〈Regular Article〉

Diagnostic accuracy of 16S ribosomal RNA gene polymerase chain reaction in bacteremia: A prospective observational study

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ABSTRACT The standard method for diagnosing bacteremia is blood culture. However, the sensitivity of blood culture is low when the number of bacteria in the blood is low or when antibiotics have already been administered. Furthermore, some bacteria are difficult to detect in blood cultures. 16S ribosomal RNA (rRNA) contains conserved sequences that are targeted for PCR amplification using universal primers. We investigated whether the threshold cycle (Ct) value of 16S rRNA real-time PCR in whole-blood samples can be used for early diagnosis of bacteremia. Ct values of the 16S rRNA real-time PCR in 307 collected specimens showed a bimodal distribution. Ct values of the blood culture-positive group were significantly lower than those of the blood culture-negative group ($P < 0.001$). The cutoff value of the receiver operating characteristic curve was 38.80, as determined using finite-mixture modeling and expectation-maximization algorithm. Analysis of the diagnostic accuracy at this cutoff value showed a sensitivity of 91.4%, specificity of 33.5%, positive predictive value of 15.0%, and negative predictive value of 96.8%. The Ct value of 16S rRNA real-time PCR shows high negative predictive value, it may be useful for excluding bacteremia when the cutoff value is set appropriately.

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Key words：Real-time PCR, Bacteremia, Early diagnosis

INTRODUCTION

Bacteremia is a condition in which bacteria are present in the naturally sterile blood. Since Schottmüller defined septicemia in 1914[1)], the relationship between bacteremia and sepsis has remained controversial until recent years. Sepsis was redefined by the US intensivists in 1992[2)]. Since then, bacteremia has become irrelevant in the diagnosis of sepsis[3)] because bacteremia and sepsis indicate different pathophysiologies[4, 5)]. Nevertheless, both conditions are closely related, and 69% of patients with septic shock yield a

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positive blood culture[6]. In patients with sepsis, delayed treatment increases the risk of death; thus, early diagnosis and treatment is crucial[7]. However, in clinical practice, physicians are hesitant to diagnose sepsis without confirming an infection. In such cases, early diagnosis of bacteremia may contribute to early diagnosis of sepsis, facilitating adequate early treatment.

The most facilitated method for diagnosing bacteremia is blood culture, a well-established method that can detect a wide range of pathogens[8]. In blood cultures, pathogens can be identified along with their respective susceptibility to antimicrobial agents; however, one limitation of blood cultures is the turnaround time[8–10]. Furthermore, the sensitivity of this test is also reduced in cases with empirical antibiotics administration prior to sample collection, low number of bacteria, and low sample blood volume[8, 11, 12]. Moreover, the detection sensitivity of blood culture is poor in cases of fastidious organisms[8, 10]. Therefore, there is no ideal method to diagnose bacteremia because blood cultures can result in false negatives that cannot be ignored in clinical practice.

The bacterial 16S rRNA gene consists of conservative and variable regions and is present in all bacteria[11]. In recent years, PCR assay using the 16S rRNA gene has been used as a diagnostic tool[8, 11, 12].

In a previous study, we investigated the diagnostic accuracy of biomarkers for bacteremia in patients with suspected infections[13]. This study aimed to investigate the utility of Ct values of 16S rRNA real-time PCR from whole-blood samples for diagnosing bacteremia in patients with suspected infections.

MATERIALS AND METHODS

Inclusion and exclusion criteria

Our original study comprised patients who had been hospitalized in the Advanced Critical Care Center at Kawasaki Medical School Hospital in Okayama, Japan, from December 2014 through September 2016 and had undergone blood cultures due to suspected bacteremia upon admission or during hospitalization[13]. When a new incidence of bacteremia was suspected, blood cultures were performed. Patients who declined to participate in the study were excluded. This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of Kawasaki Medical School (approval number: 1926-2). This study was conducted after written informed consent was obtained from each patient. Ethical approval for this secondary data analysis was approved by the Institutional Review Board of Kawasaki Medical School (approval number: 5247-00).

Study design and data collection

This single-center prospective study involved patients with suspected bacteremia at the emergency department and critical care center. The indication for blood culture testing depended on the clinical judgment of the attending physician and was not influenced by the study protocol. Data were collected for separate samples if the suspected bacteremia episodes were determined to be completely independent, even in the same patient. Additionally, a whole-blood sample was collected in an EDTA-2Na blood collection tube at the time of blood culture, and real-time PCR (16S rRNA real-time PCR) was performed to amplify partial 16S ribosomal RNA (16S rRNA) sequence. When performing blood cultures, the site of the suspected infection was recorded. The primary endpoint of this study was the diagnostic accuracy of the Ct value by 16S rRNA real-time PCR analysis using whole-blood samples when the clinician suspected bacteremia. The secondary endpoint was the evaluated mismatched results between blood cultures and 16S rRNA real-time PCR.

Blood culture and bacterial identification

Ethanol containing 1% chlorhexidine gluconate was used for skin disinfection. If alcohol disinfectants were contraindicated, a 10% povidone iodine-containing solution was used instead. For all patients, two sets of blood samples were collected from different sites and for each set, at least 16 ml of the blood sample was inoculated to BD BACTEC™ Plus Aerobic / F and BD BACTEC™ Plus Anaerobic / F bottles (Becton Dickinson, Franklin Lakes, NJ, USA) and subjected to blood culture testing using the BD BACTEC™ FX blood culture system (Becton Dickinson). All blood culture bottles were incubated at 35℃ for 7 days. When a positive signal was obtained, the culture fluid was subjected to Gram staining and processed for bacterial identification using the MicroScan WalkAway 96 SI (Beckman Coulter, Brea, CA, USA) and the API system (bioMerieux, Marcy-l' Étoile, France)[13]. Therefore, bacterial identification was based on biochemical tests, not mass spectrometry.

16S rRNA real-time PCR

Nucleic Acid Extraction

Template nucleic acid to serve as template for real-time PCR assay was extracted from EDTA-2Na whole-blood specimens. Specimens awaiting analysis and frozen at -80℃ were thawed and homogenized by gentle pipetting, from which 400 μl was subjected to nucleic acid extraction using QIAamp UCP Pathogen Mini Kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions.

Real-time PCR

Real-time PCR of 16S rRNA gene was carried out using Probe qPCR Mix (TaKaRa Bio Inc., Shiga, Japan) on a CFX96 Detection System (Bio-Rad Laboratories, Hercules, CA, USA). An established primer / probe set was adopted from the literature[14]; this set has been successfully used at the Department of Pediatrics for investigating clinical specimens[15–17]. Their sequences were: Forward primer, 5′-AGTTTGATCMTGGCTCAG-3′; Reverse primer, 5′-GGACTACHAGGGTATCTAAT-3′; probe, 5′-[VIC]-CGTATTACCGCGGCTGCTGGCAC-[MGB]-3′. The primers were ordered from Eurofins Genomics, K.K. (Tokyo, Japan), whereas the fluorescent probe was ordered from Life Technologies Japan Ltd. (Tokyo, Japan). The estimated PCR product size was 0.8kb, which corresponds to the nucleotides 10-805 of *Escherichia coli* 16S rRNA gene sequence (accession number J01859.1)[14]. Amplification was performed in triplicated 20 μl reactions containing 5 μl template, 500 nM each of forward and reverse primers, and 250 nM fluorescent hydrolysis probe. The thermal profile for amplification was 95℃ for 30 s, followed by 45 amplification cycles at 95℃ for 5 s and 60℃ for 30 s. Negative reactions were assigned a Ct value of 45 for statistical analysis.

Statistical analysis

A sample size of 232 was calculated and subsequently used to achieve a power of 1-β of 0.90, area under the curve (AUC) of 0.70, α value of 0.05, and κ (blood culture-negative / blood culture-positive) coefficient of 9. Differences in categorical variables were analyzed using Fisher's exact test. Inter-group analysis for non-normally distributed data was performed using the Mann–Whitney U-test (two-tailed).

Receiver operating characteristic (ROC) curve analysis was used to test diagnostic ability. The Youden's index and point with the shortest distance from the upper left corner of the ROC curve (closest to [0.1]) were used to determine the relevant cutoff value to identify positive blood cultures.

Bimodal data was modeled using finite-mixture modeling, and the cutoff value separating the two peaks of a given type I error was derived. The expectation-maximization algorithm was used to

estimate the cutoff value, and the Monte Carlo method was used to calculate the confidence interval. Normality, used as the basis of finite-mixture modeling, was determined using Shapiro-Wilk test. In addition, the sensitivity, specificity, positive predictive value, and negative predictive value were calculated based on the obtained cutoff value.

All statistical analyses were performed using R[18]. Bimodal data analysis was performed with bbmle[19] and cutoff[20] packages. Figures were produced using the package ggplot2[21]. A P-value of < 0.05 was considered statistically significant.

RESULTS

A total of 307 specimens collected from 210 patients were included in the study[13]. The estimated foci of infection were pneumonia (126), urinary tract infection (42), and wound infection (37) (Table 1). Of the 307 samples collected, 35 (11.4%) gave positive blood culture results. Table 2 shows the results of blood cultures.

Analysis of results

In the histogram of the Ct values of all samples (n = 307) evaluated using 16S rRNA real-time PCR, the Ct values show a bimodal distribution with a median Ct value of 34.68 (interquartile range [IQR]: 31.69-39.46) (Fig. 1).

Fig. 2 illustrates the distribution of the Ct values of the blood culture positive and negative samples. In our preceding study[13], one sample judged as false-positive based on blood culture from the clinical course or detected pathogen was analyzed as blood culture-negative. However, this study aimed to diagnose bacteremia using 16S rRNA analysis; therefore, whether the blood culture was contaminated would not affect the results. Based on this, the sample was treated as blood culture-positive. The median Ct value of the blood culture-positive group was 30.99 (IQR: 28.32-34.25), and that of the blood culture-negative group was 35.28

Table 1. Patients' characteristics

Patients (n = 210)		Suspected site of infection, n (%)	
Age at admission, years, median (IQR)	70.5 (50–80)	Lung	126 (41.4)
Sex:male, n (%)	134 (63.8%)	Urinary tract	42 (13.8)
Consecutive samples (n = 307)		Wound	37 (12.2)
Diagnosis on admission, n (%)		Skin	29 (9.5)
Trauma	176 (57.3)	Abdomen	19 (6.3)
Infectious disease	83 (27.0)	Blood infection/endocardium/catheter/implant device	11 (3.6)
Others	48 (15.6)	Central nerve	10 (3.3)
Obtained in the ER, n (%)	104 (33.9)	Osteoarticular	6 (2.0)
q SOFA score, n		Others	27 (8.9)
0	18	Prior antimicrobial treatment, n (%)	122 (39.7)
1	41	Immunosuppressive state, n (%)	
2	38	Steroid therapy	13 (4.2)
3	7	Immunosuppressant	4 (1.3)
Vital signs, median (IQR)		Anticancer agent	2 (0.7)
Body temperature (℃)	38.5 (34.4-39.0)		
Heart rate (beats/min)	96 (80-110)		
Systolic blood pressure (mmHg)	128 (109-149)		
Respiratory rate (breaths/min)	21 (17-27)		
Laboratory data, median (IQR)			
White blood cell count (/μL)	10,940 (8,110-13,920)		
Hematocrit (%)	31.05 (27.3-37.1)		
Creatinine (mg/dL)	0.66 (0.48-1.10)		
C-reactive protein (mg/dL)	8.83 (4.26-15.95)		

IQR, interquartile range; ER, emergency room; q SOFA, quick Sequential Organ Failure Assessment.

Table 2. Organisms from positive blood culture

Organisms	n
Gram-positive bacteria	18
Staphylococcus aureus (MRSA)	4
Staphylococcus aureus (MSSA)	3
Group A streptococci	1
Group B streptococci	2
Group F streptococci	1
Group G streptococci	1
Coagulase-negative staphylococci	3
Enterococcus faecium	1
Peptostreptococcus sp.	1
Corynebactrium sp.	1
Gram-negative bacteria	12
Escherichia coli	7
ESBL-producing Escherichia coli	2
Edwardsiella tarda	1
Enterobacter cloacae	1
Bacteroides fragilis	1
Polymicrobial infections	5
Clostridium perfringens, ESBL-producing *Escherichia coli*, *Alcaligenes sp.*, *Pseudomonas aeruginosa*	1
Veilonella parvula, *Bacteroides ureolyticus*, α-Streptococcus	1
Escherichia coli, *Clostridium bifermentans*	1
Enterococcus faecalis, *Staphylococcous epidermidis*	1
Bacilus sp., α-Streptococcus	1

MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*; ESBL, extended spectrum beta-lactamase.

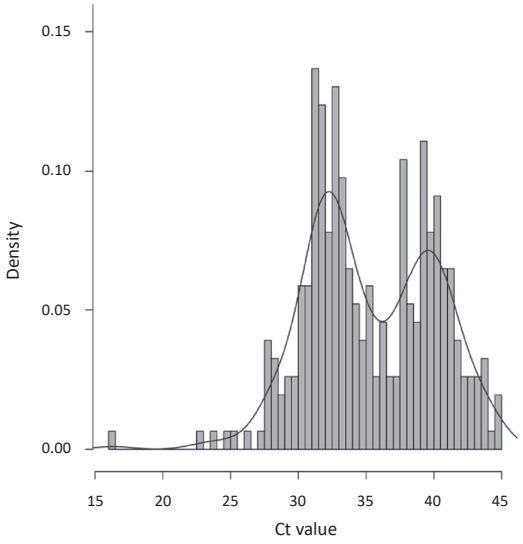

Fig. 1. Histogram and density curve of Ct value of 16S-rRNA real-time PCR of all samples (n = 307). Histogram bar width (0.5). Solid line: density curve.

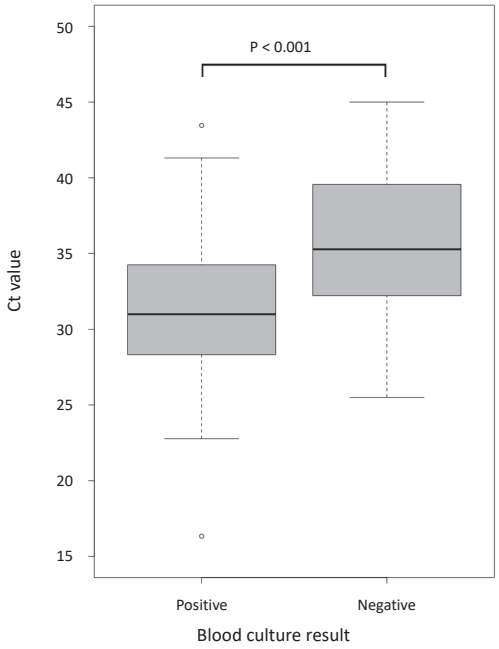

Fig. 2. Box plot of Ct values of blood culture-positive and blood culture-negative groups.

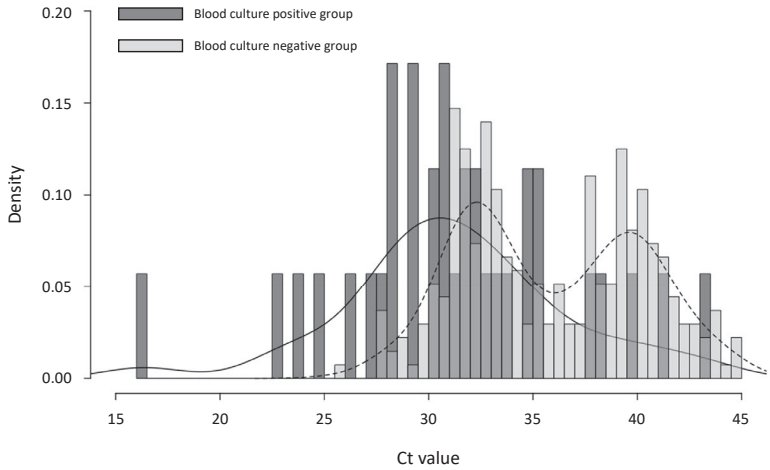

Fig. 3. Histogram of Ct values of 16S rRNA real-time PCR in blood culture-positive and blood culture-negative groups. Gray bars: blood culture-positive group. White bars: blood culture-negative group. Solid curved line: Ct value density curve of blood culture-positive group. Dotted curved line: Ct value density curve of blood culture-negative group.

(IQR: 32.22-39.56). The Ct value was significantly lower in the blood culture-positive group ($P < 0.001$).

The histogram of the Ct values obtained by dividing the collected 307 samples into blood culture-positive and blood culture-negative groups is shown in Fig. 3. The Ct values of the blood culture-positive group (gray bars, n = 35) showed a monomodal distribution; in the Shapiro-Wilk test, a normal distribution was observed with W = 0.98 and P = 0.64. The Ct values of the blood culture-negative group (white bars, n = 272) showed a bimodal distribution.

A ROC curve was drawn to derive a cutoff value for predicting positive blood cultures from the Ct values (Fig. 4). The cutoff value for Ct values of the blood culture-positive group obtained from Youden's index was 31.19 (Fig. 4A), and obtained from closest to the (0,1) was 32.65 (Fig. 4B).

The histograms of Ct values of all samples showed apparent bimodal distribution (Fig. 1). Of the two peaks in the blood culture-negative group (white bars), the peak with the lower Ct value was close to that of the blood culture-positive group (gray bars)

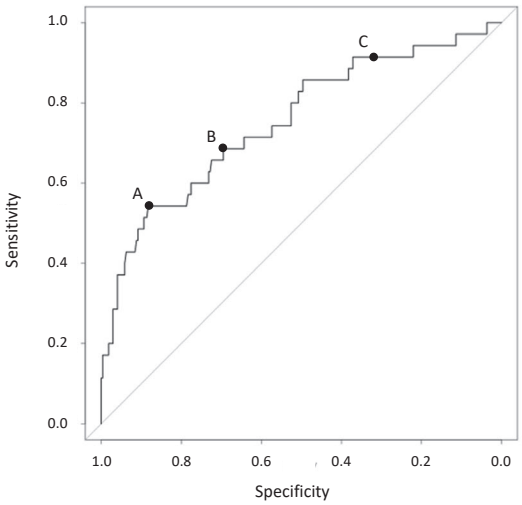

Fig. 4. Cutoff value (ROC curve) of Ct values of positive blood cultures.
AUC = 0.75, 95% CI = (0.658-0.753)
A: cutoff determined by Youden's index; cutoff = 31.19, sensitivity 54.3%, specificity 88.2%.
B: cutoff determined by Closest; cutoff = 32.65, sensitivity 68.6%, specificity 69.5%.
C: cutoff determined by finite-mixture modeling; cutoff = 38.80, sensitivity 91.4%, specificity 37.1%.

(Fig. 3). The cutoff value was estimated as 38.80 (95% CI: 38.78-38.82) (Fig. 5).

The accuracy of predicting positive blood culture

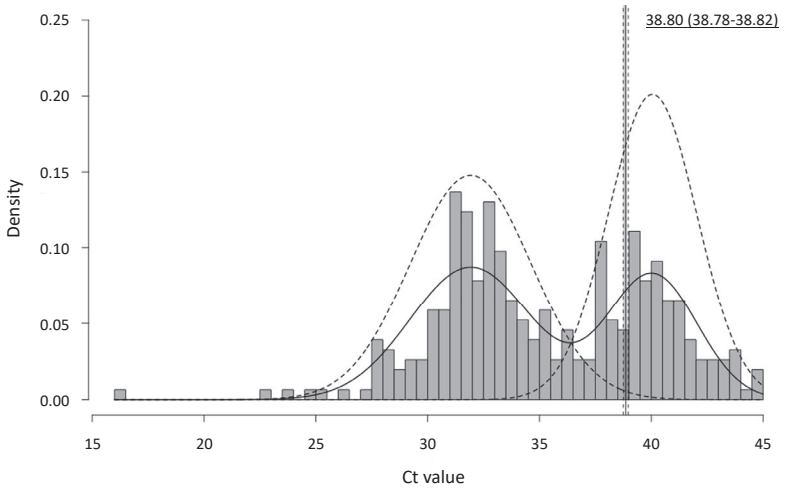

Fig. 5. Histogram of cutoff value of Ct values calculated using finite-mixture modeling of all samples (n = 307).
Solid curved line: density curve. Dotted curved line: probability distribution calculated using finite mixture modeling. Solid straight line: calculated cutoff value (38.80). Dotted straight line: 95% CI (38.78-38.82).

Table 3. Diagnostic accuracy for each cutoff value

	Threshold	Sensitivity	Specificity	PPV	NPV	TPR	FPR	TNR	FNR
Youden's index	31.19	0.543	0.882	0.373	0.938	0.543	0.118	0.882	0.457
Closest	32.65	0.686	0.695	0.224	0.945	0.686	0.305	0.695	0.314
FM modeling	38.8	0.914	0.335	0.15	0.968	0.914	0.665	0.335	0.086

PPV, positive predictive value; NPV, negative predictive value; TPR, true positive rate; FPR, false positive rate; TNR, true negative rate; FNR, true negative rate.
Youden's index, cutoff value determined from Youden's index (Fig. 4A); Closest, cutoff value determined from point with the shortest distance from the upper left corner of the ROC curve (Fig. 4B); FM modeling, cutoff value estimated using finite-mixture modeling and expectation-maximization algorithms (Fig. 4C).

results at the three cutoff values was estimated (Table 3).

Among the study group, a case presented with hemophagocytic syndrome, in which the paired serum of Scrub typhus (Tsutsugamushi disease) IgG / IgM was elevated, and the PCR of the eschar confirmed Scrub typhus. The blood culture was negative, but the Ct value of 16S rRNA real-time PCR for this case was 30.13. We detected *Escherichia coli* in the urine culture of an another case of septic shock due to acute pyelonephritis in which a previous physician had already initiated an antimicrobial agent. The blood culture of this case was negative, but the Ct value of 16S rRNA real-time PCR was 31.22. These cases were suspected blood culture false negatives.

Of the 307 samples with a Ct value over 38.80, only three cases were blood culture positive. One case was pyogenic myositis in a patient taking steroids, with two sets of methicillin-susceptible *Staphylococcus aureus* blood cultures, and the Ct value was 40.87. The second case was hematoma infection after pelvic fracture, with one set of blood cultures of *Bacteroides fragilis*, and the Ct value was 43.56. The third case was pyelonephritis, with one set of blood cultures of *E. coli*, and the Ct value was 42.04.

DISCUSSION

Current methods available for diagnosing bacteremia have several limitations. Furthermore, intracellular parasites, such as Mycoplasma, Legionella, Chlamydia, and anaerobic bacteria, are hard to detect in blood cultures, even if viable pathogens are present in the blood[8]. Therefore, blood cultures cannot detect all cases of bacteremia and may show false-negative results. Novel methods for promptly diagnosing bacteremia and identifying the pathogenic bacteria, their susceptibility to antimicrobial agents, and unaffected by prior antimicrobial treatments are urgent demands in clinical practice.

The utility of PCR as a diagnostic method for bloodstream infections has gained attention[8]. PCR can amplify a specific genomic sequence using a primer set unique to the sequence of interest. This method enables the detection of a genomic sequence indicating a specific bacterial species or specific resistance pattern from a blood sample. A real-time PCR targeting the autolysin (*lytA*) gene of *Streptococcus pneumoniae* was reported to have a sensitivity of 47% and specificity of 99% for blood samples collected at the time of suspected pneumococcal bacteremia in children[22]. For samples in which gram-positive cocci were detected in blood culture, a PCR method targeting *S. aureus* and methicillin-resistant *S. aureus* showed a sensitivity of 99.4% and specificity of 99.8% in distinguishing between *S. aureus* and non-*S. aureus* sp. and a sensitivity of 100% and specificity of 98.7% in identifying methicillin-resistant *S. aureus*[23]. PCR is a highly accurate diagnostic method for detecting specific mycetoma and bacteria with specific resistance patterns[22, 23].

When bacteremia is suspected but the pathogenic organism has not been identified, it is necessary to perform a broad-range PCR analysis targeting the 16S rRNA gene to identify the organism[8, 11]. The bacterial 16S rRNA gene consists of conservative and variable regions[8, 11] and is present in all bacteria. However, this method has not been widely used for blood samples collected from patients[9, 12, 24].

Hassan *et al*.[9] performed 16S rRNA PCR and blood culture simultaneously using blood samples from patients with suspected bacteremia. The sensitivity in predicting positive blood culture was 86.25%, and the specificity was 91.25%. Valle Jr *et al*.[24] reported that the sensitivity of 16S rRNA PCR in predicting positive blood culture was 93.75%, and specificity was 100%. However, cases of antibiotic administration were excluded, and primers for the 16S rRNA gene as well as those specific for gram-positive and gram-negative bacteria were used. Mishra *et al*.[12] studied samples from patients with suspected sepsis and reported that 16.12% of these patients had positive blood cultures, whereas 58.06% had positive PCR results; however, the diagnostic accuracy of 16S rRNA PCR was not evaluated. Most studies have focused on positive blood cultures and have not analyzed the diagnostic accuracy for true bacteremia, considering that blood culture results may include false negatives.

Zucol *et al*. evaluated a broad-range real-time PCR assay using three primer pairs targeting the 16S rRNA gene[14]. They concluded that 16S rRNA real-time PCR with the primer pair Bak11W / Bak2, giving amplicons of 796 bp in length, showed the best overall sensitivity, detecting DNA of 82% of the strains investigated at concentrations of $\leq 10^2$ CFU in water per reaction[14]. Furthermore, they reported that this protocol can detect 1-10 CFU per reaction in water, avoiding detection of background DNA[14]. In this study we applied the same primer pair.

This study evaluated the Ct value of 16S rRNA real-time PCR. The Ct values were significantly lower in the blood culture-positive group than in the blood culture-negative group (Fig. 2). The sensitivity in predicting positive blood culture was

54.3% (Youden's index) and 68.6% (Closest), and specificity was 88.2% (Youden's index) and 69.5% (Closest) (Table 3). This diagnostic performance to predict positive blood culture was insufficient for clinical application to rule in bacteremia. The histogram of the Ct values of the blood culture-positive group showed a monomodal distribution, whereas that of the blood culture-negative group showed a bimodal distribution (Fig. 3). Therefore, the blood culture-negative group was suspected to include false-negative blood culture samples. Among samples showing low Ct values in the blood culture-negative group, the Scrub typhus and urosepsis cases after antibiotic administration showed low Ct values, as mentioned earlier. *Orientia tsutsugamushi* that causes Scrub typhus is typically difficult to detect by blood culture and is considered false negative. Mishra *et al.*[12] reported that non-fermenting and anaerobic bacteria could be detected by 16S rRNA PCR even when blood cultures were negative. In addition, due to the potency of PCR in detecting and amplifying a minute amount of DNA even from dead bacteria, PCR can detect bacteremia even in blood samples collected after antibiotic administration, which give false-negative blood cultures.

In consideration of the above, the histograms of the blood culture-negative groups in this study showed a bimodal distribution because the 16S rRNA real-time PCR method is capable of detecting blood culture-undetectable bacteria.

There is no fixed method for calculating the cutoff from a bimodal distribution without an objective variable. However, Trang *et al.*[25] calculated the cutoff value of bimodal data in a study in which norovirus was detected using real-time PCR in the stool samples of children under 5 years of age with enteritis. This method uses a finite-mixture model to model bimodal data and derive a cutoff value that separates the two peaks of a given Type I error.

Previous studies[11, 26] have shown that the cutoff Ct value of predicting blood culture positivity is smaller than the 38.80 obtained from the finite mixture model in this study. Conversely, the results of the analysis using blood culture positivity as the objective variable (Fig. 4A, B) do not differ significantly from that of previous studies[11, 26]. This is because the objective variable of the analysis using the finite mixture model is the detection of true bacteremia, including blood culture false negatives. When the Ct value of 38.80 calculated using the finite-mixture modeling was used as the cutoff, its negative predictive value of 96.8% was satisfactory for excluding bacteremia (Table 3). Hassan *et al.*[9] reported that the turnaround time of blood culture-negative cases was 5 days, whereas that of PCR was 4 h. If the Ct values of the 16S rRNA real-time PCR exceeded the cutoff, clinicians can consider diagnoses other than infectious diseases earlier.

Nevertheless, PCR has some limitations. In blood culture, the bacterial species can be estimated based on information, such as the media used, colony appearance, staining pattern, and microscopic morphology. Whereas, in real-time PCR, the Ct value itself cannot determine sequence information for identifying bacterial species. Furthermore, even if clinically satisfactory aseptic techniques are used during blood collection, the procedure is not DNA-free. Thus, the collected blood samples are at risk of DNA contamination from the surrounding environment. Therefore, the risk of false positives cannot be avoided in this situation.

One limitation of this study is that this was a secondary study. The analysis was based solely on blood samples, regardless of information such as patient background, other blood test results, and biomarkers. Therefore, verification of the clinical usefulness of this method is warranted.

CONCLUSION

The Ct value of 16S rRNA real-time PCR for

whole-blood samples has diagnostic utility for evaluating bacteremia. Although the Ct value of 16S rRNA real-time PCR often shows false-positive results, it may be useful for excluding bacteremia when the cutoff value is set appropriately.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

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