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Research Article

Dithiotreitol and Next Generation Sequencing of Bacterial 16S rRNA Shows Similar Diagnostic Security as Periprosthetic Tissue Cultures to Diagnose PJI

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Abstract

Purpose: Tissue cultures are standard (std.) diagnostic procedures for identification of periprosthetic joint infections, but has some limitations. The use of Dithiothreitol (DTT) to detach the biofilm can improve the diagnostic security. The aim of this study was to compare std. diagnostic results with the microDTTect device by culturing and Next Generation Sequencing (NGS) from DTT treated endoprosthetic explants.

Methods: 66 patients (38 aseptic; 28 septic) with revision surgeries were included. We compared std. tissue cultures with microDTTect cultures and NGS of bacterial 16S rRNA isolated from DTT solution.

Results: 75% of the septic cases were in line with both culturing methods. In 8% of the aseptic cases, the microDTTect culture indicated a present pathogen. In 71% of the septic cases, the std. microbiology was concordant to NGS, while it was 79% concordant in the aseptic cohort. Polymicrobial infections were detected less frequently by culturing techniques, but with a high sensitivity using NGS.

Conclusion: Our data indicate that tissue cultures show a similar reliability compared to the other techniques. The DTT culturing had a sensitivity of 75% and specificity of 92%. NGS had a sensitivity of 71% and specificity of 79%. So far, microDTTect analysis by DTT cultivation and NGS showed promising results, but they were not superior to the standard microbiological diagnostic using tissue cultures for the identification of bacteria and illustrate the high relevance of tissue cultivation.

Introduction

The need for a revision of a total joint replacement can be caused by various complications. The most common reasons for revision surgery are aseptic loosening, malpositioning of the implant and Periprosthetic Joint Infections (PJI) [1]. The incidence of PJI is lower than the incidence of aseptic loosening [2], but the economic burden to treat PJI is higher including the costs for outpatient care [3]. The incidence of PJI varies depending e.g. on the joint, number of previous surgeries of the respective joint and comorbidities of the patient. While the risk for revision due to PJI in total hip arthroplasties (THA) within the first 2 years is approximately 1%, it is twice as high in total knee arthroplasties (TKA) [4], may increase up to 10% with multiple previous surgeries [5-7], and is even higher after a previous infection of the respective joint. The most common pathogens observed in PJI are *Staphylococcus spp.*, in particular *Staphylococcus epidermidis* and *Staphylococcus aureus* [8,9]. Other bacteria like *Streptococcus spp*. (7 - 9%) [9,10], *Enterococcus spp.* (12%) [10] and *gram-negative bacilli* (6%) [9] occur less frequently. Proteobacteria such as *Escherichia coli, Enterobacter spp., Proteus spp., Klebsiella spp*. and *Pseudomonas spp*. are also associated with PJI [11]. These pathogens can either cause a monomicrobial or a polymicrobial infection accounting for up to 10% of all PJI cases [12].

A fast and reliable test to detect PJI is of utmost importance to prevent increased bone loss and further tissue infection. A culture-negative PJI (CN-PJI) represents a serious risk for the respective patient and ranges from 7% to 15% [10,13-15]. The risk of CN-PJIs in aseptic loosening ranges from 7-15%. CN-PJIs are characterized by typical inflammatory signs at the respective joint [16], and are often associated with a low-grade infections, caused by pathogens that are difficult to diagnose due to demanding cultivation conditions, such as anaerobic bacteria [16,17]. Therefore, tissue cultivation in some cases is not sensitive enough or slow growing pathogens are not detected during the cultivation time [18,19]. In such cases, aseptic loosening is usually assumed, and these patients suffer from recurrent prosthesis loosening and pain in the respective joint due to the persisting infection [16,17]. Low-grade infections and infections caused by bacterial biofilms that are difficult to diagnose are often associated with CN-PJIs [20]. A biofilm hinders the isolation and culture of bacteria, since routine diagnostic techniques do not efficiently detach the bacteria from the implant surfaces [21]. When the bacteria are of low virulence [19] or the screening method is not sensitive enough [18], the bacteria are not detected by the diagnostic tools [22,23]. Sonication is used to improve the detaching of bacteria as it is described to destabilize biofilms [24] and therefore results in a more valid diagnosis [25-28]. However, sonication poses a risk of contamination during the incubation in the water bath [25]. This can lead to incorrect results in the standard microbiology diagnostic [25]. Furthermore, sonication may harm sensitive bacteria resulting in false negative diagnoses [24,29,30].

It was tested if the use of Dithiothreitol (DTT) solution can be an alternative to sonication [31]. DTT is a sulfhydryl compound that can reduce the disulfide bonds between polysaccharides and proteins [32]. In a previous study it was shown that DTT can destabilize biofilms. Drago et al. [31] showed that bacteria survived the DTT treatment and could be cultivated. The DTT treatment even showed a higher sensitivity for *S. epidermidis* detection in comparison to sonication. Because of the easy and cost-effective use of DTT in comparison to sonication it was suggested as a possible alternative method. A further study showed that DTT and sonication are approximately equally sensitive [14], however, a third study indicated that DTT is inferior to sonication and conventional tissue culturing methods [33]. Therefore, literature does not provide a clear indication of whether the use of DTT can improve the diagnostic security of PJI or not. Next-Generation-Sequencing (NGS) can be a useful [34] and innovative [35] complementary methodology method in the diagnosis of PJI, as the entire microbial profile can be analyzed using the bacterial 16S ribosomal RNA gene and might help identifying the correct antibiotic therapy by detecting specific antibiotic resistances of the causative pathogen [36,37]. Additional sonication of explants before applying NGS can further increase its sensitivity [38,39]. The utility of an alternative method that helps to detach bacteria from the surface of an implant that can subsequently still be sequenced would therefore be a great benefit. The present study compares the diagnostic security using the microDTTect device (NCS Lab Srl, Carpi, Italy) in comparison to standardized routine PJI diagnostic using periprosthetic tissue biopsies and NGS from microDTTect solution. The aim of this work is to determine whether DTT cultivation is equivalent to tissue cultivation and whether the use of NGS is superior to the other techniques.

Materials and Methods

Patient Cohort

66 patients undergoing revision surgery of a Total Joint Arthroplasty of the hip (THA) or The Knee (TKA) were included in this study. Informed consent was obtained prior to inclusion in the study. IRB approval for this study was provided by the Institutional Review Board (No 207/17). Written informed consent was obtained from each patient prior to inclusion in the study. Surgeries were performed in the Department of Orthopaedic Surgery. The patient history was recorded, and the macroscopic presence of inflammatory indicators at the respective joint were assessed, to give a first hint for a possible infection. The cases were also classified as aseptic and septic based on the MSIS criteria defined by Parvizi et al. [40]. Three patients included in this study exhibited other infections. One patient had a urinary tract

infection, another patient had a psoas abscess and spondylodiscitis and another patient had an ulcus ventriculi and adrenalitis. These criteria are classified as minor and major criteria. For the definition of an infection a score over six points from the minor criteria or at least one of the major criteria should apply. The major criteria comprise: (i) an infection is present when in at least two tissue cultures the same pathogen was identified, or (ii) a sinus tract with an evidence of communication to the joint or visualization of the prosthesis is present. Important factors of the minor criteria are the preoperative examination of C-Reactive Protein (CRP), Erythrocyte Sedimentation Rate (ESR) and synovial fluid White Blood Cell Count (WBC). Infections may be present if the CRP is elevated above 5 mg/l, normal ESR values range up to 20 mm/hr in men (<50) and up to 30 mm/hr in women (<50), while synovial fluid WBC count should be below 3000 cells/ microliter. Furthermore, histological sections were performed. The presence of an increased number of macrophages and neutrophils indicated the presence of an infection. Furthermore, histological sections were performed.

Using this differentiation 38 aseptic THA or TKA revisions and 28 septic THA and TKA revisions were identified. Demographic data (sex, age, implantation time, number of previous surgeries) were recorded (Table 1). Prior to surgery, serum levels of C-reactive protein (CRP, mg/l) and white blood cells (WBC, Gpt/l) were determined. Std. routine diagnostics served as controls and are well established in the study hospital and based on MSIS criteria [40]. Therefore, the results of the microDTTect device (4i for infection, Monza, NCS Lab Srl, Carpi, Italy) and next-generation sequencing (NGS) were compared with std. routine diagnostics. In cases of a discrepancy between microbiologic diagnostic and microDTTect data, the CRP levels and the implantation time were determined, the implantation time describes the time between the implantation of the prosthesis until the revision surgery. If CRP is elevated (<5 mg/l), this would indicate an infection; if implantation time is low, an infection could also be considered. The antibiotic therapy is carried out according to the in-house regime in weekly consultation with our microbiologists within the framework of Antibiotic Stewardship (ASS). For the prospective study, the treatment of the patients was based on the results of the standard microbiological examination. The results of the MicroDTTect culturing and next-generation sequencing had no influence on the patient's antibiotic therapy. However, in retrospect the selected therapeutic regime was sufficient to eradicate the pathogens causing the infection.

Cohort	N	Sex	Age [yr]	Implant type	Implant. time [m]
Septic	28	♂ 18 ♀ 10	73 ± 8	TKA: 13 THA: 15	57 ± 84
Aseptic	38	♂ 17 ♀ 21	68 ± 12	TKA: 21 THA: 17	85 ± 76

Table 1: Biometric characteristics of the study population.

Micro DTTect

MicroDTTect was used according to the manufacturer's instructions (4i for infection, Monza, NCS Lab Srl, Carpi, Italy). Briefly, explants were placed in the microDTTect bag under sterile conditions during surgery and 150 ml of the DTT solution supplied with the bags was added to the explants. The DTT solution was divided into 75 ml on each falcon tube and centrifuged according to the manufacturer's protocol. The sediment was resuspended in two ml of the supernatant for each condition. Two ml from the first Falcon tube were sent to the routine microbiology for culture and identification of pathogens. Two ml from the second Falcon tube were used for DNA isolation followed by NGS sequencing. The time to process microDTTect samples was 1h. The flowchart of the analytical process is shown in Figure 1.

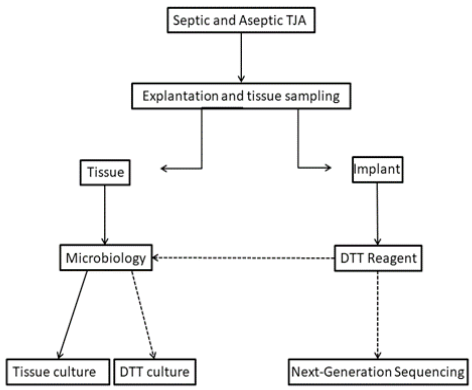


Figure 1: The analytical process in a flow-chart. Explanation of the septic and aseptic TJA were performed and tissue samplings for cultural analysis were taken. Left arm: Tissue samples which were taken during the operation are homogenized and tissue culturing was taken for the identification of the germ. Right arm: After the revision of the prostheses the implant is packed in the microDTTect bag for the treatment with DTT reagent. One part of the solution is sent to the microbiological diagnostics for culturing while the other part of the solution is used for DNA isolation and next-generation sequencing.

Microbiological Routine Diagnostic Tests

Periprosthetic tissue samples were minced and mechanically homogenized by an Ultra-Turrax Drive control disperser (IKA®-Werke GmbH & Co. KG, Germany). The homogenized samples were inoculated on agar plates: columbia agar with 5% sheep blood (Becton Dickinson, Heidelberg, Germany), chocolate agar and Schaedler agar (Oxoid, Munich, Germany) under aerobic conditions with 5% CO2 and anaerobically at 35˚C ± 1˚C. Additionally, the samples were inoculated in thioglycolate and Schaedler broth (bioMérieux, Marcy L'Etoile, France) at 35˚C ± 1˚C for 14 days. Fluid samples from MicroDTTect devices were incubated using the same conditions. The identification of pathogens was performed by MALDI-TOF MS (VITEK® MS, bioMérieux, Marcy L'Etoile, France).

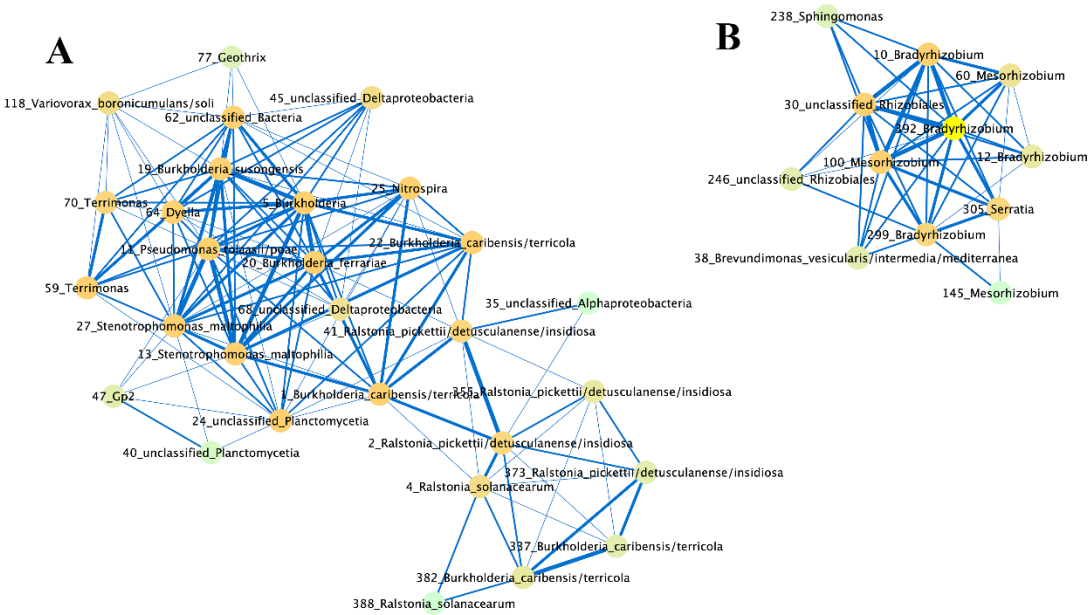
DNA Isolation and Preparation

The DTT solution was centrifuged at 3200 rpm for 10min to pellet the bacteria,. The DNA was purified from the pellet dissolved in Sodium phosphate buffer following the manufactures protocol from the FastDNA Spin Kit for Soil (MP Biomedicals™). A 3-step PCR-approach was used to amplify the V1V2 variable regions of the 16S rRNA gene. In a first PCR the primers 27FBIF (AGRGTTHGATYMTGGCTCAG) and 338R (TGCTGCCTCCCGTAGGAGT) were used for 20 cycles followed by a second PCR of 15 cycles using the 16SFw_BK (3' – ACGACGCTCTTCCGATCTAGRGTTHGATYMTGGCTCAG) and16SR_BK (GACGTGTGCTCTTCCGATCTTGCTGCCTCCCGTAGGAGT) primers. A third amplification step of 10 cycles added the two indices and Illumina adapters to amplicons [41]. Samples that failed to give a PCR product were not further analysed and defined as "aseptic" according to culture-independent analysis. MicroDTTect buffer alone was used as a negative control. Obtained products were pooled in equimolar ratios and sequenced on Illumina MiSeq (2X300 bases, San Diego, USA).

Bioinformatic Analysis

Bioinformatic processing was performed basically as previously described [42]. The raw reads were merged with the Ribosomal Database project (RDP) assembler [43], aligned and clustered (diffs = 2) in Mothur [44] and filtered and annotated as previously described [45] Correlation networks were constructed based on pairwise Spearman correlations of phylotype abundance matrices and calculated using "psych" [46] The network was visualized and analyzed using Cytoscape (version 3.7.2) [47]. The network analysis revealed three independent subnets of phylotypes showing co-occurrences with more than five neighbours. Two subnets comprised either Ralstonia and Burkholderia phylotypes which are commonly isolated from various pharmaceutical reagents and equipment, including laboratory-based purified water systems [48-50] and other contaminants in DNA extraction kits (Supplementary Figure S1a) or different *Mesorhizobium* and *Bradyrhizobium* and phylotypes (Supplementary Figure S1b) which are also documented as contaminants [51]. All members of those two subnets were deleted from the total abundance matrix. Also phylotypes from the same taxa but not included in the correlation matrix due to their low abundance (see Table S1-4) were deleted. The sequence data can be found in the PubMed repository (http://www.ncbi.nlm.nih.gov/bioproject/656723) under reference number PRJNA656723.

All samples where the read count after filtering exceeded 4000 reads or those where a single phylotype comprised >700 sequence counts were further analysed (28 of 66 samples, see Supplementary Table S2 and S3). The further 32 samples were defined as "aseptic" according to NGS analysis. Simpsons diversity indices (1-L) were calculated using PRIMER (V.7.0.11, PRIMER-E, Plymouth Marine Laboratory, UK) on rarified read count data using species level taxa (Supplementary Table S4).



Supplementary Figure 1: Co-occurrence network based on Spearman correlations. Positive correlations of $\rho > 0.575$ and $p < 10^{-7}$) were used as input. Only phylotypes present in >10% of samples and of a mean abundance > 0.01% were considered. The network was visualized and analyzed using Cytoscape (version 3.7.2). Two subnets comprising A) Ralstonia and Burkholderia phylotypes and B) Bradyrhizobium and Mesorhizobium phylotypes are shown. Co-occurrence networks with less than 3 neighbors are not visualized.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 7, GraphPad Software, San Diego, CA, USA). The plots in Figure 2 show the mean with standard deviations. Statistical significance was evaluated used the Mann-Whitney U test. P-values <0.05 were considered significant. Sensitivity and specificity were calculated using a confusion matrix. The control std. microbiology tissue culture versus (vs.) DTT cultivation and std. microbiology tissue culture versus (vs.) NGS were compared.

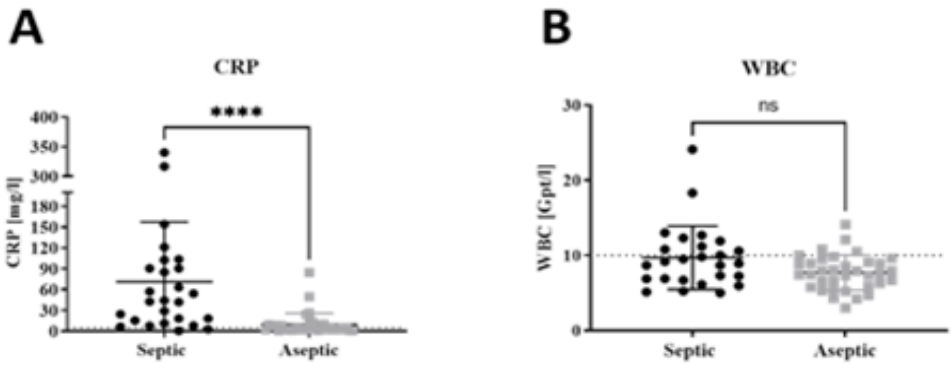


Figure 2: Significant increase in CRP, but not WBC levels in septic patients. (A) C-reactive protein (CRP) values (mg/l) in septic (black) and aseptic (dark grey) cohort. The CRP value was significantly increased in the septic cohort in comparison to the group. The pathologic threshold of 5mg/l is indicated as a black dashed line. (B) The mean white blood cells count (Gpt/l) was below the pathological threshold of 10 Gpt/l (black dashed line) for all tested groups.

Results

Demographic Data and Patient Characteristics

66 patients were included in this study and divided into two groups: aseptic and septic revisions. The average age of the patients of both cohorts was not statistically different (septic 73 ± 8 years and aseptic 68 ± 12 years). The septic group included 15 cases of septic hip revisions and 13 septic knee revisions. 17 hip revisions and 21 knee revisions were included in the aseptic group. The implantation time in the aseptic group was 85 ± 76 months, while in the septic group the implantation time was shorter (57 ± 84 months) (Table 1). The normal CRP reference value was defined as < 5 mg/l, and the pathological threshold for WBC count was defined as < 10 Gpt/l (Figure 2). Both values are indicated as red dotted line in the graphs. As expected, the CRP levels within the septic group were significantly higher compared to those of the aseptic cohort (septic: 71.1 ± 86.3 mg/l, aseptic: 7.4 ± 10.3 mg/l, ****p < .0001). However, 13 patients in the aseptic cohort exhibited an increased CRP value, and two patients in the septic cohort did not exhibit an increased CRP value. Therefore, the predictive value of CRP alone as serum parameter for PJI is low. The use of the WBC count has been proposed as an additional marker for PJI in the MSIS criteria. In our cohort there was no significant difference between both cohorts (septic: 9.7 ± 4.3 Gpt/l, aseptic: 7.1 ± 2.1 Gpt/l). 10 patients in the septic and 4 patients in the aseptic cohort exhibited a pathological increased WBC count.

Routine Microbiology and microDTTect Based Identification of Infection

To further investigate the pathogens causing PJI in the respective patients, we compared the standard microbiological diagnosis from tissue samples with microbiological diagnosis from microDTTect solution. In a first step we compared whether a pathogen was detected or not. The concordance of the results is shown in Tables 2 and 3. Routine microbiological diagnostic from tissue biopsies identified a PJI in 28 cases (Table 2). In 75% of these cases, a pathogen was also detected in bacteria culture of the microDTTect solution. Seven patients exhibited a pathogen in the standard microbiology diagnostic, but not in DTT cultures. In the aseptic cohort (Table 3), the results were concordant with both methods in 35 (92%) of all cases. However, three cases were diagnosed as aseptic in std. microbiological diagnostic, whereas bacteria were detected in the microDTTect solutions. To analyse the quality of the DTT culturing method the true positive rate (sensitivity) and the false positive rate (specificity) were calculated (Supplementary Table S5). The sensitivity of the DTT culture method compared with standard microbiological diagnostic was 75% while the specificity was 92%.

	Std. diagnostic vs. DTT culture
Septic	N = 28
Positive (+) in both methods	75% (21/28)
Negative (-) only in DTT	25% (7/28)

Table 2: Concordance of microbiology standard diagnostic vs DTT culturing for the septic cohort. The column shows the percentage of identified septic condition using std. microbiological diagnostic versus (vs.) the microDTTect culture analysed by the department of microbiology.

	Std. diagnostic vs. DTT culture
Aseptic	N = 38
- in both methods	92% (35/38)
+ only in DTT	8% (3/38)

Table 3: Concordance of microbiology standard diagnostic vs DTT culturing for the aseptic cohort. The column shows the percentage of identified aseptic condition using std. microbiological diagnostic versus (vs.) the microDTTect culture analysed by the department of microbiology.

NGS-Based Detection of Bacterial DNA

DNA isolated from microDTTect solution was used for culture-independent analysis through sequencing of the V1V2 variable region of the 16S rRNA gene, as this is thought to give insights into polymicrobial infections or the presence of bacteria difficult to culture (Tables 4 and 5). Analysis for the detection of bacteria revealed that 71% of the septic samples (Table 4) were classified as septic also by NGS. The remaining 29% were only positive in std. microbiological diagnostic, but did not show sufficient bacterial DNA that could be analysed through NGS. Interestingly, the culture-independent analysis via NGS identified a pathogen in 8 of the 38 aseptic cases (Table 5). These eight samples could indicate possible CN-PJIs or a previous infection at the respective joint with residual bacterial DNA in the tissue, but further research is needed. The sensitivity of NGS compared with standard microbiological diagnostic was 71% while the specificity was 79%.

	Std. diagnostic vs. NGS from DTT
Septic	N = 28
Positive (+) in both methods	71% (20/28)
Negative (-) only in DTT	29% (8/28)

Table 4: Concordance of microbiology standard diagnostic vs DTT treated samples examined by NGS for the septic cohort. The column of the table summarizes the percentage of septic identified by std. microbiological diagnostic vs. the NGS data from microDTTect solution.

	Std. diagnostic vs. NGS from DTT
Aseptic	N = 38
- in both methods	79% (30/38)
+ only in DTT	21% (8/38)

Table 5: Concordance of microbiology standard diagnostic vs DTT treated samples examined by NGS for the aseptic cohort. The column of the table summarizes the percentage of aseptic identified by std. microbiological diagnostic vs. the NGS data from microDTTect solution.

Detailed Analysis of Divergent Patient Samples

The following paragraph discusses in more detail the samples, which gave divergent results using different detection methods. Using the std. microbiological diagnostic with tissue cultures 28 samples were identified as septic (+). Ten of these cases were not identified as septic using DTT culturing or NGS (Figure 3a). In three cases (#18, #71, #78) the DTT culturing indicated a bacterial infection, whereas no pathogenic DNA was detected using NGS from DTT solutions. These patients had an increased CRP value (<5 mg/ml) indicating an ongoing inflammatory process that might indicate an infection. In two samples (#70, #89) a bacterium was identified using the std. microbiological routine diagnostic, no bacteria were detectable using DTT culturing, whereas the NGS from DTT solutions could identify the presence of bacterial DNA. Both patients showed an increased CRP, which indicates an active inflammation process, suggesting that DTT culturing failed in these cases. However, two samples (#5, #44) were diagnosed as septic exclusively by the std. microbiological diagnostic. In these cases, the average CRP value of the cohort was under the pathological threshold (5 mg/l). The implantation time of 55 and 382 months was also relatively long, indicating that these samples were contaminated during the culturing process in the std. microbiological diagnostic resulting in a false diagnosis of infection.

A

ID	Microbiology tissue	Microbiology DTT	DTT NGS	CRP	Implantation time [month]
5	+	-	-	2.8	55
18	+	+	-	15.4	104
27	+	-	-	85.1	0
44	+	-	-	0.5	382
56	+	-	-	42.5	1
66	+	-	-	18.2	1
70	+	-	+	41.7	3
71	+	+	-	90.4	10
78	+	+	-	7.65	16
89	+	-	+	7.8	1

B

ID	Microbiology tissue	Microbiology DTT	DTT NGS	CRP	Implantation time [month]
48	-	+	-	10.1	178
94	-	+	+	-	6
53	-	+	-	23.9	23

C

ID	Microbiology tissue	Microbiology DTT	DTT NGS	CRP	Implantation time [month]
2	-	-	+	1.7	19
9	-	-	+	2.5	23
13	-	-	+	10	140
19	-	-	+	0.6	36
23	-	-	+	2.7	189
42	-	-	+	2.2	253
50	-	-	+	3.1	66

Figure 3: Differences of diagnostic methods. Samples marked with a minus were diagnosed as aseptic and samples labelled with a plus are diagnosed as septic by the indicated method. Additionally, the respective serum CRP values, as well as the implantation time are indicated for each patient. (A) samples diagnosed as septic by classical diagnostics (B) samples diagnosed septic by culturing from microDTTect samples and (C) samples diagnosed septic by NGS.

Three samples were classified as aseptic by the routine diagnostic, but a pathogen was identified using microDTTect cultures (Figure 3b). High CRP values in two cases (#48, #53) indicate an active infection in these patients. The culture-independent NGS analysis, however, indicated a pathogen only in one case (#94), but the measurement of serum CRP levels for this patient was not performed. Figure 3c shows seven cases that were identified as aseptic in by both culture based techniques, but NGS indicated the presence of pathogens. In only one of these cases, the CRP level was elevated in the blood in the patients (#13). The implantation time was long, with a mean value of 103 ± 92 months.

Pathogen Profiles from microDTTect Samples are Comparable To Routine Diagnostic

The identified pathogens for all septic cases are summarized in Figure 4a. As expected, *Staphylococcus spp.* dominated the septic samples. The detection for *Staphylococcus spp.* was reliable and in 14 cases *Staphylococcus spp.* could be isolated both from tissue as well as from microDTTect samples. However, in four patients *Staphylococcus spp.* were identified only by std. microbiological diagnostic, but not by the other applied techniques based on microDTTect and NGS. NGS indicated the presence of a monomicrobial infection in 15 out of 28 cases that have been classified as septic by this method, based on a low simpson diversity index of <0.25 (Supplementary Figure S2). Fourteen cases were of polymicrobial nature as indicated by a simpson diversity index of >0.8. Three cases showed a simpson diversity index 0.41 – 0.73 and represented samples where typically one pathogen was dominating but accompanied by high amounts of other bacteria. Interestingly, in seven of the cases where *Staphylococcus spp.* had been identified as pathogen by both culturing methods, NGS confirmed a *Staphylococcus spp.* monoinfection.

A

ID	Microbiology Tissue	Microbiology DTT	DTT NGS
1	S. caprae	S. saccharolyticus	Polymicrobial
2			Polymicrobial
5	S. capitis		
7	S. aureus	S. aureus	S. aureus
9			Polymicrobial
11	E. faecalis	E. faecalis	E. faecalis
13			C. acnes
18	S. capitis	S. capitis	
19			Polymicrobial
23			Polymicrobial
26	S. aureus	S. aureus	Polymicrobial
27	S. epidermidis		
35	Polymicrobial	E. cloacae complex	Polymicrobial
36	S. epidermidis	S. epidermidis	S. epidermidis
40	S. sanguinis	S. sanguinis	S. sanguinis
41	S. epidermidis	S. epidermidis	S. epidermidis
42			Polymicrobial
44	S. saccharolyticus		
48		S. aureus	
50			C. avidum
53		C. avidum	
56	P. vulgaris		
66	S. epidermidis		
67	C. avidum	C. avidum	C. avidum
70	S. epidermidis		S. epidermidis
71	E. faecalis	E. faecalis	
72	S. epidermidis	S. epidermidis	S. aureus
76	S. aureus	S. aureus	S. aureus
78	S. epidermidis	S. epidermidis	
80	S. epidermidis	S. epidermidis	Polymicrobial
81	F. magna	F. magna	Polymicrobial
84	S. mitis/oralis	S. mitis/oralis	S. aureus
86	S. capitis	S. capitis	S. aureus
89	S. epidermidis		Polymicrobial
90	S. epidermidis	S. epidermidis	Polymicrobial
92	S. epidermidis	S. epidermidis	Polymicrobial
94		S. agalacticae	Polymicrobial
97	S. aureus	S. aureus	S. aureus

B

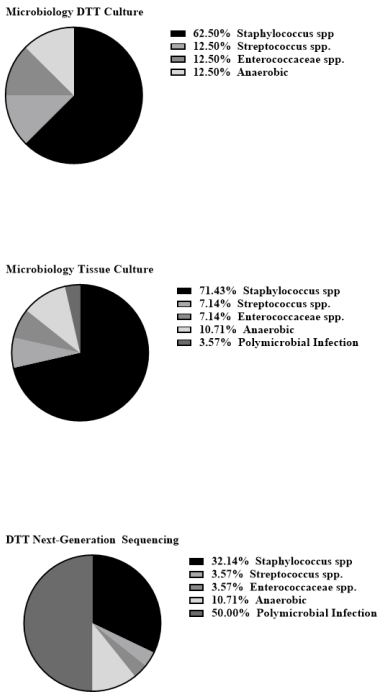
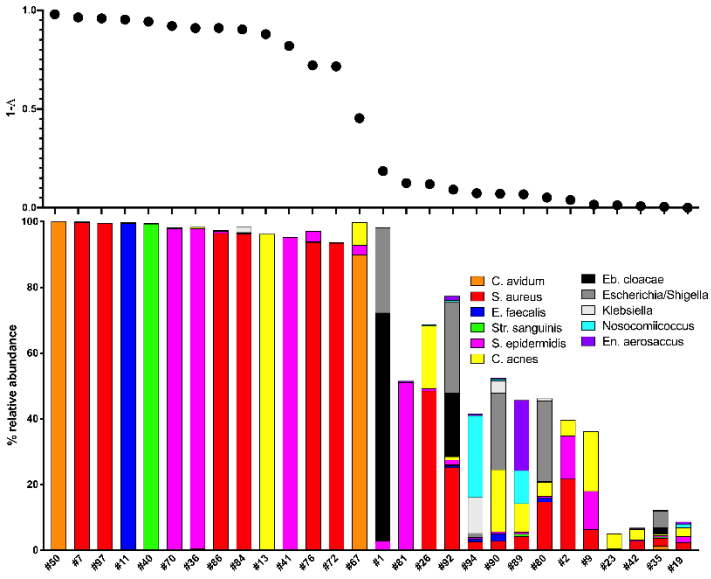


Figure 4: Pathogens identified from microDTTect samples are typically also identified by routine diagnostic. (A) The identified pathogen by the applied technique. The columns represent the results from std. microbiology tissue culturing, cultured DTT solution and DNA samples from NGS. The left column ID indicates the respective ID of the patient. The following four columns on the right hand indicate the identified pathogen. The identified pathogens are also labelled (*Staphylococcus*: black, *Streptococcus*: dark grey, *Enterococcus*: grey, Anaerobic: silvergrey, Multibacterial Infection: light grey, *Enterobacteria*: white). (B) The Pie chart of the identified pathogen spectrum. *Staphylococcus spp.* is the major pathogen and all other pathogens are less frequently detected.



Supplementary Figure 2: Diversity of microbial communities in DTT samples as indicated by the Simpson index.

Pathogens other than *Staphylococcus* were detected only rarely by culturing, making it difficult to evaluate the reliability of the different techniques. *Enterococcus spp*. was detected once (#11) and this identification was supported by culturing microDTTect samples and NGS, whereas conflicting results were observed for two other cases (#71, #35). Further, the detection of *Cutibacterium spp*. was highly variable between the applied methods. As major constituent of the skin microbiota, *C. acnes* was indicated by NGS to be present in minor amounts in various samples, however it indicated *Cutibacterium spp*. as dominating pathogen in only two cases. To further investigate polymicrobial infections and to compare NGS results with the other techniques, we listed the specific bacteria for all detection methods (Figure 4a). Interestingly, typically one bacterium of the polymicrobial infections was also identified by the other culturing techniques (Supplementary Figure S2b). Figure 4b shows the percentage of different bacteria identified by the three applied techniques. It became obvious that the std. microbiology was comparable to DTT cultures. However, NGS potentially detected more polymicrobial infections.

Discussion

PJI is amongst the most deleterious diagnoses for patients with TJA. The correct diagnosis of the pathogen causing a PJI is of utmost importance to differentiate between aseptic loosening of TJA and PJI, as well as choosing the correct antibiotic treatment Patients with PJI often require two-stage revisions with extensive tissue debridement during revision surgery as well as antibiotic spacer treatment. The identification of PJI by MSIS criteria and routine microbiologic diagnostics may be insufficient for a secure diagnosis. Various factors may compromise the sensitivity and specificity of the results. Contamination of fluid or tissue biopsies during sampling, presence of a biofilm on the explant and pre-treatment of patients with antibiotics are only some reasons modulating the reliability of PJI detection [25,52-54]. Additionally, low-grade infections, polymicrobial infections, biofilms or persisting infections in two-stage revision procedures with antibiotic spacers are difficult to diagnose and are associated with higher failure rates of revision implants [55,56,57]. Currently, the routine diagnostic of PJI is based on a combination of clinical aspects (e.g. inflammation symptoms), bacterial culture (at least 2 or more out of >5 tissue samples from different areas should be positive), blood lab test and histology, and is based on the MSIS criteria [40]. In particular for a better identification of biofilm-associated PJI, implants can be sonicated. This method can break down the biofilm [25], but this technique poses a higher risk of contamination [25] and sensitive bacteria can be damaged, which makes the detection of specific pathogens by cultivation difficult [24,29,30].

Therefore, Drago et al. indicated that the use of DTT may be helpful to break down the extracellular matrix of biofilms and result in a similar outcome like sonication, but without the increased risk of contamination. The bacteria survive the DTT treatment and can be cultivated under the std. microbiology diagnostic conditions [31]. In the current study, we examined if the diagnostic of PJI using the microDTTect device and NGS from microDTTect may be superior to the std. microbiological diagnostic using tissue biopsies. We used the microDTTect system to destabilize biofilms from the explant surface and subsequent bacteria cultures or NGS from DNA isolated from microDTTect solution. Drago et al. described that sonication of septic implants and the use of DTT had an increased sensitivity and specificity of bacteria detection on septic explants in comparison to tissue cultures. Furthermore, DTT had an increased sensitivity compared to the sonication [31]. Another study described a similar sensitivity of microbiological diagnostic using either pre-treatment of explants with DTT or sonication [14].

We included 66 patients out of these 28 were diagnosed as septic and 38 as aseptic based on the MSIS criteria [40]. Interestingly, our results indicate that the std. microbiological diagnostic might even be superior in identifying bacteria in septic cases compared to the detection using the microDTTect solution bacteria culture. Thus, our data are similar to those of Randau et al. 2021, who described that pathogen identification based on DTT cultures was less sensitive than culturing from intra-operative tissue biopsies [33]. The comparison between microDTTect based bacteria cultures and standard diagnostic from tissue biopsy showed a sensitivity of 75% and a specificity of 92% for the microDTTect

system, when taking MSIS criteria based PJI identification as reference. The use of the microDTTect device might provide additional diagnostic security as an additional diagnostic tool in case of unclear standard microbiological PJI diagnosis, since the closed system reduces the probability of contamination during sampling. We decided to use the std. diagnostic microbiology and MSIS criteria as a reference for PJI diagnosis. Ten cases were septic according to the std. microbiology diagnostic but in six cases this could not be confirmed using DTT or NGS as diagnostic technique, four samples were positive at least in one other used methods (Figure 3A). Most septic cases exhibited a pathological serum CRP level. Those two patients with a low CRP level might have been classified as false positive and the samples may have been contaminated during the culturing process. Using DTT cultivation we were able to identify pathogens in three (#48, #94, #53) of the aseptic cases (Figure 3B). The use of DTT may have destabilized the biofilm, leading to a release of the bacteria adhered on the implant [31]. As the patients had a high CRP or at least a low implantation time a possible CN-PJI could be identified using the microDTTect device based bacteria cultures. The literature describes that CN-PJI can occur in up to 7% of cases [58]. We indicated a number of 8% (3/38) of CN-PJI, therefore, it would be consistent with the literature.

In seven samples, NGS from microDTTect solution identified bacterial DNA, while the other techniques based on bacteria cultures did not detect viable pathogens (Figure 3B). The CRP of six patients was under the pathological threshold of 5 mg/l and only two patients exhibited an increased CRP value of 10 mg/l. The implantation time before revision surgery ranged from 19 month to 253 months. Again, we compared the diagnostic security of NGS from microDTTect solution with the std. microbiological diagnostic to calculate the sensitivity and specificity using a confusion matrix (Supplementary Table S5- S6). Here we indicated a sensitivity of 71% and a specificity of 79%, which is lower compared to the sensitivity and specificity of microDTTect based bacteria culture. However, NGS has been proposed to be a useful alternative method in the diagnosis of PJI [59,60]. NGS can identify bacteria that are difficult to culture, and polymicrobial infections can also be detected more precisely as no overgrowth of fast growing bacteria over slower growing bacteria can influence the pathogen identification [34,61]. Since no bacterium was identified in the culture based techniques, we would consider the eight identified samples containing residual bacterial DNA from the previous infections. Nevertheless, the additional information about the different pathogens in polymicrobial infections may be a useful information, when deciding the antibiotic therapy regime.

We further investigated whether NGS could provide more accurate taxonomic information about the pathogens in suspected polymicrobial infections. NGS has been proposed to be the technique of the future in the diagnosis of PJI and specific identification of pathogens [62]. The usefulness of NGS in microbiological diagnostic of PJI has already been described [31,32,63]. A meta-analysis of joint infections concluded that the sensitivity of 86% is lower than previously assumed [64]. However, it was possible to clearly identify pathogens in CN-PJI with the help of NGS, in cases where bacteria cultures from tissue failed to show a pathogen [65]. The high sensitivity of NGS is also a weakness, as it makes the method prone to contamination and false positive results [66-68]. Comparing the std. microbiological diagnostic with the DTT culturing the same bacteria was identified in 20 samples (20/30). The most common pathogen was *Staphylococcus spp.* which was identified in 14 samples by both methods (Figure 4A). The pathogen spectrum between std. microbiology and NGS was similar in 11 cases (11/38). NGS identified more polymicrobial infections (14/38) in comparison to the std. routine tissue culturing (1/38) or the DTT culture (0/38). Since we observed only rare cases of anaerobic or polymicrobial infections with tissue culturing, it is difficult to decide whether NGS or the microDTTect cultures could be of additional value to the routine diagnostic. There might be a need for both techniques, as NGS seems to be more sensitive in identifying polymicrobial infections than culturing based identification of pathogens.

Our data indicate that the routine microbiological diagnostic using 6 tissue samples for culture gives a very reliable result and the microDTTect based detection did not add further valuable information with respect to accuracy or specificity. However, since this study indicated 8% (3/38) possible CN-PJIs, the use of microDTTect as an additional test method for frequently recurring revision cases in which no pathogen is identified or identification of PJI using the MSIS criteria has failed, might be useful. In total, the present study shows that std. tissue culture using >2 samples is one of the best and a well-established method to diagnose PJI if sampling methods and microbiological infrastructure are well defined. Up to date, NGS and other technologies such as microDTTect show promising results but are not superior to std. microbiological diagnostic using tissue culturing. However, the cost-benefit factor of the Illumina NGS analysis almost doubling the costs should also be taken into account.

We are well aware that our study has some limitations. Most importantly, our cohort contained only few polymicrobial or anaerobic infections detected by tissue culturing, which makes it difficult to draw conclusions about a potential superiority of NGS compared to the routine diagnostic. Future studies that specify on polymicrobial infections might be able to address this question.

Conclusion

Our data demonstrate that the std. microbiological diagnostic using periprosthetic tissue sampling is a secure method and accurate in a well-established process with well-established cooperation between clinical and microbiology departments. MicroDTTect and NGS showed comparable results but are not superior to the tissue culturing. However, there is no guarantee to diagnose an infection. All applied techniques have their advantages and disadvantages that need to be considered.

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Author Contributions

AKM carried out the microDTTect reconditioning, DNA isolation, preparing for NGS and wrote the manuscript. AN provided human samples and helped with the interpretation of data, SI provided human samples and helped with the interpretation of data, JF took over the processing of the tissue and microDTTect cultivation, RVV performed the analysis of the NGS data, EM helped writing the manuscript and planned the experiments, DHP performed the analysis of the NGS data and helped writing the manuscript, JB helped writing the manuscript, planned experiments and helped with the interpretation of the data, CHL designed the experiment, provided the human tissues, helped with interpretation of the data and writing of the manuscript.

Data Availability

Sequence data can be found under reference number PRJNA656723.

Competing Interests

Ms. Meinshausen has received a personal fees payment for an educational event from KCL Focus Group at EBJIS 2019. Prof. Lohmann has received a grant for travel support for the Consensus Conference on PJI 2018, Philadelphia, USA. He is also an unpaid board member of the German Society for Orthopaedics and Traumatology. All other authors have no potential conflict of interest.

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