Introduction

Periprosthetic infection, one of the most intractable conditions, is caused by microorganisms growing in biofilms and is thus difficult to diagnose and treat. Because of the false negative results obtained by microbiological culture in cases of low-grade infection, it is especially difficult to differentiate septic from aseptic loosening. Although several diagnostic tools have been developed, this remains a challenging issue. We have utilized real-time PCR assay as a fast and sensitive method for the diagnosis of periprosthetic infection. One concern, however, is that PCR assay is able to detect not only live, but also dead bacterial DNA. Hence it is difficult to judge the status of infection in the case of PCR-positive but microbiological culture-negative results. Especially in cases of reimplantation, PCR may detect the dead bacterial DNA even after the infection has been eradicated.

We therefore address two questions in this study: (1) How long after the death of the bacteria can real-time PCR detect the dead bacterial DNA? and (2) Is there any difference depending on the species of bacteria or method of inactivation?

Materials and Methods

**<Bacterial Strains and Growth Conditions>**

Methicillin-resistant staphylococcus aureus (MRSA) (a clinically isolated strain), staphylococcus epidermidis (S. epidermidis) (ATCC 12228) and Escherichia coli (E. coli) (ATCC 23231) were used. Each bacterial strain was grown for 24 hours at 37°C in nutrition agar prior to all experiments. The concentration of each strain was adjusted to 0.5 MacFarland standard.

**<Inactivation Methods>**

Each strain was inactivated by (1) alcohol, (2) antibiotics and (3) heat shock. Five samples were prepared for each method.

(1) Alcohol: All strains were adjusted to 1×10⁶ CFU/ml with 100% ethanol (final concentration: 90%) and 0.9% NaCl.

(2) Antibiotics: MRSA was adjusted to 1×10⁶ CFU/ml with 0.9% NaCl. Vancomycin (VCM) or linezolid (LZD) were added to produce a final concentration of 100μg/ml. S. epidermidis and E. coli were adjusted to 1×10⁵ CFU/ml with 0.9% NaCl. Cefazolin (CEZ) was added for a final concentration of 100μg/ml. The minimum inhibitory concentrations of these antibacterial drugs for each bacteria were adjusted to under 2μg/ml.

(3) Heat shock: All strains were adjusted to 1×10⁶ CFU/ml with 0.9% NaCl and were inactivated for 10 minutes.

After inactivation, the solutions were maintained at 37°C for 48 hours, and for 1, 4, 8, 12, 16 and 20 weeks. All the resulting solutions were submitted to microbiological culture and real-time PCR assays.

**<Microbiological Culture for Inactivated Bacteria>**

To confirm the inactivated state, 100μl of each solution containing inactivated bacteria was grown in 20ml of Luria-Bertani broth (Difco Laboratories, Detroit, Mich.) for 48 hours. Then 200μl of these solutions was grown in MRS-specific agar for MRSA and nutrition agar for S. epidermidis and E. coli.

**<DNA Extraction and Real-Time PCR>**

DNA extraction was performed using a Bio Robot EZ1 DNA investigator kit with Bio Robot EZ1 (Quagen Inc., Valencia, CA) according to the manufacturer’s instructions. We used a Light Cycler® system (Roche Diagnostics, Mannheim, Germany) for real-time PCR assay. An MRSA detection kit (Roche Diagnostics) targeting the mecA gene was used for the solutions containing inactivated MRSA (MRS-PCR). A set of broad-range PCR primers that targeted a part of the 16S rRNA gene was used for the solutions with inactivated S. epidermidis and E. coli as a pan-bacterial PCR (universal PCR®). The difference between the detection threshold cycles of the negative control using sterile water and inactivated bacteria was calculated using the formula \( \triangle Ct = Ct_{\text{negative control}} - Ct_{\text{killed bacteria}} \).

**Results**

**<Microbiological Culture>**

Microbiological culture for E. coli inactivated by CEZ was positive only at 48 hours after inactivation, and then remained negative from 1 week onwards. All other cultures for S. aureus, S. epidermidis and E. coli were negative.

**<Real-Time PCR>**

All real-time PCR results for inactivated MRSA, S. epidermidis and E. coli are summarized in Figure 1. MRS-PCR and Universal PCR were all positive at 48 hours after inactivation. Then MRS-PCR for MRSA inactivated by VCM or heat shock revealed all negative results at 8 weeks. MRS-PCR results for LZD-inactivated MRSA were all negative at 12 weeks and for alcohol-inactivated MRSA at 16 weeks. Universal PCR for heat-treated S. epidermidis and alcohol- or CEZ-inactivated S. epidermidis showed all negative results at 4 weeks and 20 weeks, respectively. Universal PCR results for heat-, alcohol-, or CEZ-treated E. coli were all negative at 4, 8, and 20 weeks, respectively. \( \triangle Ct \) of S. epidermidis and E. coli are plotted in Figure 2. All results were significantly higher at 48 hours than at 4 weeks.

**Discussion**

In this study, we confirmed that real-time PCR is capable of detecting dead bacterial DNA regardless of the inactivation procedure. MRS-PCR detected the mecA gene from MRSA for at least 12 weeks, and Universal PCR detected the 16S rRNA from both S. epidermidis and E. coli for 16 weeks. These results suggest that positive real-time PCR results obtained 3 to 4 months after antibacterial treatment might reflect the detection of dead bacterial DNA. The detection period for dead bacterial DNA varied according to the method of inactivation and species of bacteria. The shorter MRSA detection period with VCM than with LZD may be due to the disinfectant properties of VCM and bacteriocin properties of LZD.

One limitation of this study is that it was performed in vitro, and there may be several unexpected factors in vivo. However, when considering foreign-body reactions such as phagocytosis, the PCR detection period is likely to be shorter in vivo than in vitro. Hence, with a waiting period of 4 to 5 months after appropriate antibacterial treatment, the possibility of PCR detection of dead bacterial DNA will be reduced. We thus conclude that real-time PCR enables the accurate evaluation of periprosthetic infection after sufficient duration of antibacterial treatment, but needs some improving to differentiate live from dead bacterial DNA within 4 to 5 months after antibacterial treatment.

In conclusion, we confirmed that real-time PCR was capable of detecting dead bacterial DNA, although this risk gradually decreased over time and disappeared at 20 weeks after inactivation. This indicates that real-time PCR can be an accurate tool to evaluate the eradication of infection 4 to 5 months after antibacterial treatment, but still needs further improvement.

**Reference**

