5' nuclease activity[7], resulting in the emission of a fluorescent signal (since the reporter dye is no longer suppressed by the quencher dye). Thus, with the increase in cycling of the PCR amplification, a stronger fluorescence can be detected, and such fluorescence is read in real time by a detection system.

S. marcescens in blood samples has not been assayed by real time PCR. In this study, we examined the rapid DNA diagnosis of outbreak-derived *S. marcescens* in blood samples by a real time (TaqMan) PCR assay.

MATERIALS AND METHODS

Bacterial strains

The *S. marcescens* strains used were those derived from a nosocomial outbreak at a hospital in Tokyo in 1999, and were clonally identical, as evidenced by pulsed-field gel electorophoresis[3]. They were kindly provided by Miyoko Endoh (Tokyo Metropolitan Research Laboratory of Public Health, Tokyo). The *Pseudomonas aeruginosa* strains used were isolated from the blood of patients with septicaemia at Niigata University Medical Hospital.

Media and bacterial growth

For bacterial growth, we used LB broth (Difco Laboratories, Detroit, Mich., USA) as a liquid medium, which was inoculated and incubated at 37℃ to a log phase with agitation. Nutrient agar (Eiken Chemical, Tokyo) was used as a solid medium.

Extraction of DNA

S. marcescens cells, grown in LB broth, were suspended in phosphate-buffered saline (PBS, pH 7.4) at 0.13 OD_{600} units; this concentration corresponded to 2×10^8 CFU/ml. A series of 10-fold dilutions was made from this bacterial suspension, and 10 μl of each dilution was mixed with 200 μl of blood from healthy adults.

Bacterial DNA was prepared from these bacterial suspensions (ranging from 1 CFU/ml to 1×10^7 CFU/ml) using the QIAamp DNA mini kit (QIAgen, Japan), following the manufacturer's instructions. Briefly, the samples were suspended in 180 μl of buffer ATL (from the kit) and 20 μl of proteinase K (600 mAU/ml). The mixtures were incubated at 56℃ for 10 min. Subsequently, 200 μl of buffer AL (from the kit) was added and the mixtures were incubated at 72℃ for 10 min. The DNA extracted was dissolved in 200 μl of Tris-hydrochloride, pH 8.0.

This procedure extracted DNA from both bacteria in serum and blood cells, in the case of blood samples.

PCR primers and the probe

The primers, UF2 and UR2, have been described previously[8]. The probe (designated SM1) was designed based on the 16S rRNA gene sequence of *S. marcescens*, as shown in Fig. 1. This probe contained the reporter dye FAM (6-carboxyfluorescein) at the 5'-end and the quencher dye TAMRA (6-carboxytetramethylrhodamine) at the 3'-end.

Real time (TaqMan) PCR assay

The PCR was performed with a ABI 7700 sequence

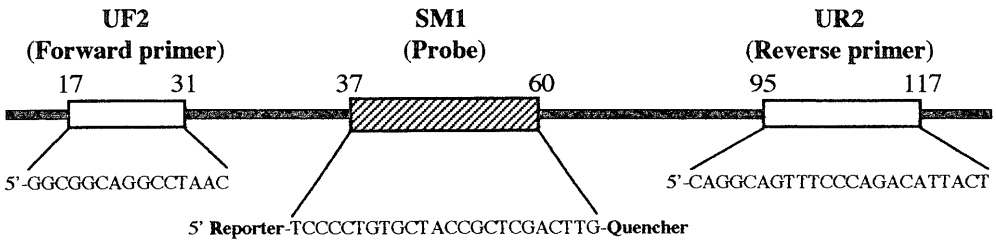

Fig. 1. Nucleotide position for the primers (UF2 and UR2) and the probe (SM1) in the 16S rRNA gene sequence for *S. marcescens*.

The *S. marcescens* 16S rRNA gene sequence was from GenBank Accession Number AJ233431. The numbers on the map represent DNA sizes (in base pairs), and correspond to the numbering of the 16S rRNA gene sequence from the 5'-end (1). The primers UF2 and UR2[8] amplify a fragment of 101 bp spanning nucleotides 17 to 117 of the 16S rRNA gene. The TaqMan probe (SM1) is located at positions 37 to 60 (reverse complement), and was designed for this study. This probe contains the reporter dye FAM (6-carboxyfluorescein) at the 5'-end and the quencher dye TAMRA (6-carboxytetramethylrhodamine) at the 3'-end.

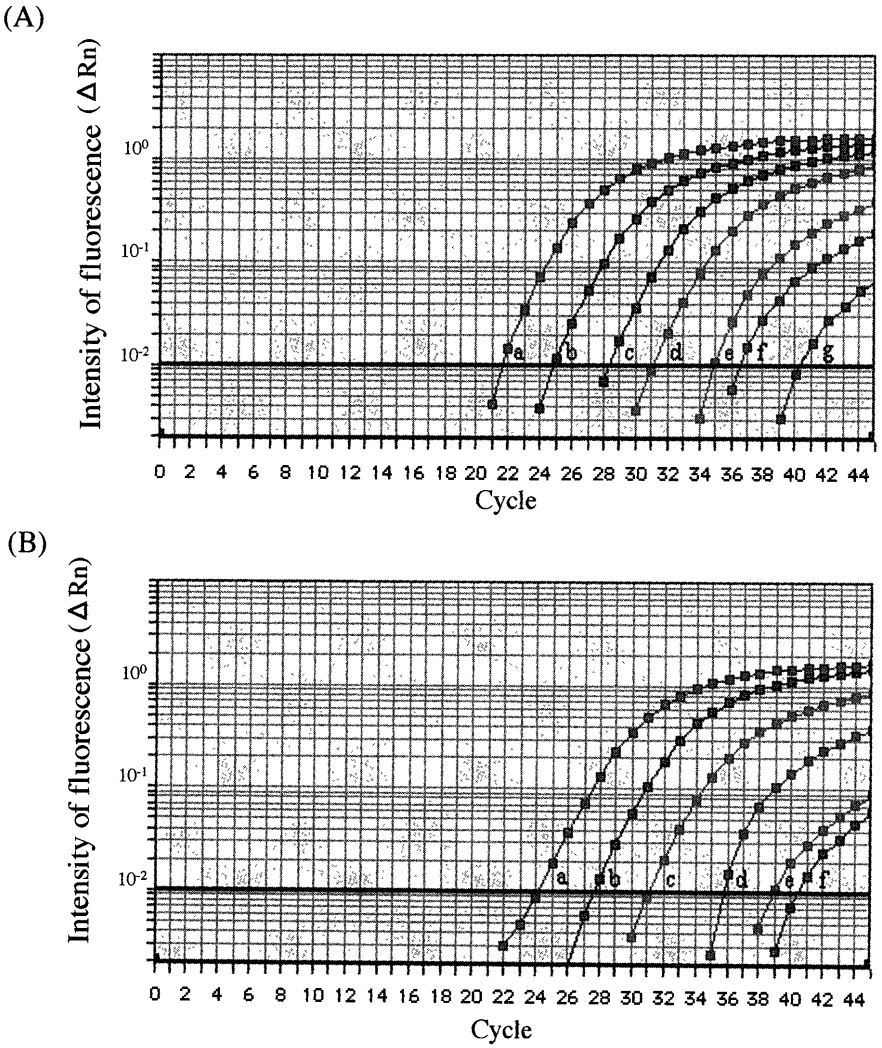

Fig. 2. Real time (TaqMan) PCR assay of *S. marcescens*.

Freshly cultured *S. marcescens* cells serially diluted in PBS in (**A**) and in blood from healthy adults in (**B**). The bacterial concentrations are 1×10^7 CFU/ml for (a), 1×10^6 CFU/ml for (b), 1×10^5 CFU/ml for (c), 1×10^4 CFU/ml for (d), 1×10^3 CFU/ml for (e), 1×10^2 CFU/ml for (f) and 5×10^1 CFU/ml for (g). No fluorescence signals are detected in samples containing less than 5×10^1 CFU/ml. When the same bacterial concentration was employed, the C_T values for bacterial blood samples were 2 or 3 cycles later than for bacterial PBS samples. When *P. aeruginosa* strains were employed instead of *S. marcescens*, no fluorescence signals were detected in either (**A**) or (**B**).

detector (Applied Biosystems, Foster City, CA), following the manufacturer's instructions. The reaction mixture (50 μl) contained 400 nM of primers, 80 nM of the probe, 25 μl of Taqman universal prepared mixture, and 20 μl of the template. The cycling conditions were an initial single cycle for 10 min at 95℃ (to activate AmpliTaq Gold) and 45 cycles of two-temperature cycling consisting of 15 s at 95℃ (for denaturation) and 1 min at 60℃ (for annealing and polymerization). The intensity of the fluorescence (ΔRn) was calculated by subtracting the baseline fluorescence from the actual fluorescence signal data. The threshold cycle (C_T) was defined as the cycle number at which the reporter fluorescence exceeded the threshold value, a parameter defined as 10 standard deviations above the baseline fluorescence. The

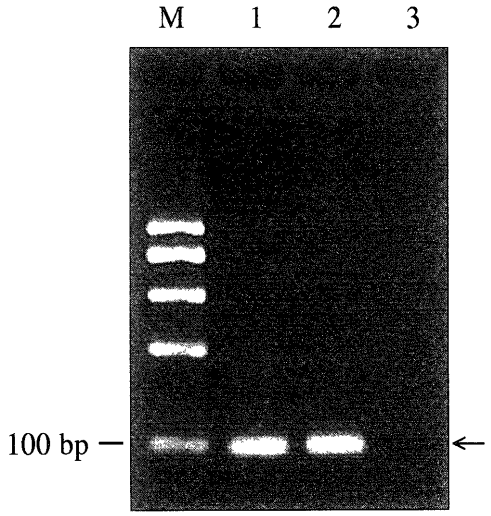

Fig. 3. Agarose gel electrophoresis of PCR products. The PCR was conducted with the primers UF2 and UR2, the PCR products were analyzed by electrophoresis in 3% agarose gel, and the gels were stained with ethidium bromide. Lanes: M, 100bp DNA ladder (molecular size standards); 1, *S. marcescens* in PBS; 2, *S. marcescens* in blood; 3, blood without bacteria. The expected 101 bp fragments indicated by an arrow are seen in both lanes 1 and 2

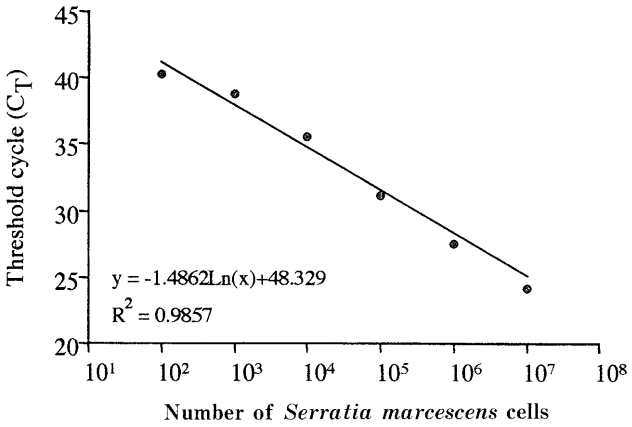

Fig. 4. Correlation between the C_T values and bacterial concentrations (log10) in *S. marcescens* blood samples.

A linear correlation is observed over the range of 1×10^2 CFU/ml to 1×10^7 CFU/ml (a significant coefficient, $R^2=0.9857$).

$\log_{10}$ of the number of targets (bacterial DNA) initially present was proportional to the C_T and could be measured with a standard curve.

PCR assay and sequencing

The primers, UF2 and UR2, are described above. The cycling conditions were denaturation for 45 s at 94℃, annealing for 45 s at 55℃, and polymerization for 45 s at 72℃ (30 cycles). The amplified PCR products were analyzed by gel electrophoresis with 3% agarose and stained with ethidium bromide. A 100 bp DNA ladder (Wako, Tokyo) was used as the molecular size standard.

The DNA sequences of the amplified PCR products were directly determined using the Taq dyedeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and a 373A DNA sequencer (Applied Biosystems) according to the manual.

RESULTS

Real time (TaqMan) PCR assay

Outbreak-derived *S. marcescens* cells, freshly cultured in LB broth, were serially diluted in PBS at different concentrations (1 CFU/ml to 1×10^7 CFU/ml). Bacterial DNA was then extracted from each dilution and used for the real time PCR assay (Fig. 2A). Typical amplification curves were observed, and the minimum level of bacterial concentration in the original suspension, which could be detected by the real time PCR assay, was determined to be 5×10^1 CFU/ml.

Next, *S. marcescens* cells were suspended in blood from healthy adults, instead of PBS. DNA was extracted, and the DNA solution was assayed by the real time PCR (Fig. 2B). Again, typical amplification curves were observed. The minimum level of bacterial concentration in the original suspension, which could be detected by the real time PCR assay, was 1×10^2 CFU/ml. When the same bacterial concentration was employed, the C_T value for the bacterial suspension in blood was 2 or 3 cycles later than for the bacterial suspension in PBS. No fluorescence signal was detected when blood itself (without bacteria) was employed.

The amplified PCR products were analysed on agarose gels (Fig. 3). Both the PBS samples and blood samples of *S. marcescens* gave a single band of the same size (101 bp), as shown in Fig. 3 (lanes 1 and 2). The sequence of the amplified PCR products was confirmed by direct sequencing (data not shown). Blood itself (without bacteria) gave negative results in the PCR assay. (Fig. 3, lane 3)

P. aeruginosa strains were also employed in the real time PCR assay, but produced negative results. (Fig. 2)

Quantitative detection of S. marcescens in blood samples

By using the amplification curves for *S. marcescens* in blood samples (as shown in Fig. 2B), the C_T values were plotted against the $\log_{10}$ of the *S. marcescens* concentration. A good linearity was observed over the range from 1×10^2 CFU/ml to 1×10^7 CFU/ml (Fig. 4). A significant coefficient of the correlation was repeatedly found for the C_T values and concentration ($R^2=0.9857$).

DISCUSSION

S. marcescens is an opportunistic bacterium that has been linked to hospital-acquired infections. The resulting bacteremia must be seriously considered because the mortality is reportedly 39-50%[9,10]. Recently, three large-scale outbreaks of *S. marcescens* infections occured in Japan in 1999, 2000 and 2002, and several patients rapidly deteriorated and died while waiting for blood culture results[3,4]. Clinically, the most rapid method of determining bacteremia is gram-staining, but the blood must be incubated for at least several hours to produce the large numbers of bacteria required for detection[11], and blood cultures take even more time. Newer rapid and sensitive methods are therefore needed to detect bacteremia caused by *S. marcescens*.

The detection of bacterial pathogens by PCR based on bacterial 16S rRNA gene amplification has been achieved with specimens of various body fluids[12,13]. Recently, several investigators reported that the real time (TaqMan) PCR assay was more rapid and sensitive in detecting bacteria in blood samples than an ordinary PCR method[6,8,14]. No assay of *S. marcescens* in blood samples by real time (TaqMan) PCR has been reported.

In this study, outbreak-derived *S. marcescens* strains were suspended in blood from healthy adults, and examined using the real time PCR assay. The *S. marcescens* strains in blood were successfully detected by real time PCR. In addition, a good linearity was observed with the C_T values and bacterial counts over a range of 1×10^2 CFU/ml to 1×10^7 CFU/ml.

When the same bacterial concentration was employed, the C_T value for the bacterial suspension in blood was 2 or 3 cycles later than for the bacterial suspension in PBS in the real time PCR assay. Also, the detection limit for *S. marcescens* in blood was 1×10^2 CFU/ml, while that for *S. marcescens* in PBS was 5×10^1 CFU/ml. This was due to less effective DNA extraction for blood samples compared with that for PBS samples.

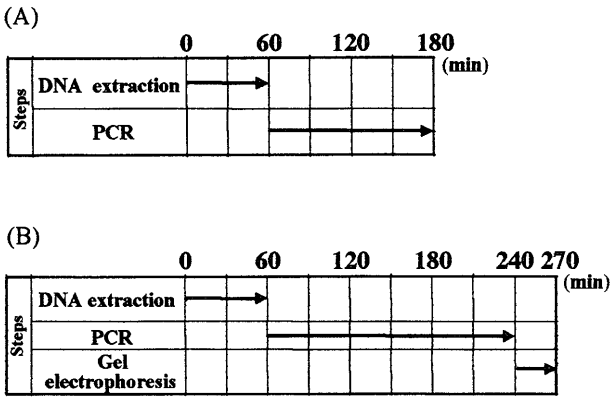

Fig. 5. Comparison of time and steps needed to complete the real time (TaqMan) PCR assay (**A**) and the ordinary PCR assay (**B**).

Sabui et al. reported that 79.3% of blood cultures from septic newborns accounted for more than 1×10^2 CFU of bacteria per ml[15]. In contrast, Kellogg et al. pointed out bacteremia cases with less than 10 CFU of bacteria per ml[16]. Thus, more sensitive methods are clinically needed. The reverse Transcriptase PCR (RT-PCR) method was reported as 100 times more sensitive than the ordinary PCR method for detecting the 16S rRNA gene of *Treponema*[17]. Therefore the real time (TaqMan) RT-PCR method can possibly improve sensitivity dramatically, and we are currently trying to improve its detection limit.

In this study, we used *S. marcescens* and *P. aeruginosa* as test bacteria. The primer-probe set, used in this study, produced negative results for *P. aeruginosa* but positive results for *S. marcescens* in the real time PCR assay. This primer-probe set, however, also showed positive results for *S. liquefaciens* (data not shown). We are now trying to develop *S. marcescens*-specific primers. For this, we designed two primers, SMF (5'-GGTGAGCTTAATACGTTCATCAATTG) and SMR (5'-GCAGTTCCCAGGTTGAGCC), based on the 16S rRNA gene sequence of *S. marcescens*. We want to utilize this primer set for a further real time PCR assay for the specific detection of *S. marcescens* in clinical blood samples.

The time required to complete the real time (TaqMan) PCR assay was only 3 hours, and only 200 μl of blood sample was needed, making it quicker than the ordinary PCR assay, as shown in Fig. 5. A real time (TaqMan) PCR assay should be useful in the rapid detection of bacteremia caused by *S. marcescens*.

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