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Simulated Joint Infection Assessment by Rapid Detection of Live Bacteria with Real-Time Reverse Transcription Polymerase Chain Reaction

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Background: Although microbiological bacterial culture is currently considered the gold standard for diagnosis of septic arthritis, many studies have documented substantial false-negative and false-positive rates. The objective of this study was to determine whether real-time quantitative reverse transcription polymerase chain reaction can be used to detect bacterial messenger RNA (mRNA) in synovial fluid as a way to distinguish live and dead bacteria as an indicator of active infection.

Methods: Synovial fluid samples were obtained from twelve consecutive patients who presented with knee pain and effusion but no evidence of infection. Following assurance of sterility with plate cultures, each sample was inoculated with clinically relevant bacteria and incubated for twenty-four hours to simulate septic arthritis. Bacterial viability and load were assessed with cultures. Selected samples were also treated with a single dose of a combination of two antibiotics, vancomycin and gentamicin, and sampled at several time points. Total RNA isolated from each sample was analyzed in triplicate with one-step real-time quantitative reverse transcription polymerase chain reaction to detect mRNA encoding for the genes *groEL* or *femC*. Controls included sterile, uninoculated samples and inoculated samples analyzed with quantitative polymerase chain reaction without reverse transcription. mRNA content was estimated on the basis of detection limits as a function of serial dilutions and was expressed as a function of colony number in bacterial cultures and RNA content as determined spectrophotometrically.

Results: All synovial fluid samples that had been inoculated with one of the four bacterial species, and analyzed in triplicate, were identified (distinguished from aseptic synovial fluid) with real-time quantitative reverse transcription polymerase chain reaction; there were no false-negative results. All inoculated samples produced bacterial colonies on culture plates, while cultures of the aseptic samples were negative for growth. The detection limit of the one-step bacterial mRNA-based real-time quantitative reverse transcription polymerase chain reaction varied depending on the bacterial species. A time-dependent decrease in the concentration of detectable bacterial mRNA was seen after incubation of bacteria with antibiotics.

Conclusions: The direct quantification of the concentration of viable bacterial mRNA with real-time quantitative reverse transcription polymerase chain reaction allows identification of both culture-positive bacterial infection and so-called unculturable bacterial infection in a simulated septic arthritis model. In contrast to conventional polymerase chain reaction, real-time quantitative reverse transcription polymerase chain reaction minimizes false-positive detection of nonviable bacteria and thus provides relevant information on the success or failure of antibiotic therapy.

Clinical Relevance: Because real-time quantitative reverse transcription polymerase chain reaction detects live bacteria, its application in combination with other polymerase chain reaction-based methods for speciation could dramatically improve the way that joint infections are diagnosed and treated.

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Septic arthritis in adults, both in native joints and at the sites of prosthetic joints, is associated with substantial morbidity. The accurate and rapid diagnosis of a true infection is imperative because the best outcomes occur when infection is correctly diagnosed early¹. Although microbiological bacterial culture is currently considered to be the gold standard for diagnosis, many studies have documented substantial rates of false-negative and false-positive results². Misdiagnosis with standard microbiological testing has led to chronic complications³, such as persistent infection, conversion of acute infection to chronic infection, and even death¹. Bacterial cultures of synovial fluid from native joints are positive in only 90% of cases of nongonococcal arthritis and 25% of cases of gonococcal arthritis, with the latter being the most common form of septic arthritis in otherwise healthy adults. Cultures of samples taken from the sites of prosthetic joints during revision arthroplasty have rates of false-negative results ranging from 27% to 50%⁴⁻¹¹ and rates of false-positive results of up to 50%¹²⁻¹⁸. In addition, the rate of false-negative cultures for patients who have begun an antibiotic regimen approaches 60%^{6,19}. Standard cultures for common pathogens (*Staphylococcus aureus* and *Staphylococcus epidermidis*) take approximately two to three days to produce growth. However, other pathogens implicated in joint infections are more difficult to grow on culture and can take up to several weeks to grow^{1,20-23}. Additional complications, such as sepsis, may arise during longer wait times for the characterization of bacterial sensitivities to antibiotics²⁴. False-negative cultures, the absence of a modality to accurately diagnose infection in a patient receiving antibiotics, and long bacterial culture incubation periods undermine timely decision-making regarding proper treatment of a suspected septic joint. These issues have societal as well as individual ramifications in terms of potentially unnecessary treatment and hospitalizations, longer hospital stays, more operations, increased cost, and increased antimicrobial resistance because of broad-spectrum empiric treatment of unknown bacteria²⁵⁻²⁷.

Recent investigations support the use of polymerase chain reaction for the detection of bacterial DNA as a more sensitive method of identifying joint infection²⁸⁻³¹. Polymerase chain reaction involves a process of selective enzymatic amplification and detection of known target DNA or RNA sequences present in the suspected organism. Amplified DNA is then analyzed for identification and quantification of bacteria. Several research groups have developed polymerase chain reaction-based tests using the highly conserved bacterial 16S rRNA gene to identify joint infections. We have previously reported a minimum detection level of ten bacterial cells per 100 μ L of synovial fluid, with unequivocal identification of bacterial titers within twenty-four hours²⁸⁻³¹. The need to know a specific target sequence is a limitation. However, information from genomic catalogues of pathogenic species has permitted identification of multiple species^{32,33} and allowed the assessment of antibiotic sensitivity³⁴ from a single tissue or fluid sample.

Two methods of polymerase chain reaction analysis have been widely used. Standard polymerase chain reaction involves

the use of labeled DNA, with a given complementary sequence, as a probe to identify the amplified DNA in hybridization assays. Real-time quantitative polymerase chain reaction uses either a nonselective DNA dye or a target-specific fluorescently-labeled DNA probe to measure signal intensity, and thus the quantity, of amplified DNA³⁵. In addition to permitting measurement of the amount, as opposed to the simple presence or absence, of a given sequence, real-time quantitative polymerase chain reaction can be performed rapidly, in five hours or less.

Although polymerase chain reaction-based bacterial detection methodology is faster and more sensitive than bacterial culture, it has a high false-positive rate³⁶⁻⁴¹. It has been hypothesized that these false-positive results are due to exogenous sources of DNA, either from the environment or from the reagents used in the process itself (e.g., many of the enzymes used are recombinant bacterial proteins)³⁹. In addition, the finding that polymerase chain reaction can produce a positive result after antimicrobial treatment of the bacteria suggests that the rRNA genes of both live and dead bacteria can be detected^{23,36,40,41}.

The disadvantages of a polymerase chain reaction-based detection system can be overcome by selection of a target sequence for a viability gene that undergoes active transcription only in live bacteria. Messenger RNAs (mRNAs) are produced only in live bacteria, and most have an average half-life of a few minutes²³. Recently, detection of mRNA with real-time reverse transcription quantitative polymerase chain reaction has been used to assess the viability of heat and chemically killed bacteria, with a detection limit for live bacteria of 125 to 1×10^4 colony-forming units (CFU) per milliliter^{23,42}.

In this study, we employed real-time quantitative reverse transcription polymerase chain reaction to assay for bacterial mRNA in a simulated septic arthritis setup for the purpose of detecting live bacteria as an indicator of active infection. Real-time quantitative reverse transcription polymerase chain reaction was also applied to detect mRNA in antibiotic-treated samples, with the hypothesis that the detected mRNA concentration would decrease over time in conjunction with bacterial death.

Materials and Methods

Simulated Septic Arthritis

After we obtained institutional review board approval (number 120414) and informed consent from the patients, synovial fluid samples (approximately 20 mL from each patient) were obtained from twelve consecutive patients who presented with knee pain and effusion with no evidence of infection. The samples were cultured in standard bacterial nutrient broth, and their sterility was verified with both polymerase chain reaction and plate culture. The twelve synovial fluid samples were each inoculated with the following bacteria obtained from the American Type Culture Collection (ATCC, Manassas, Virginia): *Escherichia coli* (ATCC 35218), *Staphylococcus aureus* (ATCC 29213), methicillin-resistant *Staphylococcus aureus* (ATCC BAA-41), or *Staphylococcus epidermidis* (ATCC 14990), as described below. A single colony from a

TABLE I Detection of Bacteria in Synovial Fluid by Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction*

Bacteria	Ct Values†			
	Aseptic Synovial Fluid		Inoculated Synovial Fluid	
	Mean and Standard Deviation	95% Confidence Interval	Mean and Standard Deviation	95% Confidence Interval
<i>Staphylococcus epidermidis</i>	41.3 ± 0.8	±0.45	27.0 ± 1.6	±0.91
<i>Staphylococcus aureus</i> ‡	36.6 ± 1.5	±0.85	19.3 ± 0.7	±0.40
<i>Escherichia coli</i>	37.9 ± 1.6	±0.91	19.9 ± 0.4	±0.23

*Synovial fluid samples were inoculated with *Staphylococcus epidermidis*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, or *Escherichia coli* and were analyzed with real-time quantitative reverse transcription polymerase chain reaction for femC (*Staphylococcus*) or groEL (*Escherichia coli*) mRNA. Detection was based on Ct (cycle number or threshold cycle) values and the standard deviation. †Ct, the threshold cycle, is defined as the cycle number at which fluorescence passes a fixed threshold and corresponds to the number of cycles of target amplification necessary for detection. Samples with lower concentrations of a target require more cycles of amplification for detection, so a larger Ct number indicates a lower concentration. ‡The results for methicillin-resistant *Staphylococcus aureus* were identical to those for *Staphylococcus aureus*.

plate culture of each of the bacterial species was inoculated into 1 mL of each synovial fluid sample, and this was followed by incubation for eighteen to twenty-four hours at 37°C under aerobic or anaerobic conditions according to ATCC protocols. Forty-eight samples (twelve samples for each of the four bacterial species) were inoculated. As a control, samples were also cultured on agar plates (according to ATCC protocol) to assess bacterial activity and viability. RNA was isolated from all samples after eighteen to twenty-four hours of incubation, when all cultures had reached a stationary phase of bacterial concentration (approximately 10⁸ bacteria/mL).

RNA Isolation

Total RNA was isolated from 100-µL aliquots of simulated septic synovial fluid with a RiboPure-Bacteria kit (Ambion, Austin, Texas) according to the manufacturer's instructions and was treated with DNase I (included in the kit) to remove any background contaminating DNA. Concentration of isolated RNA samples was determined spectrophotometrically on the basis of absorbance at 260 nm. Total RNA isolation procedures were also done on sterile synovial fluid samples to be used as negative controls.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Two genes were selected as candidate targets for analysis of viable bacteria with the real-time quantitative reverse transcription polymerase chain reaction. The groEL gene encodes a 60-kDa heat shock protein that is essential for bacterial growth⁴³; the femC gene of *Staphylococcus* is functionally required for methicillin resistance⁴⁴. Recent work has shown that the expression of groEL is closely linked to the viability of the bacteria^{42,45} and that femC is greatly downregulated in response to bacterial death⁴⁶. Using primer and probe sets encoding groEL (*Escherichia coli*) or femC (*Staphylococcus epidermidis*, *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus aureus*), as described below, respective total bacterial RNA was

reverse transcribed and amplified in a one-step iScript RT-PCR Kit for Probes (Bio-Rad, Hercules, California) on the iCycler Thermal Cycler (Bio-Rad) to detect mRNA. For each sample, the cycle number at which the fluorescence became logarithmic (Ct) was determined. Each of the forty-eight samples was analyzed in triplicate.

Forward and reverse primers and probes for groEL and femC were used with a final concentration of 18 µM for primers and 5 µM for probes. The cycling conditions for groEL were 50°C for ten minutes, 95°C for five minutes (cDNA synthesis), forty-five cycles of denaturation at 95°C for five seconds, annealing at 58°C for fifteen seconds, and extension at 72°C for twenty seconds. The cycling conditions for femC were 50°C for ten minutes, 95°C for five minutes (cDNA synthesis), forty-five cycles of denaturation at 95°C for fifteen seconds, and annealing at 60°C for one minute. The total processing time from RNA extraction to real-time quantitative reverse transcription polymerase chain reaction analysis was approximately five hours. The polymerase chain reaction primer sequences for groEL (*Escherichia coli*) were forward, 5'-TGA-AACGYGGTATCGACAAA-3'; reverse, 5'-CTGCATACCTT-CAACMACGTCC-3'; and probe, 5'-[6-FAM]CCTTCTTTA-CCGACTTTTCCATCGCTT[TAMRA]-3'. The sequences for femC (*Staphylococcus aureus*) were forward, 5'-GTGGATATT-TCGATTTAGCACCTACAGA-3'; reverse, 5'-CTTCAATATCG-AAGCCCATATCCTCTA-3'; and probe, 5'-[FAM]ACGACGA-CAGTTTTTC[NFQ]-3'. The sequences for femC (*Staphylococcus epidermidis*) were forward, 5'-CTTTGATATTGAAGCAAGCC-ACCA-3'; reverse, 5'-TGCTGTAACGGCATCTGCATATTTA-3'; and probe, 5'-[FAM]ACCTGGCGCTACTTCA[NFQ]-3'.

Antibiotic Treatment and Viability Test

Synovial fluid samples inoculated with *Escherichia coli* or *Staphylococcus aureus* were treated with a single dose of vancomycin and gentamicin (20 mg/mL of each), followed by incubation under the described conditions, and sampled for real-time quantitative reverse transcription polymerase chain

TABLE II Detection Limit for *Escherichia coli*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* with Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Bacteria*	Detection Limit†			Ct‡
	pg/mL	CFU/mL	Bacteria/100 μ L	
<i>Escherichia coli</i>	400	60,000	1000	36.65
<i>Staphylococcus aureus</i> §	500	110,000	1000	32.39
<i>Staphylococcus epidermidis</i>	30	18,000	100	37.17

*Detection was based on real-time quantitative reverse transcription polymerase chain reaction analysis of mRNA of groEL (*Escherichia coli*) and femC (*Staphylococcus*). †The bacteria number is based on the assumption that the stationary phase of bacterial growth represents 10^8 bacteria/mL. ‡The average cycle number at the detection limit. §The results for methicillin-resistant *Staphylococcus aureus* were identical to those for *Staphylococcus aureus*.

reaction analysis at one, two, three, and ten days for *Escherichia coli* and at twenty-four hours for *Staphylococcus aureus*.

Determination of Detection Limit

Simulated septic (*Escherichia coli*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*) and sterile culture aliquots for each synovial fluid sample were serially diluted down to 10^{-6} and spread-cultured on agar plates. Plates were incubated for twenty-four hours at 37°C , after which growth was determined

by colony counting. Each diluted sample was also analyzed with real-time quantitative reverse transcription polymerase chain reaction to establish a standardization curve for quantification of sample mRNA content. The cycle number at which the increase in fluorescence became logarithmic (Ct) (minimum detection limit) was determined and compared between the aseptic and simulated septic samples. Simulated septic samples were additionally analyzed by amplification without reverse transcription of RNA to DNA (i.e., in the absence of the reverse transcriptase enzyme) to test for any potential background DNA contamination and to further substantiate the minimum detection limit of mRNA.

Statistical Analysis

A two-tailed Student t test assuming equal variances was used for statistical analysis and to calculate 95% confidence intervals for detection values of septic compared with aseptic samples.

Results

Effectiveness of Bacterial Detection with Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

All twelve synovial fluid samples inoculated with each of the four bacteria (forty-eight samples), and analyzed in triplicate, were identified with real-time quantitative reverse transcription polymerase chain reaction; there were no false-negative results (Table I). All inoculated samples showed colony growth on culture plates, whereas similar plate cultures of the aseptic samples were negative for growth.

Detection Limit

Using one-step real-time quantitative reverse transcription polymerase chain reaction, the detection limit for *Escherichia*

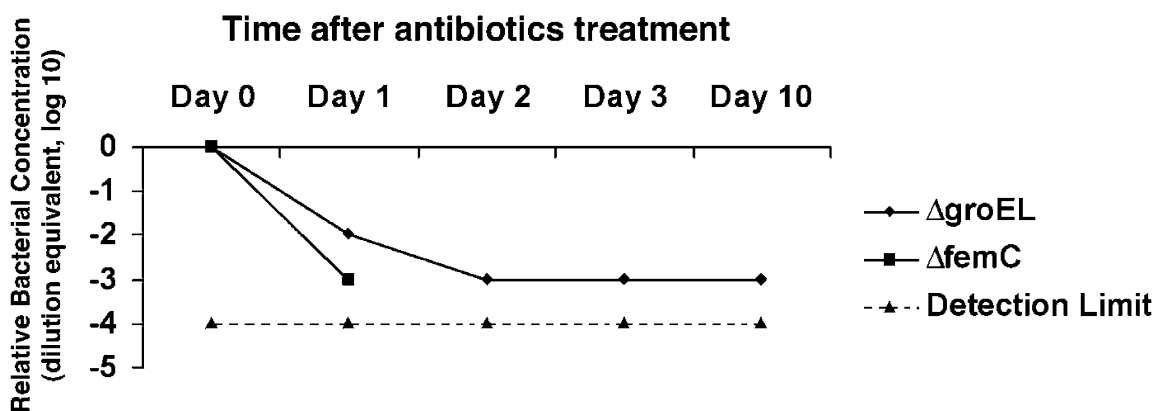


Fig. 1

Real-time quantitative reverse transcription polymerase chain reaction detection of the decrease in bacterial mRNA in bacterial cultures treated with antibiotics. Bacterial cultures containing *Escherichia coli*, *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus aureus* were treated with vancomycin and gentamicin (20 mg/mL of each), and total RNA was isolated after one, two, three, and ten days. Detection of groEL mRNA in *Escherichia coli* and femC mRNA in *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* was carried out by real-time quantitative reverse transcription polymerase chain reaction. The respective mRNA levels are expressed as equivalent of dilutions of the original inocula (data for methicillin-resistant *Staphylococcus aureus* not shown). The detection limit is indicated by the dotted line.

coli groEL mRNA was 400 pg/mL, 6×10^4 CFU/mL, or 1000 bacterial cells/100 μ L. The detection limit for *Staphylococcus aureus* femC mRNA was 500 pg/mL, 11×10^4 CFU/mL, or 1000 bacteria/100 μ L, and the detection limit for *Staphylococcus epidermidis* femC was 30 pg/mL, 18×10^3 CFU/mL, or 100 bacteria /100 μ L (Table II). Detection limits were based on quantitative polymerase chain reaction analysis of the sterile controls and mRNA samples amplified without prior reverse transcription. For the sterile controls, minimum detection represented where sterile samples were indistinguishable from diluted septic samples. In the absence of reverse transcription, detectable DNA for the target primer/probes used represents the background DNA present in the sample unrelated to the content of mRNA. On the basis of these measures, the detection limit for *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, and *Escherichia coli* is equivalent to detection of a 1:10,000 dilution, and the limit for *Staphylococcus epidermidis* is equivalent to detection of a 1:100,000 dilution.

Viability Test

Cultures containing *Escherichia coli* and *Staphylococcus aureus* were treated with antibiotics (vancomycin and gentamicin), and RNAs were extracted and analyzed at various time points. The concentration of detectable *Escherichia coli* groEL mRNA decreased by a factor of 100 after one day of incubation with antibiotic and decreased by a factor of 1000 after two days of incubation with antibiotic (Fig. 1). Bacterial mRNA concentrations remained at this level for up to ten days. In antibiotic-treated *Staphylococcus aureus* samples, the femC mRNA concentration dropped by a factor of 1000 after one day of incubation. All culture plates of antibiotic-treated bacterial samples showed no colony growth, indicating the antibacterial activity of the antibiotic treatment.

Discussion

A major goal of this investigation was to determine whether a real-time quantitative reverse transcription polymerase chain reaction detection of mRNA is an effective, rapid, and reliable method for identifying simulated septic arthritis. We showed that it can be used to detect and quantify the presence of bacteria in simulated bacteria-infected synovial fluid samples. Bacterial samples treated with antibiotics and found to have no growth on culture were also detectable and quantifiable with real-time quantitative reverse transcription polymerase chain reaction, albeit with a time-dependent decrease in signal. This phenomenon has been referred to in the literature as a "viable but not-culturable state" of bacteria^{47,48}. However, in the setting of septic arthritis, this state should more appropriately be called a septic but unculturable bacterial infection or "S.U.B. infection." In fact, it is the inability of traditional culture to detect this dormant state that contributes to the high rates of false-negative results that have been observed⁴⁷.

Real-time quantitative reverse transcription polymerase chain reaction analysis can also quantify the time-dependent decrease in the concentration of the bacterial population over time as a result of antibiotic treatment and thus provides in-

formation on the reduction in the number of viable bacteria. Interestingly, the detectable concentration of these so-called unculturable, antibiotic-treated bacteria was at least one order of ten above the detection limit on day 10, strongly suggesting that a viable number of bacteria still existed, even with no growth detected on a culture plate. The clinical relevance of this observation is that it might represent an effective means of obtaining a snapshot of the viable bacterial load during the course of antibiotic therapy. It is noteworthy that bacterial culture has a false-negative rate of up to 50% in the setting of periprosthetic infection^{6-10,13}. In addition, the false-negative rate approaches 60% for patients who have started antibiotic therapy¹⁹, illustrating the unreliability of the culture method for bacterial detection.

The reliability of standard polymerase chain reaction assay, based on the detection of bacterial 16S rRNA gene, as an indicator of periprosthetic joint infection is also compromised by false-positive results^{37,38}. Because of the extended persistence of bacterial DNA and rRNA, standard polymerase chain reaction analysis with use of universal 16S ribosomal RNA primers has been shown to detect bacterial DNA even after twenty-two days of antibiotic treatment³⁶.

Despite the problem of false-positive results, polymerase chain reaction, or amplification of gene markers, has been shown to be an effective way to detect septic arthritis with extreme sensitivity. We previously showed that polymerase chain reaction analysis could detect a threshold of ten bacterial cells per 100 μ L of synovial fluid, a threshold below which a specimen was not considered to be infected²⁸. By applying this criterion, some of the false-positive results could be designated as clinically unimportant. In our present study, the detection limits were 1000 bacteria/100 μ L for *Escherichia coli* (groEL), 1000 bacteria/100 μ L for *Staphylococcus aureus* (femC), and 100 bacteria/100 μ L for *Staphylococcus epidermidis* (femC). This one-to-two order-of-magnitude difference in detection is likely related to the fact that the concentration of 16S rRNA gene (DNA) is approximately 1000-fold higher than that of any specific mRNA.

While it is not possible to directly compare the detection limit and sensitivity of polymerase chain reaction with those of real-time quantitative reverse transcription polymerase chain reaction, it is reasonable to speculate, on the basis of the findings described above, that the two methods provide equivalent detection limits for the same amount of starting bacterial nucleotide material. We previously showed that polymerase chain reaction detection of 16S rRNA successfully identified 100% of septic failures in one series of revision total knee arthroplasties (thirty-one of fifty revisions were determined to be septic failures on the basis of preoperative and intraoperative clinical data)²⁹. However, it should be noted that the real-time quantitative reverse transcription polymerase chain reaction procedure in the experimental pilot study reported here has been tested only in the laboratory setting on simulated septic samples and not on clinically infected samples. On the basis of this study of twelve samples with no false-negative results, the true rate of false-negative results that can

be applied to a greater population (on the basis of the 95% confidence interval) should lie somewhere between 0% and 25%. The clinical detection limit and applicability of the methodology will need to be established before it is adopted as a standard clinical test.

To our knowledge, this is the first study in which real-time quantitative reverse transcription polymerase chain reaction was used to detect mRNA as an indicator of infection in a simulated septic arthritis model. The results reported here suggest that this one-step method can be used rapidly and accurately to detect the presence of several clinically relevant bacteria both before and after the initiation of antibiotic treatment. The direct quantification of viable bacterial concentration, by the analysis of mRNA, allows identification of potentially clinically relevant infections. In addition, the use of this method eliminates the false-positive detection of nonviable genomic DNA and ribosomal RNA, which has been a major limitation of standard polymerase chain reaction analysis.

The ability to identify and quantify live bacteria with high sensitivity and reliability would allow determination of the appropriate course and duration of treatment of infection. The real-time quantitative reverse transcription polymerase chain reaction method described here has many potential applications, including the timely diagnosis and appropriate treatment of septic arthritis and related orthopaedic compli-

cations, such as periprosthetic infections. Although further clinical study is clearly needed, this method of detecting live bacteria, in combination with previously described methods of using polymerase chain reaction-based molecular detection to determine species³² and antibiotic sensitivity³⁴, could dramatically improve the way septic arthritis is diagnosed and treated. In particular, such practice would improve outcomes for patients with a periprosthetic infection. ■

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