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BASIC RESEARCH

Detecting the Presence of Bacterial DNA and RNA by Polymerase Chain Reaction to Diagnose Suspected Periprosthetic Joint Infection after Antibiotic Therapy

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Objective: To explore the diagnostic efficiency of DNA-based and RNA-based quantitative polymerase chain reaction (qPCR) analyses for periprosthetic joint infection (PJI).

Methods: To determine the detection limit of DNA-based and RNA-based qPCR *in vitro*, *Staphylococcus aureus* and *Escherichia coli* strains were added to sterile synovial fluid obtained from a patient with knee osteoarthritis. Serial dilutions of samples were analyzed by DNA-based and RNA-based qPCR. Clinically, patients who were suspected of having PJI and eventually underwent revision arthroplasty in our hospital from July 2014 to December 2016 were screened. Preoperative puncture or intraoperative collection was performed on patients who met the inclusion and exclusion criteria to obtain synovial fluid. DNA-based and RNA-based PCR analyses and culture were performed on each synovial fluid sample. The patients' demographic characteristics, medical history, and laboratory test results were recorded. The diagnostic efficiency of both PCR assays was compared with culture methods.

Results: The *in vitro* analysis demonstrated that DNA-based qPCR assay was highly sensitive, with the detection limit being 1200 colony forming units (CFU)/mL of *S. aureus* and 3200 CFU/mL of *E. coli*. Meanwhile, The RNA-based qPCR assay could detect 2300 CFU/mL of *S. aureus* and 11 000 CFU/mL of *E. coli*. Clinically, the sensitivity, specificity, and accuracy were 65.7%, 100%, and 81.6%, respectively, for the culture method; 81.5%, 84.8%, and 83.1%, respectively, for DNA-based qPCR; and 73.6%, 100%, and 85.9%, respectively, for RNA-based qPCR.

Conclusions: DNA-based qPCR could detect suspected PJI with high sensitivity after antibiotic therapy. RNA-based qPCR could reduce the false positive rates of DNA-based assays. qPCR-based methods could improve the efficiency of PJI diagnosis.

Key words: Antibiotic therapy; Bacterial DNA; Bacterial RNA; Periprosthetic joint infection; Real-time PCR

Introduction

Periprosthetic joint infection (PJI) is a serious complication of arthroplasty. The incidence rates are 1%–3% in patients with primary arthroplasty and 4%–6% in revision patients[1,2]. Infection often leads to multiple surgeries, prolonged use of antibiotics, extensive consumption of medical resources, and a series of social, economic, and psychiatric effects[2].

Rapid and accurate diagnosis is crucial for the treatment of PJI, but the diagnosis of infection remains challenging.

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Bacterial culture is the gold standard for diagnosis. However, some microorganisms are fastidious, and in our region, patients often undergo antibiotic treatment before specimens are obtained, resulting in decreased bacterial proliferation ability. All of these factors might result in false negative cases[3]. In addition, it often takes several days to obtain culture results, and these results may, therefore, not be available to guide timely treatment. Because of these problems, most surgeons use a combination of clinical symptoms, microbial evidence, imaging evidence, and serological evidence for diagnosis[4,5], but the sensitivity and specificity of imaging evidence are weak[6]. Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and other serum inflammation indicators are highly sensitive but have low specificity, especially in patients with inflammatory arthritis, whose levels of these indicators may be higher than the diagnostic threshold before surgery[7].

Many molecular diagnostic studies on PJI have aimed to improve these shortcomings. 16S rRNA is highly conserved in bacterial species, abundant in number, and not expressed in humans. Amplification and detection by polymerase chain reaction (PCR) is commonly performed using specific primers for the 16S rRNA gene. Most of the studies extract bacterial DNA, which has a higher sensitivity than culture, because even with the decline in bacterial activity and death after the use of antibiotics, the DNA level remains and can be measured by PCR[8–10]; however, as a side effect, the false positive rate is also high[11]. Recent studies have suggested that RNA can easily degrade in the natural environment. Therefore, bacterial RNA can be extracted for reverse transcription and amplification, which might reduce exogenous contamination[12]. *In vitro* studies conducted in 2006 compared the ability of DNA-based and RNA-based quantitative PCR (qPCR) to detect the same microbial specimen and found that RNA-based detection could reflect the bioactivity changes of bacteria after the use of antibiotics[13]. Clinical studies have also extracted RNA from the synovial fluid of patients with PJI. These studies found that the sensitivity of RNA-based qPCR was similar to that of culture and had lower false positive rates[14,15]. However, the number of cases involved in these clinical studies was small, and the diagnostic efficiency of DNA-based and RNA-based qPCR was not directly compared in these clinical studies. Thus, the evidence remains insufficient.

The aim of our study was to evaluate the utility of DNA-based and RNA-based qPCR for PJI diagnosis. We collected synovial fluid from patients undergoing revision arthroplasty and extracted DNA and RNA from each sample. qPCR was performed using universal primers to identify the 16S rRNA gene sequence. The diagnostic efficiency of both PCR methods was compared to that of the culture method.

Material and Methods

In Vitro DNA-based and RNA-based Quantitative Polymerase Chain Reaction Detection Limits

Prior to clinical sample testing, to verify the sensitivity of the two methods, *Staphylococcus aureus* and *Escherichia coli* strains were cultured overnight, and each sample was added to 2 mL sterile synovial fluid obtained from a patient with knee osteoarthritis and then divided into two equal portions for DNA and RNA extraction. Each sample was diluted with a concentration gradient of 10– 1×10^9 colony forming units (CFU)/mL. DNA and RNA were extracted, and real-time qPCR was performed on diluted samples to detect the limits of the two methods *in vitro*.

Clinical Sample Analysis

Patients who underwent revision arthroplasty in our hospital from July 2014 to December 2016 were screened. All operations were approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University (Ethics Number: [2014] 047). The inclusion criteria were as follows: (i) patients who were suspected of having PJI according to their medical history, symptoms and clinical data and who eventually underwent revision surgery; (ii) both DNA-based and RNA-based PCR analyses were performed on joint fluid samples obtained preoperatively or intraoperatively; and (iii) the residual samples were sufficient for culture after PCR detection. The exclusion criteria were as follows: (i) incomplete clinical and laboratory information; (ii) specimen contamination or suspected contamination; (iii) acute infectious diseases, such as pneumonia, that might affect the accuracy of qPCR and culture; and (iv) tuberculous infection or fungal infection. Patients who met the criteria described by the Musculoskeletal System Infection Association (MSIS) were categorized as having PJI[16]. According to MSIS criteria, a total hip arthroplasty or total knee arthroplasty (THA/TKA) was considered infected if it had a draining sinus tract communicating directly with the joint; two separate specimens with positive culture results; or four of five minor criteria were met, including (i) elevated ESR and CRP, (ii) elevated synovial white blood cell count, (iii) elevated polymorphonuclear cell differential, (iv) a single positive culture result, and (v) >5 neutrophils per high-powered field on tissue histology.

Preoperative joint cavity puncture or intraoperative collection was performed on patients who met the above criteria to obtain synovial fluid. The synovial fluid samples were dispensed and packed immediately after collection. The same volume from each patient was used for DNA and RNA extraction, and real-time qPCR was subsequently performed. The demographic characteristics, medical history, laboratory tests, and culture outcomes of each patient were recorded.

Preoperative and Intraoperative Cultures

The preoperative puncture procedures were conducted in a sterile operating room according to described guidelines[17]. Intraoperatively, the joint fluid samples were aspirated before articular capsule incision. Aerobic and anaerobic BACTEC Plus culture bottles (442023 and 442022, Becton-Dickinson GmbH, Heidelberg, Germany) were used for bacterial culture immediately after the synovial fluid samples were obtained.

We extended the culture time from the traditional 1 week to 2 weeks to ensure the growth of fastidious bacteria[18].

DNA and RNA Extraction

The *in vitro* and *in vivo* samples underwent the same extraction protocol. For DNA extraction, each sample was centrifuged at 14 000 *g* for 10 min. After removal of the supernatant, a lysate containing 20 mg/mL of lysozyme and protease K (L004504, Sigma-Aldrich, St. Louis, Missouri, USA) was added and incubated at 37°C for 30 min. Genomic DNA samples were extracted using a DNeasy Blood & Tissue Kit (69504, QIAGEN, Valencia, California, USA). Briefly, the lysate was loaded onto the DNeasy Mini spin column. During centrifugation, DNA selectively bound to the DNeasy membrane, and the contaminants passed through. The remaining contaminants and enzyme inhibitors were removed in two efficient wash steps and DNA was then eluted in water or buffer, ready for use.

Each sample for RNA extraction was supplemented with an RNA protection reagent (76163, QIAGEN, Valencia, California, USA) immediately after collection and then centrifuged and lysed by the same procedure used for the DNA samples. Total RNA was extracted by an RNeasy Mini Kit (74102, QIAGEN, Valencia, California, USA). Briefly, ethanol was added to the lysate to promote selective binding of RNA to the RNeasy membrane. The sample was then applied to the RNeasy Mini spin column. Total RNA bound to the membrane, and contaminants were efficiently washed away. High-quality RNA was eluted in RNase-free water. Prior to the elution of RNA, DNase-I was used to remove DNA contamination.

Real-time Quantitative Polymerase Chain Reaction Process

For RNA samples, 1 μg of total RNA was reverse-transcribed using random primers according to the manufacturer's instructions. After the cDNA samples were obtained, qPCR was performed according to standard protocols using a SYBR Green Kit (RR820A, Takara, Dalian, China) in an -ABI7500-PCR instrument. Briefly, 1 μL of cDNA was added to 19 μL of reaction mixture containing 0.5 μmol/L primer sets and 0.5X SYBR Green. We used a universal primer sequence to identify the bacterial 16S rRNA gene as follows: forward 5′-ATTAGATACCCTGGTAGTCCACGCC-3′; reverse 5′-CGTCATCCCCACCTTCCTCC- 3′. The internal reference primers for the GAPDH sequence were as follows: forward 5′-TCCCTGAGCTGAACGGGAAG-3′; reverse 5′-CGCCTGCTTCACCACCTTCT-3′. The following cycling conditions were used for qPCR: 50°C for 10 min, 95°C for 5 min, 40 cycles of 95°C for 10 s, and 60°C for 30 s. For DNA samples, 1 μL of total DNA was added to the SYBR Green reaction system, and the same protocol used for RNA-based qPCR was performed. Sterile synovial fluid was used as a negative control for both *in vivo* and *in vitro* detection. The standard strain of *E. coli* was added to sterile synovial fluid, at a concentration of 1×10^{10} CFU/mL, as a positive control. For both the DNA-based and RNA-based methods, a two-cycle difference from the sterile baseline was considered a detectable result.

Statistical Analysis

Quantitative data are represented as means ± SD. All statistical calculations were performed using SPSS v.23.0 (SPSS, Chicago, IL, USA). The sensitivity, specificity, accuracy, and positive and negative predictive values (PPV, NPV) of each diagnostic method (culture, DNA-based and RNA-based qPCR) were calculated and a 95% confidence interval was also determined for each test. The statistical significance threshold was set at $P = 0.05$ (two-tailed).

Results

In Vitro *RNA and DNA Detection Limits*

In vitro, in the *S. aureus* dilution series, the detection limits of DNA-based and RNA-based qPCR were 1200 CFU/mL and 3200 CFU/mL, respectively. The detection limits of *E. coli* DNA and RNA were 2300 CFU/mL and 11 000 CFU/mL, respectively. The melting temperature of all amplicons after serial dilution was similar between groups. The results were confirmed by gel electrophoresis.

Analysis of Included Cases

According to the inclusion and exclusion criteria, 73 patients were eligible for the study. Two patients were excluded because the internal reference primers could not be amplified during qPCR processing. Therefore, a total of 71 patients were included in the study. According to the MSIS PJI diagnostic criteria, 38 cases were diagnosed with PJI. Among these 38 cases, 21 cases were confirmed by two positive cultures, and 4 cases were diagnosed by a single positive culture combined with other diagnostic criteria. The remaining 13 cases were diagnosed with PJI based on sinus formation, purulent joint fluid, intraoperative pathology, serological indicators, and other factors according to the MSIS standards. The remaining 33 cases were classified as non-PJI.

The subjects included 11 men and 27 women in the PJI group, aged 47–78 years (mean, 63.7 ± 12.7 years); 9 patients underwent hip arthroplasty, and 29 patients underwent knee arthroplasty. A total of 9 men and 24 women aged 51–76 years (mean, 67.1 ± 13.9 years) were included in the non-PJI group. A total of 7 patients underwent hip arthroplasty and 26 patients underwent knee arthroplasty. The reasons for the primary arthroplasties are listed in Table 1.

Comparison of the Diagnostic Efficiency by Culture and Both Polymerase Chain Reaction Methods

The results of qPCR and culture assays of joint fluid samples are listed in Table 2. Besides PJI patients, non-PJI patients were also included to determine the diagnostic efficiency (sensitivity, specificity, positive predictive value, and negative predictive value) of qPCR and culture assays. The analysis of

TABLE 1 Clinical data on patients with suspected infection status

Diagnosis	Age (mean ± SD, years)	Sex (M/F)	Surgical type (hip/knee)	Initial arthroplasty reason (cases)				
				Knee osteoarthritis	Hip osteoarthritis	Rheumatoid arthritis	Femoral head necrosis	Hip dysplasia
PJI (38 cases)	63.7 ± 12.7	11/27	9/29	24	5	5	2	2
Non-PJI (33 cases)	67.1 ± 13.9	9/24	7/26	23	3	3	3	1

F, female; M, male; PJI, periprosthetic joint infection.

TABLE 2 PCR and culture results of joint fluid samples (cases)

Final diagnosis	Culture		DNA-based PCR		RNA-based PCR	
	Positive	Negative	Positive	Negative	Positive	Negative
PJI (38 cases)	25	13	31	7	28	10
Non-PJI (33 cases)	0	33	5	28	0	33

PCR, polymerase chain reaction; PJI, Periprosthetic joint infection.

these results showed that the sensitivity, specificity, positive predictive value, and negative predictive value were: 65.7%, 100%, 100%, and 71.7%, respectively, for conventional culture; 81.5%, 84.8%, 86.1%, and 80.0%, respectively, for DNA-based qPCR; and 73.6%, 100%, 100%, and 85.9%, respectively, for RNA-based qPCR. The accuracy of culture and DNA-based and RNA-based qPCR were 81.6%, 83.1%, and 85.9%, respectively (Table 3).

The 25 cases with positive culture results included 7 multiple bacterial infections, 8 *S. epidermidis* cases, 3 *S. aureus* cases, 3 *E. coli* cases, 2 *Pseudomonas aeruginosa* cases, 1 hemolytic staphylococcus case, and 1 anaerobic infection. In all 25 cases, the qPCR results were positive for DNA and RNA detection using universal primers for 16s rRNA.

Utility of Polymerase Chain Reaction in Antibiotic Therapy Cases

Among the 13 clinically diagnosed PJI but culture-negative cases, 8 patients underwent antibiotic treatment before sample collection. Although most patients had significant symptoms of inflammation and visible intraoperative purulent synovial fluid, the results of the culture were still negative. Of these 8 patients, 3 were positive according to both DNA-based and RNA-based qPCR detection, 3 were positive according to DNA-based qPCR only, and 2 were negative according to both DNA-based and RNA-based qPCR detection. In cases 1 and 2, redness and pain occurred 2 weeks after arthroplasty, and antibiotics were administered. The treatment periods were both less than 1 week. Inflammatory indicators and joint fluid examinations were positive (according to the MSIS criteria), and culture results were negative. A large volume of purulent synovial fluid was observed during the operation. The intraoperative pathology was positive, and DNA and RNA detection analyses were positive. Cases 3–6 included patients who experienced a long period of swelling after arthroplasty; long-term antibiotics were used before obtaining specimens. All of these cases were culture-negative and DNA detection-positive, but the RNA detection was positive only in case 3. In cases 7 and 8, the results of all three detection methods were negative. However, due to the obvious swelling and pain, the joint fluid

TABLE 3 Comparison of diagnostic efficiency of three detection methods

Methods	Sensitivity (95% *CI*)	Specificity (95% *CI*)	PPV (95% *CI*)	NPV (95% *CI*)	Accuracy (95% *CI*)
Culture	0.66 (0.48–0.80)	1.00 (0.89–1.00)	1.00 (0.86–1.00)	0.72 (0.57–0.84)	0.82 (0.73–0.91)
DNA PCR	0.82 (0.66–0.92)	0.85 (0.68–0.95)	0.86 (0.71–0.95)	0.80 (0.63–0.92)	0.83 (0.74–0.91)
rRNA PCR	0.74 (0.57–0.87)	1.00 (0.89–1.00)	1.00 (0.88–1.00)	0.77 (0.61–0.88)	0.86 (0.79–0.94)

CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value.

white blood cell count was increased, intraoperative pus was observed, and sinus formation occurred, the diagnosis of PJI was established for cases 7 and 8 (Table 4).

Discussion

Advantage of Polymerase Chain Reaction Methods in Sensitivity

The accurate diagnosis of PJI is critical to treatment. The sensitivity and accuracy of the existing detection methods remain insufficient. Molecular diagnosis based on PCR has become an important method for the diagnosis of bone and joint infections. Currently, most methods involve extracting bacterial DNA to detect the 16S rRNA gene. DNA is still present in dead bacteria, which makes this method more sensitive, but exogenous bacterial DNA introduced during sample processing may cause contamination, leading to false positives[19]. RNA can easily degrade and is closely related to cell activity. Studies have suggested that detection of bacterial mRNA could reduce the false positive rate. However, the proportion of mRNA in total RNA was less than 1%, resulting in an increased specificity of mRNA detection, although the sensitivity was low[12,20]. rRNA accounts for more than 95% of the total RNA components. During the extraction process, rRNA does not degrade as easily as mRNA, and it is easy to acquire a high concentration of rRNA[21]. *In vitro* experiments can even detect pg levels of 16S rRNA[14]. Therefore, we extracted and reverse transcribed the total RNA and then performed the same qPCR protocol used for DNA-based detection to identify the 16S rRNA gene.

Unlike in previous studies, the diagnostic efficiency of DNA-based and RNA-based qPCR methods was compared in the same sample in this study. *In vitro*, DNA detection was more sensitive than RNA detection in the same strain of *S. aureus* or *E. coli*. In clinical samples, DNA-based detection sensitivity was also higher than that of RNA-based detection and culture, although some false positive cases occurred. The advantage of RNA-based assays is that these assays were more specific than DNA assays, and they maintained a higher sensitivity than culture methods.

Advantage of Polymerase Chain Reaction Methods in Detecting Antibiotic Therapy Cases

Although some false positive cases occurred, DNA-based qPCR has its advantages. Because the diagnosis and treatment of PJI is still not standardized, many suspected cases of PJI are treated with antibiotics before a definite diagnosis. Therefore, we also examined cases in which antibiotics were administered before specimen collection. All 8 cases resulted in negative cultures. DNA-based detection was positive in six of the 8 cases, and RNA-based detection was positive in 3 of the 8 cases. After the application of antibiotics, the bacteria were usually not culturable within 1 week, and as an indicator of cell activity, rRNA became undetectable 1 week after the bacteria became unculturable[13]. DNA could be detected even after bacterial death[22]. This feature may explain our results. Antibiotics were used for a short time before the sample was obtained in cases 1 and 2, and rRNA was still detectable. In cases 4–6, after the long-term application of antibiotics, only DNA could be detected. Therefore, the detection of DNA remains an effective tool for patients with prolonged antibiotics use.

In case 3, long-term antibiotics had been used, and RNA-based detection was still positive, indicating that although bacterial growth was subject to inhibition and was not culturable, the bacteria still exhibited biological activity. In such cases, the preoperative antibiotic regimen should be adjusted, and the administration time should be extended postoperatively. For patients diagnosed with PJI, two-stage arthroplasty (including extensive debridement and delayed revision) is currently considered a standard procedure. The timing of the secondary surgery depends on the infection control situation. The presence of live bacteria is a contraindication for revision surgery. Surgeons need to make decisions based on existing clues, including whether to execute a revision or to continue to extend the use of antibiotics. Several features, including wound conditions, blood inflammation, synovial fluid culture, bone scan results, and frozen pathology results, are often used to determine the extent of infection clearance[23–25]. Nevertheless, many flaws remain in these examinations. False negatives will lead to repeated surgery and increased complications. Because RNA-based

TABLE 4 The cases with antibiotics therapy before specimen collection

Case	Age(years)/Sex	Diagnosis	Symptom duration	Antibiotic duration	Sinus	Culture	DNA-PCR	rRNA-PCR
1	67/M	THA infection	2 weeks	1 week	Absent	–	+	+
2	53/F	TKA infection	1 weeks	3 days	Absent	–	+	+
3	62/M	TKA infection	2 months	3 weeks	Present	–	+	+
4	72/F	THA infection	1 month	2 weeks	Absent	–	+	–
5	51/F	TKA infection	2 months	3 weeks	Present	–	+	–
6	55/M	TKA infection	1 month	2 weeks	Absent	–	+	–
7	77/M	TKA infection	3 weeks	1 week	Present	–	–	–
8	61/F	THA infection	2 months	2 weeks	Present	–	–	–

–, negative; +, positive; THA, total hip arthroplasty; TKA, total hip arthroplasty.

detection could reflect the biological activity of bacteria in the tested samples, RNA-based qPCR could be used to determine the biological activity of bacteria[13,14]. Compared to inflammatory markers and biomarkers, this method has better sensitivity and specificity. Compared to culture, RNA-based detection can obtain positive or negative results in a few hours[26–29]. The synovial fluid of patients who had been treated with debridement and antibiotic bone cement implantation could be monitored to determine the appropriate time for revision surgery, as a possible future application.

Limitations of this Study

In this study, a variety of bacteria were identified, but even with the high sensitivity of DNA detection, some false negatives still occurred (cases 7 and 8). The reason for these false negatives may be that the qPCR method for bacterial 16S rRNA identification is not applicable to all strains, and studies have shown that this method is sensitive to *S. aureus* and *P. aeruginosa* but is not sensitive to coagulase-negative staphylococcus[30]. The universal primers used in our study could only detect the presence of bacteria and could not distinguish species to guide the use of antibiotics. Notably, although 16S rRNA is highly conserved, it also contains variable regions. Studies have been conducted using rRNA-based qPCR to detect hypervariable regions of 16S-rRNA and distinguish between different staphylococcus species[14], suggesting that in a follow-up study[31,32], it could be possible to design species-specific primers for bacterial identification at the species level[33].

As a single center study, the incidence of PJI was low, and the number of specimens obtained was relatively small, which may lead to bias. Multicenter studies with greater numbers are needed to confirm the above findings.

Conclusion

Compared to culture results, the results of qPCR could be obtained within a few hours. DNA-based qPCR detection could detect suspected PJI with high sensitivity after antibiotic therapy. RNA-based qPCR could help reduce the false positive rates of DNA-based assays. Combined use of both methods could help improve the diagnostic efficiency of PJI.

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