

Nosocomial Outbreak-Derived *Serratia marcescens*: Rapid and Quantitative Detection of the Agent in the Blood by a Real Time PCR Assay

Akira IWAYA^{1,2}, Saori NAKAGAWA¹, Nobuhiro IWAKURA¹, Ikue TANEIKE¹,
Katsuyoshi HATAKEYAMA² and Tatsuo YAMAMOTO¹

¹Division of Bacteriology, Department of Infectious Disease Control and International Medicine, and ²Department of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

Received July 11 2002, accepted October 1 2002

Summary. There have recently been three large nosocomial outbreaks of *Serratia marcescens* with serious septicaemia and high mortality in Japan. In these blood stream infections, several patients died before the blood culture results were obtained, suggesting that rapid and sensitive methods are needed to detect *S. marcescens* in the blood. In this study, we examined a rapid DNA diagnosis of outbreak-derived *S. marcescens* in blood samples by a real time (TaqMan) PCR assay. The minimum level of sensitivity achieved was 5×10^1 CFU/ml in a buffer, and 1×10^2 CFU/ml in blood samples. The real time PCR assay was completed within 3 h from only 200 μ l of blood samples. There was a linear correlation between the bacterial cell number (in a range of 10^2 to 10^7 CFU/ml) and the minimum number of cycling to yield positive results in the assay. The data suggest that a real time (TaqMan) PCR assay is useful for the rapid and quantitative detection of *S. marcescens* in blood samples.

Key words—*Serratia marcescens*, Real time (TaqMan) PCR, Bacteremia.

INTRODUCTION

Serratia marcescens, a gram-negative rod, is an important opportunistic pathogen responsible for nosocomial infections^{1,2}. Three large nosocomial outbreaks of *S. marcescens* infections recently occur-

ed in Japan in 1999, 2000, and 2002^{3,4}. In these outbreaks, the mortality of the bacteremia was more than 50%, and several patients died before the blood culture results became clear. Since a traditional blood culture takes several days to detect bacteremia, newer rapid and sensitive methods are needed to detect bacteremia caused by *S. marcescens*.

Molecular DNA assays based upon the polymerase chain reaction (PCR) offer promise as sensitive and rapid detection methods for bacterial pathogens, and are widely used⁵. More recently, a rapid and sensitive DNA assay, known as a real time PCR assay (alternatively called 5' nuclease fluorogenic TaqMan PCR assay or TaqMan PCR assay), has been described⁶.

This assay system consists of PCR primers and a labeled oligonucleotide probe with a trick, which binds to the sequence present between the two PCR primers⁶. The probe has a reporter fluorescent dye at the 5'-end and a quencher dye at the 3'-end. The probe itself is not able to emit a fluorescent signal because of the proximity of the reporter and quencher dyes. When the target DNA sequence is present in the samples, the first primer (forward), the probe and the second primer (reverse), respectively, bind to the target (template) DNA strand from the 5'-side to 3'-side. When PCR starts, Taq polymerase present in the reaction mixture extends the first primer along the template, and on the way, Taq polymerase cleaves off the reporter dye bound to the probe by the

Correspondence: Tatsuo Yamamoto, Division of Bacteriology, Department of Infectious Disease Control and International Medicine, Niigata University Graduate School of Medical and Dental Sciences, 757 Ichibanchou, Asahimachidori, Niigata, Japan.

Abbreviations—bp, base pairs; CFU, colony forming unit; LB broth, Luria-Bertani broth; OD, optical density; PBS, phosphate-buffered saline; PCR, polymerase chain reaction

5' nuclease activity⁷⁾, 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 electrophoresis³⁾. 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°C 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 OD₆₀₀ 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°C for 10 min. Subsequently, 200 µl of buffer AL (from the kit) was added and the mixtures were incubated at 72°C 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 previously⁸⁾. 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

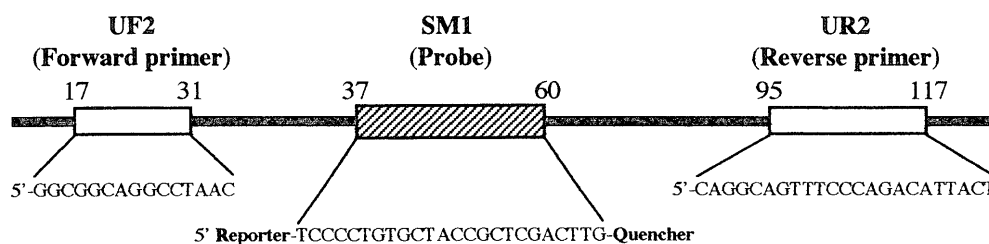


Fig. 1. Nucleotide position for the primers (UF2 and UR2) and the probe (SM1) in the 16S rRNA gene sequence for *S. marcescens*.

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 UR2⁸⁾ 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.

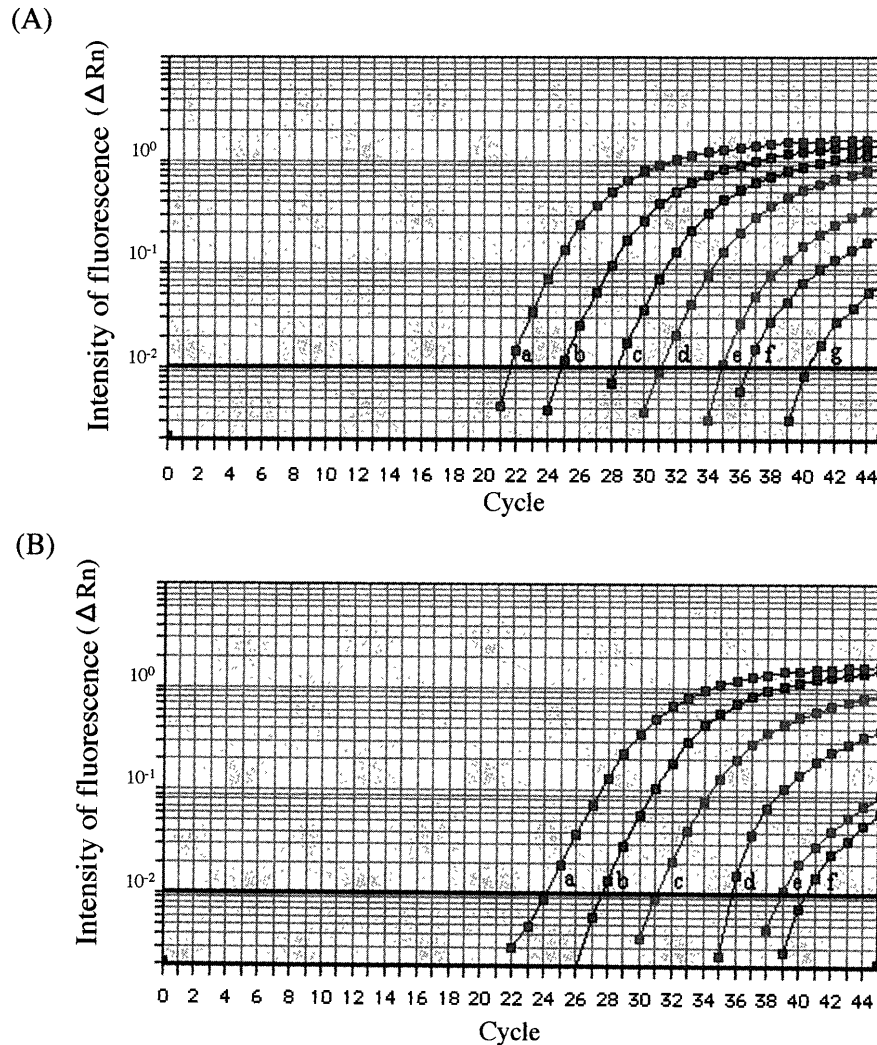


Fig. 2. Real time (TaqMan) PCR assay of *S. marcescens*.

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 ($50 \mu\text{l}$) contained 400 nM of primers, 80 nM of the probe, $25 \mu\text{l}$ of Taqman universal prepared mixture, and $20 \mu\text{l}$ of the template. The cycling conditions were an initial single cycle for 10 min at 95°C (to activate AmpliTaq Gold) and 45 cycles of two-temperature cycling consisting of 15 s at 95°C (for

denaturation) and 1 min at 60°C (for annealing and polymerization). The intensity of the fluorescence (ΔR_n) 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

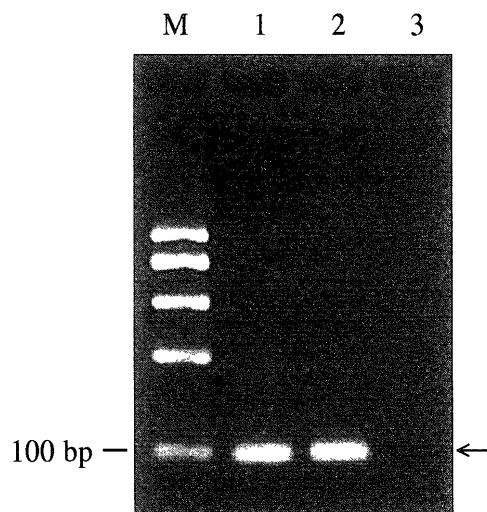


Fig. 3. Agarose gel electrophoresis of PCR products. The PCR was conducted with the primers UF2 and UR2, the PCR products were analyzed by electrophoresis in 3% agarose gel, and the gels were stained with ethidium bromide. Lanes: M, 100bp DNA ladder (molecular size standards); 1, *S. marcescens* in PBS; 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

\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°C, annealing for 45 s at 55°C, and polymerization for 45 s at 72°C (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 dyedexy 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/

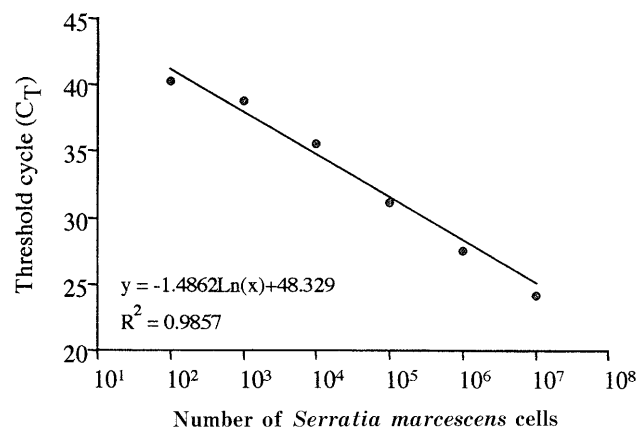


Fig. 4. Correlation between the C_T values and bacterial concentrations (\log_{10}) in *S. marcescens* blood samples.

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

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 occurred 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 detection¹¹, 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

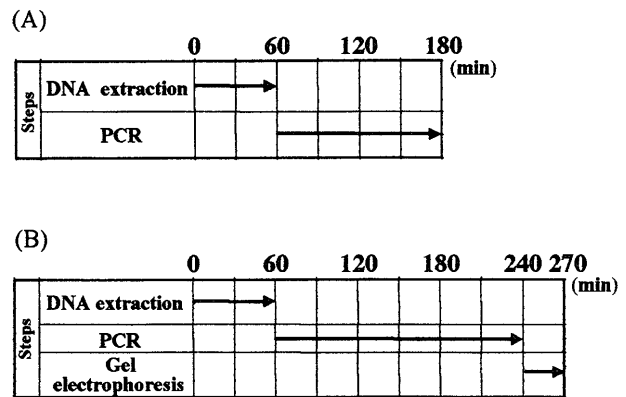


Fig. 5. Comparison of time and steps needed to complete the real time (TaqMan) PCR assay (A) and the ordinary PCR assay (B).

PBS samples.

Sabui et al. reported that 79.3% of blood cultures from septic newborns accounted for more than 1×10^2 CFU of bacteria per ml¹⁵. In contrast, Kellogg et al. pointed out bacteremia cases with less than 10 CFU of bacteria per ml¹⁶. 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'-GGTGAGCTTAATACGTT-CATCAATTG) and SMR (5'-GCAGTTCCCAGG-TTGAGCC), 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 μ l 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*.

REFERENCES

- 1) Hejazi A, Aucken HM, Falkiner FR: Epidemiology and susceptibility of *Serratia marcescens* in a large general hospital over an 8-year period. *J Hosp Infect* **45**: 42-46, 2000.
- 2) Hejazi A, Falkiner FR: *Serratia marcescens*. *J Med Microbiol* **46**: 903-912, 1997.
- 3) Report of the study group for disease of unknown cause. Tokyo: Bureau of Public Health Tokyo Metropolitan Government 2000. (in Japanese)
- 4) The report of nosocomial *Serratia marcescens* outbreak in Sakai city. Sakai city government public health and welfare bureau 2000. (in Japanese)
- 5) Mullis K, Faloona F, Scharf S, Saiki R., Horn G, Erlich H: Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harbor Symposia on Quantitative Biology* **51**: 263-273, 1986.
- 6) Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K: Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl* **4**: 357-362, 1995.
- 7) Holland PM, Abramson RD, Watson R, Gelfand DH: Detection of specific polymerase chain reaction product by utilizing the 5'→3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc Natl Acad Sci USA* **88**: 7276-7280, 1991.
- 8) Sen K, Asher D: Multiplex PCR for detection of *Enterobacteriaceae* in blood. *Transfusion* **41**: 1356-1364, 2001.
- 9) Yu WL, Lin CW, Wang DY: *Serratia marcescens* bacteremia: clinical features and antimicrobial susceptibilities of the isolates. *J Microbiol, Immuno Infect* **31**: 171-179, 1998.
- 10) Wong WW, Wang LS, Cheng DL, Lin SJ, Chin TD, Hinthorn DR, O'Connor MC, Huang WK: *Serratia marcescens* bacteremia. *J Formos Med Assoc* **90**: 88-93, 1991.
- 11) Wilson ML: Blood cultures. Introduction. *Clin Lab Med* **14**: 1-7, 1994.
- 12) Zhang Y, Isaacman DJ, Wadowsky RM, Rydquist-White J, Post JC, Ehrlich GD: Detection of *Streptococcus pneumoniae* in whole blood by PCR. *J Clin Microbiol* **33**: 596-601, 1995.
- 13) Lu JJ, Perng CL, Lee SY, Wan CC: Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. *J Clin Microbiol* **38**: 2076-2080, 2000.
- 14) Sen K: Rapid identification of *Yersinia enterocolitica* in blood by the 5' nuclease PCR assay. *J Clin Microbiol* **38**: 1953-1958, 2000.
- 15) Sabui T, Tudehope DI, Tilse M: Clinical significance of quantitative blood cultures in newborn infants. *J Paediatr Child Health* **35**: 578-581, 1999.
- 16) Kellogg JA, Manzella JP, Bankert DA: Frequency of low-level bacteremia in children from birth to fifteen years of age. *J Clin Microbiol* **38**: 2181-2185, 2000.
- 17) Centurion-Lara A, Castro C, Shaffer JM, Van Voorhis WC, Marra CM, Lukehart SA: Detection of *Treponema pallidum* by a sensitive reverse transcriptase PCR. *J Clin Microbiol* **35**: 1348-1352, 1997.