysis of periprosthetic tissue and sonication fluid by molecular methods

Method	TP	FP	TN	FN	Sensitivity	Specificity	PPV	NPV
BR-PCR PT ≥ 2 [a]	22	4	54	7	76 (56–89)	93 (83–98)	85 (64–95)	89 (77–95)
BR-PCR SF	27	4	54	2	93 (76–99)	93 (83–98)	87 (69–96)	96 (87–99)

TP: true positive; FP: false positive; TN: true negative; FN: false negative; SE: sensitivity; SP: specificity; PPV: positive predictive value; NPV: negative predictive value; PT: periprosthetic tissue; SF; sonication fluid.
[a] Bacteria were considered significant if the same species was present in at least 2 samples of periprosthetic tissue; 95% confidence intervals are given in parentheses. Clinical Calculator 1 (Lowry 2001) was used for calculation of sensitivity, specificity, positive and negative predictive value, and confidence intervals.

molecular methods failed to detect bacterial DNA of clinical significance.

One reason for this could be that detection of bacterial DNA with our BR-PCR assay has a lower analytical sensitivity (in our case, 10^3 CFU/mL) than culture. Reasons for the low limit of detection could be the lower amount of sample used for PCR in comparison to culture. Furthermore, the presence of residual bacterial DNA in PCR reagents and human DNA from tissue samples has a negative effect on the detection limit (Borst et al. 2004, Moojen et al. 2007, Horz et al. 2008, Cherkaoui et al. 2009).

The problem of detection limit was shown in a study by Bjerkan et al. (2012), where colonies identified from periprosthetic tissue and sonicate fluid were quantified. These authors

showed that analysis of periprosthetic tissue with molecular methods was negative in cases where low quantities of bacteria were detectable with culture. Furthermore, most of the results of molecular analysis were negative when low-virulence bacteria were identified with culture. We did not quantify periprosthetic tissue, so we cannot claim that a low amount of bacteria was present, but we can confirm that low-virulence bacteria were present in 4 of 5 cases with negative periprosthetic tissue and positive sonication fluid (by molecular methods).

Bemer et al. (2014) suggested that low-virulence bacteria may be present in low bacterial inocula in chronic infections. In the present study, we failed to detect low-virulence bacteria in acute infections also, indicating that low bacterial inocula could also be present in acute infections.

Another explanation for BR-PCR-negative results of periprosthetic tissue could be presence of inhibitors in tissue samples. The study by Bemer et al. (2014) showed that PCR was inhibited in 5% of all samples tested. Our method for detection of bacterial DNA also included a PCR inhibition test, but the reaction was only considered to be inhibited if there was no amplification of the control gene. However, other studies have shown that "all or none" amplification is not a good indicator of PCR inhibition (Bustin et al. 2005, King et al. 2009). Thus, using an external DNA control and measuring the shift in delta Ct relative to an uninhibited reaction would be a more reliable control.

On the other hand, molecular analysis of samples from patients with AF showed the presence of bacterial DNA in periprosthetic tissue in 9 patients who had negative results from sonication fluid. Only 3 of 9 bacteria were considered to be significant based on criteria for interpretation of BR-PCR results of periprosthetic tissue analysis (Rak et al. 2015). However, the origin of these bacteria is not known for 2 of the 3 cases (patients 16 and 17), because culture of periprosthetic tissue was negative. Although culture was negative in patient 16 and 17, histology showed the presence of less than 5 polymorphonuclear neutrophils per high-power field. From the results of histology in these 2 cases, we cannot exclude infection; the results were therefore inconclusive. We believe that, based on our definition, these 2 patients were misclassified as having AF and should have been classified as having PJI.

Bacteria that were also detected in sonication fluid in patients who were receiving antibiotic therapy were considered significant. However, the viability of these bacteria is questionable, as it is possible that the bacteria were not viable, and would not grow in culture. Without any additional steps, the BR-PCR method does not discriminate between dead and living bacteria. Thus, an additional step with pretreatment of the samples—to remove or inactivate DNA from dead bacteria—would greatly ease interpretation of the BR-PCR results.

In summary, our results suggest that in case of PJI, sonication fluid may be more appropriate material for molecular analysis than periprosthetic tissue. However, analysis of sonication fluid by molecular methods in patients with AF is still a problem, probably because of the low quantities of bacteria in clinical samples. Further investigations and technical advances are needed to to solve this problem.

MR: design of the study, preparation of the manuscript, BR-PCR analysis, and interpretation of data. MK: culture analysis and supervision. RT: conception and design of the study, surgery, and collection of clinical data. AC: conception and design of the study, preparation of the manuscript, histopathology, supervision, and interpretation of data.

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