Evaluation of the Time Period for Which Real-Time Polymerase Chain Reaction Detects Dead Bacteria

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Abstract

Real-time polymerase chain reaction (PCR) is currently widely used for the diagnosis of infections. We evaluated the time after treatment during which real-time PCR can detect dead bacteria. The presence of bacterial DNA was identified by real-time PCR through methicillin-resistant Staphylococcus (MRS)-PCR and universal PCR. Methicillin-resistant Staphylococcus aureus (MRSA), Staphylococcus epidermidis, and Escherichia coli were each killed with alcohol, antibiotics, or heat treatment in vitro. The detection periods of MRS-PCR for MRSA treated by alcohol, vancomycin, linezolid, and heat were found to be less than 16, 8, 12, and 8 weeks, respectively. The detection period of universal PCR for S. epidermidis treated by alcohol, cefazolin, and heat was less than 20, 20, and 4 weeks, whereas that for E. coli was 8, 20, and 4 weeks, respectively. The presence of detectable bacterial DNA in infected arthroplasty patients before and after successful treatment was also assessed by MRS- and universal PCR. MRS-PCR was positive in 6 patients before treatment and all became negative after a mean interval of 20.8 weeks (95% confidential interval, 13.2 to 33.7) after treatment. Universal PCR detected remnant bacterial DNA in 4 patients at a mean of 15.2 weeks (95% CI, 12.4 to 18.0) after treatment and was negative in 7 patients at a mean of 17.3 weeks (95% CI, 10.6 to 24.0) after treatment. Our studies revealed that real-time PCR detects dead bacteria for several weeks, but this capability decreases with time and is likely lost by 20 weeks after treatment.

Key words: dead bacterial DNA, MRS-PCR, real-time PCR, universal PCR

Introduction

Clinically infected but culture negative cases are difficult to make definitive diagnoses of infection. Although the conventional laboratory method of determining bacterial cell viability by visible colonies on a suitable solid medium is a universal method for the determination of the causative organism, it is unreliable for the diagnosis of viable but non-culturable (VBNC) bacterial infection (Trevors, 2011; Trevors, 2012). Elevated C-reactive protein (CRP) levels and erythrocyte sedimentation rate (ESR) are recognized as good indicators of infections (Sanzen et al., 1989); these are non-specific markers for the diagnosis of infection. Several novel diagnostic tools based on real-time PCR have been developed recently and used for the diagnosis of infections (Choe et al., 2013, Moojen et al., 2010, Morre et al., 1998, Nilsson et al., 2008). It is a rapid and sensitive method (Kobayashi et al., 2009), however, one major concern is that PCR can detect not only live but also dead bacteria (Keer et al., 2003, McKillip et al., 1999, Workowski et al., 1993).

Appropriate antibiotic therapy is usually useful for eradicating bacterial infection, however, some patients may develop a VBNC bacterial condition when the microorganisms propagate in biofilms that facilitate bacterial survival despite antibiotic treatment (Trevors, 2011). Consequently, it is occasionally difficult to judge the infection status in antibiotic-initiated patients with PCR-positive but culture-negative results. In these patients, it is unclear whether PCR detects remnant dead bacterial DNA, even in patients after the infection has been successfully treated or if the positive PCR reflects viable bacteria in a VBNC condition (McKillip et al., 1999, Workowski et al., 1993). The exact period for which real-time PCR can detect remnant bacterial DNA from dead bacteria is therefore important, but has not been well evaluated. In the present study, we addressed the following 2 questions: the time period for which real-time PCR can continue to
detect and whether the capability of real-time PCR for determination of dead bacterial DNA will decrease overtime.

**Experimental**

**Materials and Methods**

**Bacterial Strains and Growth Conditions in vitro.** Methicillin-resistant *Staphylococcus aureus* (MRSA) (a clinically isolated strain), *Staphylococcus epidermidis* (ATCC [America Type Culture Collection] 12228), and *Escherichia coli* (ATCC 23231) were tested in this study. Each bacterial strain was grown for 24 h at 37°C in a Luria-Bertani (LB, Difco Laboratories, Detroit, MI) agar plate before analysis. Single colony of MRSA, *S. epidermidis*, and *E. coli* were isolated and inoculated into 20 ml of LB broth. LB broth was incubated at 37°C and 300 μl of overnight culture were incubated in the 30 ml of LB broth for 3 hours. Bacterial suspensions of each strain were adjusted to 0.5 McFarland standards.

**Methods of treatment in vitro.** Each strain was separately treated using (1) alcohol, (2) antibiotics, and (3) heat, and 5 samples were prepared for each treatment method. The reaction conditions were as follows: (1) Alcohol, all strains were adjusted to 1 x 10⁸ CFU/ml using 95% ethanol (final concentration, 90%) and 0.9% saline; (2) Antibiotics, MRSA was adjusted to 1 x 10⁵ CFU/ml using 0.9% saline. Vancomycin (VCM) or linezolid (LZD) were added to produce a final concentration of 100 μg/ml. *S. epidermidis* and *E. coli* were adjusted to 1 x 10⁵ CFU/ml using 0.9% saline. Cefazolin (CEZ) was added to a final concentration of 100 μg/ml. The minimum inhibitory concentration (MIC) of each strain was determined using MIC determination kit (Micro Scan; Dade Behring Inc, Deerfield, IL), and MIC of these antibacterial drugs for each bacterium were confirmed to be below 2 μg/ml (3). Heat shock, all strains were adjusted to 1 x 10⁶ CFU/ml using 0.9% saline and were treated at 100°C for 10 min.

After each treatment procedure, the bacterial suspensions were maintained at 37°C for 2 days and for 1, 4, 8, 12, 16, and 20 weeks. All of the resulting bacterial suspensions were proceeded for microbiological culture and real-time PCR analysis.

**Microbiological culture for treated bacteria in vitro.** 100 μl of each culture sample was grown in 20 ml of LB broth for 48 hours. Subsequently, the treated state of the bacteria was confirmed by growing 100 μl aliquots of these bacterial suspensions in either MRSA-specific agar for the MRSA strain or LB agar for the *S. epidermidis* and *E. coli* strains.

**DNA Extraction and Real-Time PCR.** For DNA extractions, 200 μl aliquots of solutions were taken from each sample. DNA extractions were performed using a Bio Robot EZ1 DNA investigator kit with a Bio Robot EZ1 (Qiagen Inc., Valencia, CA) in accordance with the manufacturer’s instructions. We used a Light Cycler system (Roche Diagnostics, Mannheim, Germany) to perform the real-time PCR assay. Two different primer and probe sets were used: a commercially available MRSA specific detection kit (Roche Diagnostics, Mannheim, Germany) targeting the meca gene (MRS-PCR) and a set of primers (forward: 5’-CAAACAGGATTAGTA.TACCCTG-3’ and reverse: 5’-GGTAAGGTTCCTGCG-3’) and hybridization probes designed for broad range detection that targeted a part of the 16S rRNA gene (universal PCR) (Choe et al., 2013, Kobayashi et al., 2006, Kobayashi et al., 2009). MRS-PCR was used for the solutions containing treated MRSA and universal PCR were used for the solutions with treated *S. epidermidis* and *E. coli*. We placed 5 μl of DNA extract into a final reaction volume of 20 μl for each tube. The cycling conditions were 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 12 s. The amplified products were then quantified using the LightCycler software. The differences between the threshold cycles (Ct) for the negative control (sterile water) and the treated bacteria were calculated using the formula:

\[ \Delta Ct = Ct_{\text{negative control}} - Ct_{\text{treated bacteria in universal PCR results}} \]

\[ \Delta Ct > 2.0 \]

cycles was defined as a positive result in universal PCR (Miyamae et al., 2012).

**Clinical cases** The current clinical study was approved by our institutional review board. Samples were harvested from total hip or knee arthroplasty patients who were diagnosed with an infection by a positive result in microbiological culture or histopathology. A histopathological diagnosis of infection was made when the infiltration of ≥ 10 neutrophils per high-power visual field (× 400) was confirmed (Lonner et al., 1996; Tsaras et al., 2012). All patients underwent the two-stage revision surgery that consisted of the first surgery (surgical debridement and implant removal), an interval period with antibacterial treatment, and a second surgery (revision arthroplasty). At the time of first and second surgery, tissue samples were harvested and underwent MRS- and/or universal PCR analysis. Eradication of bacteria was confirmed in all patients by negative results in microbiological culture and histopathology at the time of second surgery and the absence of clinical and radiographic evidence of infection. All patients completed at least one year of follow-up period after second surgery. Real-time PCR results obtained for each patient before and after treatment (at the time of first surgery and second surgery) were compared.

**Statistical analysis.** Statistical significance of difference among in vitro longitudinal data of Ct val-
values in MRS-PCR or ΔCt values in universal PCR at each time point after treatment were determined by repeated measures analysis of variance (ANOVA) followed by Bonferroni t-test or by Friedman repeated-measures ANOVA on ranks followed by Scheffe test when assumptions of normality or homogeneity of variances were not verified. Statistical significance of difference between clinical samples harvested at 1st and 2nd surgery of Ct values in MRS-PCR or ΔCt values in universal PCR were determined by paired t test or Wilcoxon signed ranks test when the data was not normally distributed. All tests were reported as significant differences if p value was less than 0.05.

Results

In our in vitro study, all cultures containing treated MRSA, S. epidermidis, and E. coli showed no growth of colonies after 1 week. MRS-PCR results were positive in all 5 samples for MRSA at 2 days after each treatment (Figure 1-A). The mean Ct values of MRS-PCR for MRSA at 2 days after treatment were significantly lower than those at 1 week and 4 weeks for each treatment (Figure 1-B). Heat- and VCM-treated MRSA were not detected by MRS-PCR at 8 weeks after treatment. LZD-treated and alcohol-treated MRSA were all negative in MRS-PCR by 12 weeks and 16 weeks, respectively (Table I). Universal PCR results were positive in all 5 samples for S. epidermidis and E. coli at 2 days after each treatment. The mean ΔCt values of S. epidermidis at 2 days after alcohol-, CEZ-, and heat-treatment were 4.5, 4.6, and 4.0, respectively. The ΔCt values of S. epidermidis showed a gradual decrease up to the 4-week time point. The mean ΔCt values of the CEZ- and heat-treated S. epidermidis were significantly higher at 2 days than at any other time point and that value of the alcohol-treated S. epidermidis was significantly higher than the negative control (sterile water) (Figure 1-B).

Table I

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MRS-PCR</th>
<th>Universal PCR</th>
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<tr>
<td>Alcohol</td>
<td>16 weeks</td>
<td>20 weeks</td>
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<tr>
<td>LDA</td>
<td>12 weeks</td>
<td>20 weeks</td>
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<td>Heat</td>
<td>8 weeks</td>
<td>4 weeks</td>
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Fig. 1. Threshold cycle (Ct) values of methicillin-resistant Staphylococcus (MRS)-PCR for treated methicillin-resistant Staphylococcus aureus (MRSA) increased overtime after each treatment in vitro.

MRSA were processed for MRS-PCR analysis at indicated time points after the treatments. (A) At 48 hours after each treatment, all samples showed positive results in MRS-PCR excluding the negative control (sterile water). (B) The mean Ct values of MRSA at 2 days after each treatment gradually increased over time. As a result, the detectable period of MRS-PCR for MRSA treated by alcohol (Alc), vancomycin (VCM), linezolid (LZD), and heat were less than 16, 8, 12, and 8 weeks, respectively. All experiments were conducted with 5 samples. *'s denotes p<0.05 and **'s denotes p<0.01 in repeated measures ANOVA followed by Bonferroni t-test.
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Universal PCR results for alcohol-, CEZ-, and heat-treated *S. epidermidis* were negative in all 5 samples by 20, 20, and 4 weeks, respectively (Table I). The mean ΔCt values of *E. coli* at 2 days after alcohol-, CEZ-, and heat-treatment were 7.6, 8.6, and 5.3, respectively. The mean ΔCt value of the CEZ-treated *E. coli* was significantly higher at 2 days than at 12 weeks. The mean ΔCt values for the alcohol- or heat-treated *E. coli* were significantly higher at 2 days than at 4 weeks (Figure 2-B). The ΔCt values of *E. coli* decreased and all universal PCR results were negative at 8 weeks, 20 weeks, and 4 weeks after alcohol-, CEZ-, and heat-treatment, respectively (Table I).

In clinical cases, MRS-PCR was positive in 6 patients with mean Ct values of 31.4 at the time of first surgery. All 6 patients were negative at the time of second surgery after a mean 20.8 week interval (95% confidential interval, 13.2 to 33.7) of interval period (Figure 3-A). Universal PCR, performed in 11 patients, decreased from a mean ΔCt value of 4.9 at the time of first surgery to 1.8 at the time of second surgery after a mean 16.5 weeks interval (Figure 3-B). Universal PCR detected remnant bacterial DNA in 4 out of 11 patients at the time of second surgery after a mean interval of 15.2 weeks (95% CI, 12.4 to 18.0) and was negative in 7 patients after a mean interval of 17.3 weeks (95% CI, 10.6 to 24.0). In any cases at 2 days than at 4 weeks (Figure 2-A). Universal PCR results for alcohol-, CEZ-, heat-treated *S. epidermidis* were negative in all 5 samples by 20, 20, and 4 weeks, respectively (Table I). The mean ΔCt values of *E. coli* at 2 days after alcohol-, CEZ-, and heat-treatment were 7.6, 8.6, and 5.3, respectively. The mean ΔCt value of the CEZ-treated *E. coli* was significantly higher at 2 days than at 12 weeks. The mean ΔCt values for the alcohol- or heat-treated *E. coli* were significantly higher at 2 days than at 4 weeks (Figure 2-B). The ΔCt values of *E. coli* decreased and all universal PCR results were negative at 8 weeks, 20 weeks, and 4 weeks after alcohol-, CEZ-, and heat-treatment, respectively (Table I).

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with longer than 20 weeks of interval period, remnant bacterial DNA was not detected in both MRS-PCR and universal PCR (Figure 3-A and 3-C).

Discussion

Antibacterial treatment is sometimes initiated in patients with a strong suspicion of infection before detection of causative bacteria, although determination of the organism is important for appropriate antibacterial treatment. When bacteria are not eradicated successfully by the initial treatment, determination of the organism often becomes difficult because microbiological culture frequently shows negative results after the antibiotics (Choe et al., 2013; Ilharreborde et al., 2009; Parviz et al., 2014; Trevors, 2012). Real-time PCR detects a part of specific bacterial DNA that enables etiological diagnosis even in this VBNC bacterial condition (Ilharreborde et al., 2009; Maas et al., 2007). However, clinical use of this novel tool as a routine examination is still controversial (Parviz et al., 2014). One problem is its capability to detect bacterial DNA from dead bacteria and may possibly lead to a false positive result by detecting remnant bacterial DNA in successfully treated infection cases (Maas et al., 2007; McKillip et al., 1999; van der Heijden et al., 1999). Evaluation of the time duration for which real-time PCR can detect dead bacterial DNA is, therefore, of clinical importance because this helps to judge the status of bacteria in culture negative but real-time PCR positive cases for which antibacterial treatment had been initiated.

In the current study, real-time PCR based on a previously established MRS specific PCR and/or pan bacterial universal PCR (Choe et al., 2013; Choe et al., 2011; Kobayashi et al., 2006) was used to detect dead bacterial DNA from successfully treated bacteria in vitro and in clinical samples. Our in vitro study mimicked the clinical situation in which bacterial DNA remains after successful treatment. We demonstrated that the time period for which real-time PCR can detect the mecA gene from dead MRSA was less than 16 weeks and that for 16S rRNA gene from both dead S. epidermidis and E. coli were less than 20 weeks. These data were in a wide range consistent with our clinical results of successfully treated arthroplasty infection cases, which demonstrated the PCR detects remnant dead bacterial DNA for several weeks but less than 20 weeks. Our in vitro study revealed that the reduction of the amount of dead bacterial DNA starts in 1 week after treatment and gradually decreases overtime. This gradual reduction suggests that the bacterial DNA was not degraded directly by the treatment, but subjected to digestion by DNases following bacterial death. Accordingly, the capability of real-time PCR for detecting dead bacterial DNA decreased over time.

In our clinical cases, all mecA genes becomes undetectable in MRS-PCR in a mean 20.8 weeks after treatment. The capacity of universal PCR for the detection of dead bacteria also decreased over time. Although universal PCR detected remnant dead bacterial DNA after treatment in 4 patients, this capacity did not last beyond 20 weeks in any case. This is likely because universal PCR is designed for pan-bacterial detection, and therefore, its capacity to detect bacterial DNA is dependent upon on the causative strain. Current in vitro data have shown that the duration of the real-time PCR detection period in treated bacterial DNA varied depending on the pathogen and treatment method i.e. alcohol-treated E. coli was detectable for a shorter period as compared with alcohol-treated S. epidermidis and in VCM-treated MRSA was detectable for a shorter period as compared with LZD-treated MRSA. These differences may be due to structural differences between gram-positive and negative strains and their mode of action (the disinfectant properties of VCM versus the bacteriostatic properties of LZD). A limitation of our in vitro study is that we only investigated three strains of bacteria. Since there are numerous other strains that cause clinical infection, the real-time PCR detectable period for strain of interest should be individually assessed. Our in vitro method to assess the dead bacterial DNA would be suitable for that purpose as a future study. Consistent results between current in vitro and clinical data indicate that the capacity of real-time PCR for the detection of dead bacteria will decrease overtime and is lost by 20 weeks after successful treatment. On the other hand, shortly after the first initiation of treatment, alive-dead discrimination assay would be required to judge the bacterial viability in culture negative but real-time PCR positive cases.

In conclusion, to properly evaluate the result of real-time PCR in patients after initiation of antibacterial treatment, the presence of DNA from dead bacteria must always be considered because real-time PCR can detect the DNA of treated bacteria for several weeks. However, the amount of dead bacterial DNA gradually decreased over time and the real-time PCR capability for detecting the dead bacterial DNA is most likely lost in 20 weeks after successful treatment.

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Literature


