Exploration for the rapid detection method for bacterial pathogen in pediatric clinical specimens using the melting temperature mapping method.

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Abstract

The melting temperature (Tm) mapping method is a novel technique that uses seven primer sets without sequencing to detect dominant bacteria. Without using conventional culture methods, this method can identify pathogenic bacteria in adults within 3 h following blood collection. However, no studies have examined whether pathogenic bacteria can be detected in clinical specimens from pediatric patients with bacterial infections. Here, I designed a new primer set for commercial use, constructed a database with more bacterial species, and examined the agreement rate of bacterial species *in vitro*. Furthermore, I investigated the bacterial species in the blood culture bottles that tested positive for bacteria in my children's hospital and the percentage of these bacteria listed in the database. Moreover, I investigated whether my system could detect pathogenic bacteria from pediatric patients using the Tm mapping method and compared the detection rates of the Tm mapping and culture methods. The coverage was 588/605 (99%) and 42/46 (91%) for the number of culture bottles and bacterial species, respectively (1). Overall, 256 pediatric clinical specimens from 156 patients (94 males and 62 females; median age, 2 years [< 18 years of age]) were used. The observed concordance rates between the Tm mapping method and the culture method for both positive and negative samples were 76.4% (126/165) and 79.1% (72/91) in blood samples and other

clinical specimens, respectively. The Tm mapping detection rate was higher than that of the culture method using both blood and other clinical specimens. Additionally, using the Tm mapping method, causative bacteria in pediatric clinical specimens were rapidly identified compared to when using blood cultures. Therefore, the Tm mapping method could be a useful adjunct for diagnosing bacterial infections in pediatric patients and valuable in antimicrobial stewardship for patients with bacterial infections, particularly in culture-negative cases.

Introduction

Traditional culture-based methods require several days to identify and examine microorganisms from clinical specimens, during which patients may receive ineffective or unnecessary broad-spectrum antibiotics, potentially leading to a poor prognosis (2, 3). Although recent advancements in novel diagnostic platforms for bacterial infections, including matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, multiplex polymerase chain reaction (PCR) assays, 16S ribosomal DNA sequencing, and metagenomic deep sequencing, have improved diagnosis, rapid pathogen identification could promote the early administration of appropriate antimicrobial therapy, thus, decelerating the emergence of antimicrobial-resistant organisms, lowering medical expenses, and improving

clinical outcomes (4, 5). Previously, Niimi et al. reported a novel “melting temperature (Tm) mapping method” for rapidly identifying the dominant bacteria in a clinical sample using the 16S rRNA gene (6). Notably, using seven primer sets and no sequencing, this method can identify unknown pathogenic bacteria using 2 mL of whole blood within 3 h after blood collection and without performing a culture test. Furthermore, this method can prove the absence of bacteria because it uses a universal bacterial primer. However, it cannot identify multiple bacteria. Conversely, whether this method can be used for samples from pediatric patients is yet to be determined.

The circulating blood volume of a child is significantly lower than that of an adult. In newborns, particularly premature infants, collecting a large amount of blood may decrease blood pressure. Therefore, the lower the volume of blood required for the test, the lesser the burden on the circulatory system. The Clinical Laboratory Standards Institute guideline (7) recommends collecting less than 1% of the total blood volume for blood cultures. In contrast, since several pediatric patients have low-level bacteremia (10 CFU/mL or less), recent guidelines from the Infectious Diseases Society of America and the American Society for Microbiology recommend that 3%–4% and 1.8%–2.7% of the total blood volume should be collected for a child with a body weight < 12 kg and > 12 kg, respectively (8). Therefore, the

optimal blood volume that should be collected from children for culture studies is not clearly defined. Similarly, the number of clinical specimens required for bacterial identification using the Tm mapping method is not well defined, and no comprehensive reports are available on its usefulness. Hence, in this study, I prospectively evaluated the efficacy of the Tm mapping method in pediatric patients with bacterial infections. This doctoral dissertation is based on a paper (9).

Materials and Methods

Sensitivity test

I performed a sensitivity test for commercial use using newly constructed primer sets and a new database. The procedures were as follows: each bacterium was cultured purely in Luria Bertani (BD Difco™, Franklin Lakes, NJ, USA) medium, and the number of microbial cells per milliliter in the culture solution of each sample was measured using a flow cytometer (Beckman Coulter CytoFLEX (Product No: B53019)). Subsequently, the microbial cell density in the culture solution was adjusted by inoculating 2 mL of blood with each bacterial culture solution. The type strain was obtained from JCM (Japan Collection of Microorganisms, RIKEN BioResource Research Center, Tsukuba, Japan). The minimum

detectable concentrations for each bacterium include the following: *Escherichia coli* (JCM 1649^T), *Enterobacter cloacae* (JCM 1232^T), *Enterococcus faecalis* (JCM 5803^T), and *Klebsiella pneumoniae* (JCM 1662^T) were identified at 10–20 cells/mL, whereas *Pseudomonas aeruginosa* (JCM 5962^T), *Staphylococcus aureus* (JCM 20624^T, JCM 2151, JCM 8704, and JCM 16555), and *Staphylococcus epidermidis* (JCM 2414^T) were identified at 20–40 cells/mL. Next, the bacterial DNA was extracted from the collected bacterial pellets by crushing them with glass beads (high-purity PCR template kit; Roche, Mannheim, Germany). Finally, the bacteria were identified using the Tm mapping method.

In addition, the coverage of the bacterial species in the database was investigated. This was done by determining the bacterial species when culturing of blood collected from 2011 to 2015 at Saitama Children's Medical Center tested positive for bacterium and comparing the bacterial species with those listed in the database (1).

Setting and participants

This is a prospective single-center study conducted at the Saitama Children's Medical Center from January 2015 to April 2020. Eligible participants included patients less than 18 years of age suspected of bacterial infection. However, when I started the study, the Center for Disease Control/National Healthcare Safety Network Surveillance Definitions for Specific Types of

Infections 2014 stated that the definition of an episode of the same infection was yet to be established. The attending physician and the infection consulting team clinically prospectively determined one bacterial infection event, one case at a time, depending on the type of infection, including signs and symptoms, laboratory results, and completion of antimicrobial therapy. Furthermore, blood was collected from the participants and aseptically incubated in BacT/ALERT® PF plus (approximately 4 mL) and BacT/ALERT® FN plus (approximately 10 mL) (bioMérieux, Marcy-l'Étoile, France) bottles for culture (pediatric and anaerobic bottles). The attending physician determined the blood sample volume for culture based on clinical settings, including medical conditions and weight. The attending physician collected one or two sets of blood cultures based on the patient's general condition. For Tm mapping, 0.5–2 mL of whole blood was collected in sterile tubes (Neotube; NIPRO, Osaka, Japan) free of DNA contamination and sent to the laboratory. Other clinical samples were collected in sterile screw-capped tubes for both the traditional culturing and Tm mapping methods. There was a delay between sample collection for blood culture and Tm mapping. I limited my comparisons to samples collected from the same patient within 24 h since no difference was found in the positive rate within 24 h of a positive blood culture bottle in febrile patients (10). After collecting specimens for culturing, blood specimens for Tm

mapping were collected. Clinical specimens, including cerebrospinal fluid, ascites, and pus, were divided into two sterile screw-capped tubes: one for culturing and the other for Tm mapping. Clinical specimens that did not meet the above criteria (e.g., if the blood volume was less than 0.5 mL or the time between the culture and Tm mapping specimen collection exceeded 24 h) were not considered comparable between methods; therefore, these specimens were excluded from the analysis. However, when one or both bottles of a blood culture set tested positive, it was counted only once as a positive blood culture set. Therefore, if several samples collected simultaneously yield positive results, the time for extracting pathogenic microorganisms would be shorter.

Culture

After collection, the specimens were sent to a laboratory, and the blood was cultured using a BacT/Alert three-dimensional (bioMérieux) automated blood culture system. In the case of blood culture, Gram staining was performed directly within the blood culture bottle. In addition, aliquots of in-bottle fluid were aseptically removed from positive bottles where the bacteria had developed using standard methods and inoculated onto sheep blood agar, chocolate agar, and bromothymol blue lactose agar media. However, when Gram staining of

the culture medium indicated anaerobic bacteria, the laboratory technician added isolation media for anaerobic bacteria. Therefore, blood culture bottles that did not test positive in the system for 6 days were defined as negative. For clinical specimens other than blood, general bacterial isolation and culture intensification were performed by inoculating the specimen directly into the medium with sterile platinum ears. Since the bacterial species detected differed depending on the specimen, the clinical technologist selected different media, including selective enrichment broths, in addition to the common basic media, based on smear results and clinical information. The bacterial isolates were subjected to biochemical tests for identification and classification (8). The isolates were identified at the hospital laboratory using the MicroScan WalkAway 40 SI between 2014 and 2016 and the MicroScan WalkAway 96 Plus between 2017 and 2020.

DNA isolation

Bacterial DNA was isolated from the clinical specimens using DNA extraction kits (high pure PCR template kit [Roche, Mannheim, Germany] used from January 2015 to November 2018 and DNA Extraction Kit [Mitsui Chemicals, Tokyo, Japan] used from December 2018 to April 2020) following the manufacturer's instructions, and eluates were stored at −20ºC.

Furthermore, DNA isolation was performed in a laminar flow biosafety cabinet decontaminated daily by UV radiation, and strict separation from the PCR workstation was maintained to prevent DNA contamination.

Tm mapping method

The procedure of the Tm mapping method has been previously described in detail (6). Briefly, the first PCR was performed using the eukaryote-produced thermostable DNA (Taq) polymerase (Mitsui Chemicals), which was not contaminated with bacterial DNA, and one universal bacterial primer (a primer for the bacterial conserved region of the 16S rRNA gene, which is a primer for PCR-based detection of all bacteria). A negative control sample containing sterile water (non-template sample) and a positive control sample (e.g., *E. coli* ATCC 25922) were included in each experiment. The amplification protocol included the following: 95°C for 5 min, followed by 30 cycles of 94°C, 65°C, 72°C, and 85°C for 10 s, 20 s, 30 s, and 2 s, respectively. Subsequently, the PCR product was diluted 500-fold using molecular-grade distilled water (water deionized and sterilized for molecular biology; Nacalai Tesque Inc., Japan) and used as a template for the second (nested) PCR procedure. Next, seven universal bacterial primers targeting conserved regions of the 16S rRNA genes

were used in the second PCR. The primers developed for commercial use included the following: Region 1 primers (forward: 5′-GCAGGCTTAACACATGCAAGTCG-3′, reverse: 5′-CGTAGGAGTCTGGACCGT-3′); Region 2 primers (forward: 5′-GTCCAGACTCCTACGGGAG-3′, reverse: 5′-CCTACGTATTACCGCGG-3′); Region 3 primers (forward: 5′-AGCAGCCGCGGTAATA-3′, reverse: 5′-GGACTACCAGGGTATCTAATCCT-3′); Region 4 primers (forward: 5′-AACAGGATTAGATACCCTGGTAG-3′, reverse: 5′-AATTAAACCACATGCTCCACC-3′); Region 5 primers (forward: 5′-TGGTTTAATTCGATGCAACGC-3′, reverse: 5′-GAGCTGACGACAGCCAT-3′); Region 6 primers (forward: 5′-GTTAAGTCCCGCAACGAG-3′, reverse: 5′-CCATTGTAGCACGTGTGTAG-3′); and Region 7 primers (forward: 5′-GGCTACACACGTGCTACAATGG-3′, reverse: 5′-AGACCCGGGAACGTATTC-3′). The amplification protocol used during the second step was similar to the previous one: 95°C for 5 min followed by 35 cycles of 94°C, 60°C, 72°C, and 85°C for 10 s, 20 s, 30 s, and 2 s, respectively. For the Tm analysis, the resulting amplicons were first heated at 95°C for 10 s and subsequently cooled at 72°C for 90 s. Afterward, the temperature was gradually increased from 72°C to 95°C at a rate of 0.5°C/step. The data profile was analyzed using Rotor-Gene Q® (Qiagen, Germany). Subsequently, the

Tm values of the seven PCR amplicons were measured and mapped onto two dimensions. I identified the bacteria by comparing them with the bacterial species registered in the database (Rapid Diagnostic System for Bacterial Identification; Mitsui Chemicals). The accuracy of the identification was evaluated using the difference value (D).

Interpretation criteria for discrepancies in the results

Two or more physicians, including the treating physician, evaluated several criteria while considering the patient's clinical status. A pathogen whose nucleic acid was detected using Tm mapping was defined as a “true pathogen” when cultured from additional specimens collected from a similar infectious site during the same infectious episode and/or if the species was specific to the type of infection found in the patient. A pathogen was considered a “possible pathogen” if it had been previously reported as a causative agent of infection and was detected using only one method. Alternatively, if the nucleic acid of a common contaminant was isolated from the clinical specimen using the Tm mapping method without a positive result of blood culture that was judged by the attending physician and Infectious Diseases Consultant to be a contaminant and with no treatment initiated, then pathogen was termed “contamination pathogen.” The definition of contamination was based on the previous

report (11). The microorganisms that were detected by PCR but met none of the other criteria were designated as "indeterminate." A pathogen identified only by culturing was regarded as a "true pathogen" since the culturing method is considered the gold standard for identifying microorganisms.

Statistical analyses

I compared the Tm mapping and culturing methods using McNemar's test to detect pathogens from clinical specimens. Statistical analyses were performed using the EZR v. 1.54 software. Differences were considered statistically significant at two-tailed $P < 0.05$.

Results

Table 1 shows the sensitivity test results. A D of $0 < D \leq 0.26$ was a perfect match (37/37 = 100%), as was $0.26 < D \leq 0.53$ (34/35 = 97%), with a 1/35 = 2.9% mismatch. For $0.53 < D$, there was a perfect match (2/4 = 50%), with a genus match (1/4 = 25%) and a mismatch (1/4 = 25%). For $0.53 < D$, complete agreement, genus agreement, and discrepancies were 50% (2/4), 25% (1/4), and 25% (1/4), respectively. However, the two samples in which the bacterial counts could not be detected had low bacterial counts. Therefore, the cut-off value

of D for identifying bacteria in the clinical samples in this study was set to $0 < D < 0.53$. An examination of database coverage indicated that the database covered 588 of the 605 positive bottles (99%). However, the number of bacterial species in the positive bottles was 46, of which 42 (91%) were listed in the database (Figure 1) (1). Overall, 256 specimens from 156 patients (94 male and 62 female patients) with a median age of 2 years (range: 0–17 years) were collected for this study; nine samples from six patients were discarded from the analysis process due to contamination (eight and one blood and urine samples, respectively). In addition to blood samples (165), cerebrospinal fluid (41), abscess (16), synovial fluid (8), urine (7), ascites (7), and other specimens (12) were collected (Table 2). Of the 256 samples, 42 (18 blood and 24 other specimens) from 34 patients were culture-positive. Positive results were obtained using the Tm mapping method for 97 samples from 70 patients. Furthermore, the time interval of blood samples between the standard culture and the Tm mapping methods was at a median of 0 h (interquartile range [IQR]: 0–0 h). Among the 165 blood samples, 18 (10.9%) and 53 (32.1%) tested positive when the culture and Tm mapping methods were used, respectively (Table 3A). Among the 91 other clinical specimens from conventionally sterile body sites, 24 (26.4%) and 43 (47.3%) tested positive using the culture method and Tm mapping methods, respectively (Table 3B). Therefore, the detection rate of the Tm

mapping method was higher than that of the culture method ($P < 0.01$). The overall time from filling the automated blood culture device to reporting the results was as follows. The median time to report the results of the analysis of blood and non-blood samples was 6.39 days (IQR: 6.15–7.01 days) and 3.07 days (IQR: 1.95–4.40 days), respectively. However, the average time from DNA extraction to reporting the results of the Tm mapping method was 3.6 h (range: 2.22–3.37 h).

Congruence of the culture and Tm mapping method results

Overall, 198 sample results were congruent positive or negative, across the two methods. A total of 40 specimens (a + e) showed concordant positive results. Of the 35 specimens identified as positive using the Tm mapping method ($D \leq 0.53$), 33 were identified and matched, whereas two showed discordance after species identification. The organisms identified were *Staphylococcus aureus* (n = 12), *Streptococcus pyogenes* (n = 4), *Klebsiella oxytoca* (n = 3), *Staphylococcus epidermidis* (n = 3), *Enterococcus faecalis* (n = 2), *Escherichia coli* (n = 2), *Streptococcus intermedius* (n = 2), *Enterobacter aerogenes* (n = 1), *Enterococcus faecium* (n = 1), *Klebsiella pneumoniae* (n = 1), *Pseudomonas aeruginosa* (n = 1), and *Streptococcus gallolyticus* subsp. *pasteurianus* (n = 1). Overall, 158 specimens from

108 patients showed negative results with both methods. Therefore, the overall agreement between the Tm mapping method and the culture method for positive and negative samples was 77.3% (Table 2: [a + d + e + h]/total = 198/256). In addition, including the contamination results, 3.3% of samples were determined to be false positive using the Tm mapping method (contamination/(total + contamination) = 9/265). In comparison, 0.75% of samples were false negatives (c/(total + contamination) = 2/265).

Tm mapping method-positive, culture method-negative specimens

Of the 56 specimens (Table 3: [b + f]) in which bacterial species were identified using the Tm mapping method ($D \leq 0.53$), 32 specimens from 26 patients were culture-negative (Table 4). These samples included 21 blood, four abscess, two urine, two CSF, and two ascites specimens and one synovial fluid sample. In addition, a "true pathogen" was detected in 13 specimens from 11 patients. These findings were supported by detecting a similar pathogen in eight specimens from the same infectious site and identifying a similar pathogen in five specimens from other sites. Thirty-two specimens were from 38 patients who had received adequate antimicrobial treatment before collecting clinical specimens.

Tm mapping-negative and culture-positive results

Two blood samples from two patients tested positive for pathogens when the culture method was used; however, they were negative when the Tm mapping method was used. One sample contained *Streptococcus pneumoniae,* whereas the other contained *Salmonella enteritidis*.

Discordance in species identification

In two samples from two patients, different organisms were identified using the culture and Tm mapping methods; I found consistency in one of these samples at the genus level (*Staphylococcus aureus* vs. *Staphylococcus cohnii*). However, the culture method identified *Staphylococcus aureus* from multiple samples collected from the same infectious site as that of the other sample with discordant species identification. In contrast, the Tm mapping method identified the pathogen to be *Prevotella bivia*.

Discussion

To the best of my knowledge, this is the first study to investigate the detection rate of bacterial pathogens in clinical specimens collected from children using the Tm mapping method. This prospective study revealed that the Tm mapping method is associated with a higher pathogen

detection rate in pediatric patients than the classical culture method. Moreover, the higher detection rate was not restricted to blood samples. Comparing my findings to those of a previous study (6), the accuracy of the Tm mapping method using whole blood samples was slightly lower than that of the culture method (76.4% vs. 85.5%). However, two main reasons can justify this discrepancy. First, since the rate of positive results using the culture method was lower in this study than that of the previous study (6) (10.9% vs. 22.5%), it is assumed that there are differences in the patient background information, including differences in the collection timing and the proportion of patients administered antibiotics. Second, in this study, the primers and databases used for the Tm mapping method were those developed for commercial use. The databases were based on bacterial species obtained from adult blood specimens. My study was also based on clinical specimens other than blood specimens. My test of blood culture-positive bottles from a children's hospital showed a 99% concordance rate, whereas that for the bacterial species was 91%. Rarely positive organisms were not included in the database, so the detection sensitivity might be lower than that in previously published data (1). Therefore, it is necessary to verify whether the expansion of the database will enable the identification of rare species of bacteria.

I used specific criteria to classify the detected pathogen as a true, possible, contamination,

or indeterminate pathogen for culture-negative specimens that tested positive using the Tm mapping method. Of the 41 specimens detected using the Tm mapping method, only 13 contained true pathogens. Simultaneously, 16, 9, and 3 specimens were categorized as samples harboring possible, contamination, and indeterminate pathogens, respectively. Upon further investigation of the culture method results from the same infectious site, eight samples from seven patients were considered to contain true pathogens. Five specimens from five patients were verified using the culture method analysis of samples from other sites. Furthermore, I identified 16 specimens from 14 patients as harboring possible pathogens, of which the results associated with two specimens were confirmed by identifying a similar pathogen at the same infectious site using 16S rDNA sequencing. The results of 14 specimens were supported by case reports of infections due to *Corynebacterium xerosis* (12-14), *Cutibacterium acnes* (*Propionibacterium acnes*) (15-17), *Staphylococcus haemolyticus* (18-20), and *Staphylococcus warneri* (21-23) in patients with sepsis or bacteremia. Additionally, fastidious or non-cultivable organisms, including *Clostridium butyricum* (24, 25), *Finegoldia magna* (26, 27), and *Fusobacterium nucleatum* (28, 29), were detected in necrotizing enterocolitis, urinary tract infection, and sepsis, respectively. Moreover, I identified *Streptococcus pneumoniae* (30, 31), *Acinetobacter baumannii* (32, 33), *Streptococcus*

intermedius (34, 35), and *Staphylococcus aureus* (36-38) in a patient with septic arthritis, perforated appendicitis, subdural empyema, and lymphadenitis, respectively. All specimens with true and possible pathogens (except one blood specimen from a patient with septic arthritis) were collected after antibiotic treatment. However, this could explain the negative culture results in these specimens. The initiation of empirical antibiotic pretreatment among patients with sepsis significantly reduces the likelihood of obtaining positive blood culture results from blood drawn shortly after treatment initiation (39). Culture tests do not detect dead bacteria due to leukocyte phagocytosis or antibiotic administration. Conversely, in this test, since a buffy coat containing many white blood cells with phagocytosed bacteria was collected, dead bacteria can also be detected. Opota et al. (40) stated that one of the limitations that could be faced in detecting bacteria via PCR amplification of DNA from blood samples is the presence of DNA from dead microorganisms. The Tm mapping method also uses PCR-based amplification of bacterial DNA. It is a testing method that also detects dead bacteria. Therefore, it is crucial to avoid defining dead bacteria as infectious and subjecting patients to unnecessary antibiotic therapy. However, the continued use of broad-spectrum antimicrobials without de-escalation because of the inability to identify the pathogenic microorganisms after antimicrobial administration is concerning. Thus, I believe that

detecting bacterial DNA, a sign of infection and usually undetectable in a sterile area, whether from viable or dead bacteria, is a significant additional piece of information for me as clinicians. I reported a case in which the nucleic acid of *Streptococcus pneumoniae* was detected in the cyst of a patient with an active, infected simple renal cyst after antimicrobial therapy. Although the culture tested negative for the bacteria, antimicrobial de-escalation could be performed based on the results of the Tm mapping method (Figure 2, 3) (41). Additionally, I reported a case of a boy in whom *Streptococcus intermedius* was rapidly identified from the drainage of a brain abscess using the Tm mapping method, which supported empiric therapy (1). Furthermore, Sato et al. reported a case in which *Klebsiella pneumoniae* was rapidly identified in the blood of a child with liver abscess and bacteremia, and appropriate antimicrobial agents were promptly started (42). Therefore, the Tm mapping method can be beneficial for obtaining some clinical information regarding bacterial infections, particularly in identifying the nucleic acids of dead bacteria in patients who have received prior antimicrobial therapy. Moreover, rapid identification of pathogenic microorganisms hours after specimen collection would be useful in supporting empiric therapy in the clinical setting.

Among the organisms classified as contamination pathogens, *C. acnes* (*P. acnes*) was the

most common, followed by Staphylococcal bacteria and *Corynebacterium xerosis*. The organisms most frequently detected as contaminants in culture tests are coagulase-negative staphylococci, *Corynebacterium* spp., *Bacillus* spp. streptococci, and *Clostridiium perfringens* (43). Therefore, more than one blood culture sample should test positive for the same isolate to avoid being considered contaminated by commensal microorganisms and to distinguish contamination from true pathogens involved in skin and bloodstream infections. However, the PCR-based method exhibits a higher detection rate of *C. acnes* than the culture method (44). Thus, contamination will likely occur regularly since the Tm mapping method is based on PCR with high detection sensitivity. Clinical specimens testing positive for microorganisms can affect decisions on appropriate treatment, remarkably increasing patients' exposure to unnecessary antimicrobial agents. Various intervention methods have been proposed to prevent contamination, including patient selection, standardized collection techniques, and appropriate blood volume (45). A thorough sterilization during sample collection, DNA extraction, and mechanization of the Tm mapping method are required to prevent contamination with commensal bacteria from the environment.

However, I could not determine the association between the detected pathogen and the disease in the indeterminate cases. *C. acnes* can be a true pathogenic microorganism for

infections in bones and joints, while it also has a triggering aspect for autoinflammatory diseases. For example, although *C. acnes* has been detected in patients with chronic recurrent multifocal osteomyelitis, it is yet to be determined whether *C. acnes* is a pathogenetic organism (46, 47). Although *C. acnes* could produce acute infections (48), the infection was alleviated without antibiotics. The Tm mapping method also detected *Clostridium perfringens*, whereas *Kocuria spp.* was detected using the 16S rDNA. Therefore, a mixed infection without dominant species was identified as the likely cause.

The two culture-positive specimens that tested negative after using the Tm mapping method may be considered false negatives. This false-negative result could be explained using human serum DNases, which are known to degrade bacterial DNA. Heininger et al. (49) reported that PCR-based detection of *E. coli* in serum was reduced by 10% after antibiotic treatment. Therefore, residual bacterial DNA may be detected using the PCR method after antibiotic treatment, even at low levels of bacterial DNA. The bacterial DNA extracted from 2 mL of blood is ultimately concentrated into 50 μL, of which 2 μL is used for the Tm mapping method. Therefore, theoretically, if there is even one bacterial species in the 2 μL sample, it will be amplified and detected. Thus, I estimate that small bacterial DNA might not be inserted into the Tm mapping method but only into the culture method.

Using the Tm mapping method, dominant bacteria from clinical specimens were amplified because PCR amplifies nucleic acids. Moreover, the seven Tm values overlap when specimens contain a similar number of multiple bacteria, making it challenging to identify the causative organism. Therefore, the Tm mapping method is primarily suitable for identifying a single bacterium in a sterile sample. When identifying multiple infection-inducing bacteria, particularly from sputum and perianal abscesses, only the dominant bacteria can or cannot be identified based on the ratio of the bacterial mass, which was discovered to be the case for some of the indeterminate cases. Additionally, this method can only identify bacteria because PCR is performed using universal bacterial primers. Since Tm mapping can directly identify bacteria from clinical samples without a culture assay, it could be used, particularly in cases where rapid testing is required or the detection of dead bacteria following antibiotic treatment is desired. However, this method could be generalized by simplifying and mechanizing it to ensure that contamination does not occur during the testing process.

My study had some limitations. First, this study had confounding factors and biases, including the ages of pediatric patients from whom clinical specimens were collected and the small number of clinical specimens obtained. Second, most blood samples for the Tm

mapping method were collected after antibacterial drug administration. Therefore, the detection rate of bacteria using Tm mapping could be underestimated because it was compared with that of the culture method, which involved testing samples collected before antibiotic administration. Third, since no data are available on the amount of blood filled in the blood culture bottles, the possibility cannot be ruled out that the higher the amount of blood, the higher the positive rate of blood culture. Fourth, not all 16S rDNA phylogenetic analyses were performed on all specimens. Fifth, as of July 2019, 162 pathogenic bacterial strains from adult patients with sepsis had been registered in the sepsis-causing bacterium identification system database using the Tm mapping method, with each bacterial species including 2–3 mutant strains. In addition, this Tm mapping method does not provide information on antibiotic susceptibility and colony counts, which culture methods can confirm. Therefore, it is incomplete as a guide to treatment and is not a replacement for a conventional culture test. Sixth, in the conventional culture method, fungi may be detected in the culture medium; however, no fungi were detected in this method. Therefore, consideration should be given to how to intervene in treatment based on the clinical course and how to avoid unnecessary antimicrobial exposure due to detecting dead bacteria. Finally, this study was limited to patients at a single center. However, my hospital is located in the

center of the prefecture, and as a tertiary medical institution, healthy and immunocompromised patients with various diseases visit the hospital. Although the false-positive rate was not shown in the previous study conducted at a different hospital using adult clinical specimens, the false-negative rate was comparable (0.75% vs. 1%).

In conclusion, the Tm mapping method may be useful for diagnosing various bacterial infections in children. Although various testing tools have been developed, appropriate treatment strategies should be selected according to the patients' clinical information and test results. For example, antibiotics were changed to narrow-spectrum antibacterial drugs, or the treatment was supported after identifying the bacterial species in some cases. Therefore, more cases should be studied in detail using this method to identify the optimal treatment method for bacterial infections and the appropriate use of antibiotics.

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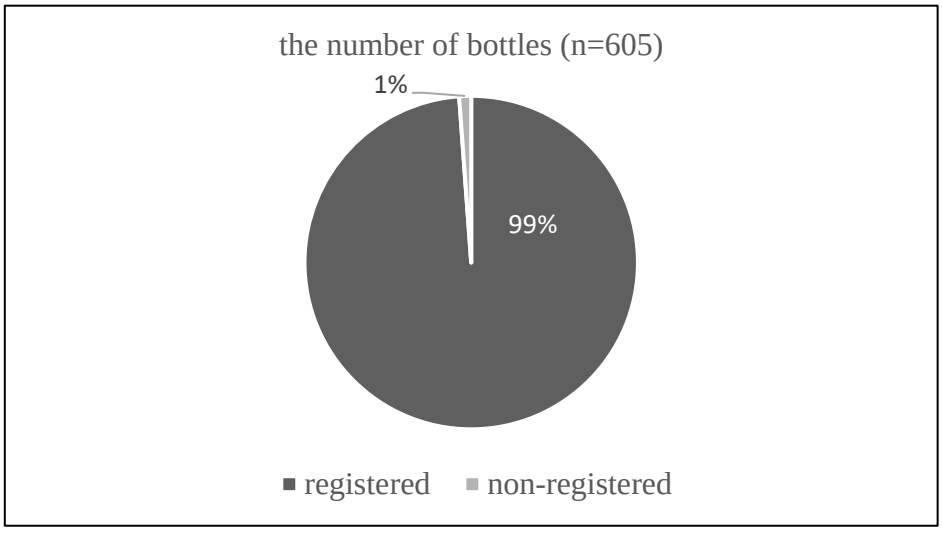
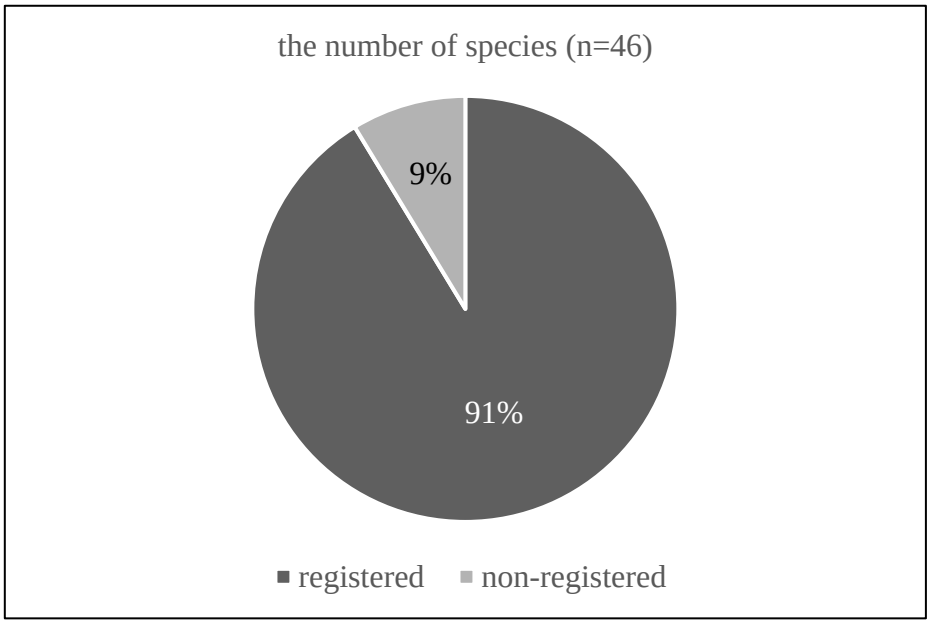


Figure 1: (A) Coverage compared to the database based on the number of bacterium-positive bottles



(B) Coverage compared to the database based on the number of bacterial species

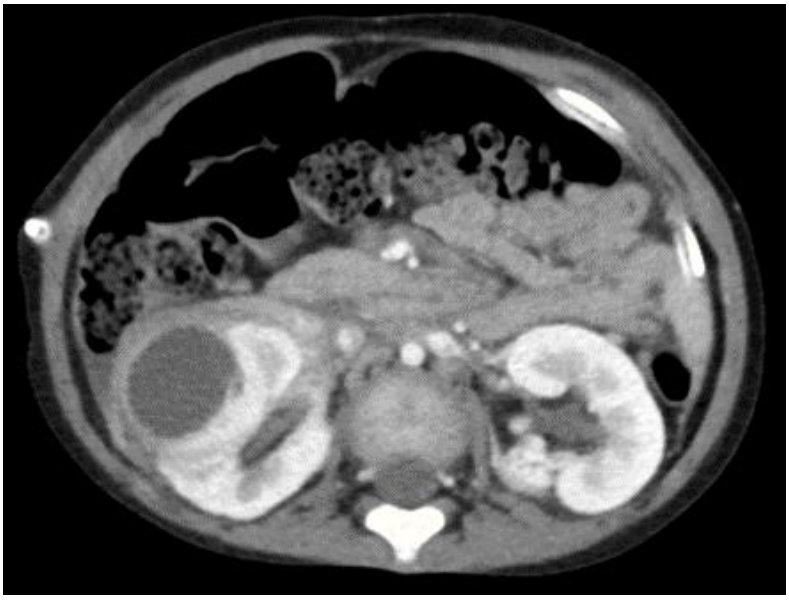
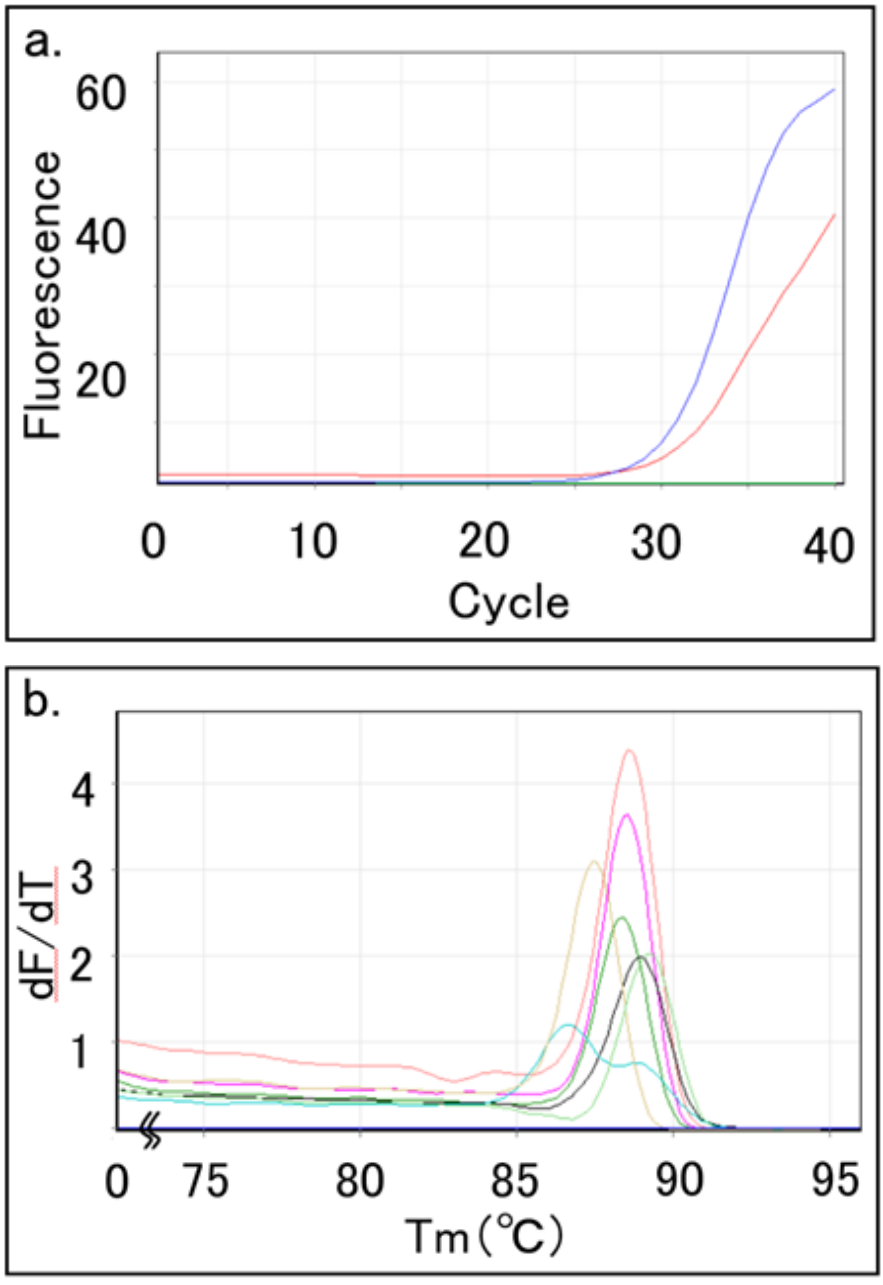


Figure 2　Enhanced computed tomography showing compression of the renal parenchyma, pelvis, and ureter due to the enlargement of the right renal cyst and enhancement and thickening of the wall around the cyst, with perirenal inflammatory changes


a.
Fluorescence
60
40
20
0
10
20
30
40
Cycle
b.
dF/dT
4
3
2
1
0
75
80
85
90
95
Tm(℃)

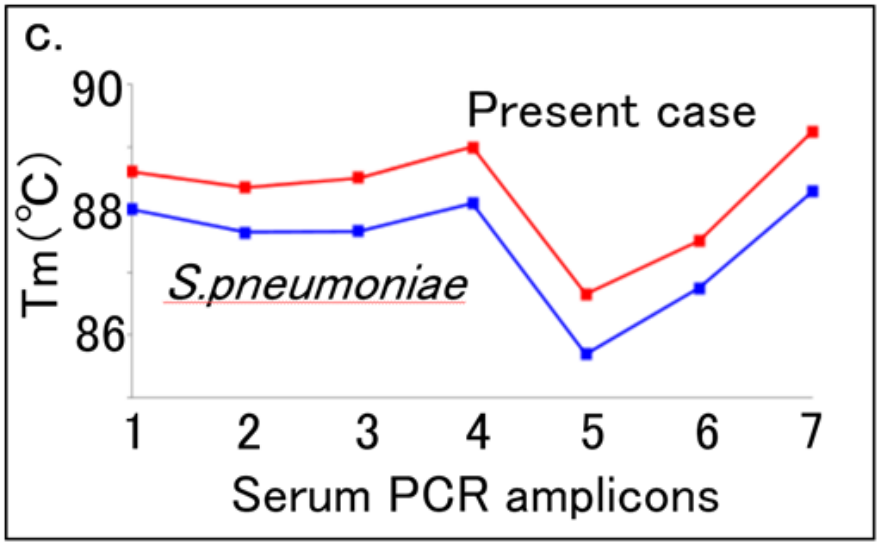


Figure 3 Identification of bacterial pathogens using the melting temperature mapping method. (a) Amplification of the 16S rDNA gene extracted from pus, positive control (Escherichia coli) and negative control (highly pure water) indicated by the red line, blue line and green line, respectively, in the 1st PCR. (b) Melting curves of the seven amplicons in the 2nd PCR. (c) The seven melting temperatures of the amplicons were mapped, and their plot matches the plot of Streptococcus pneumoniae in the database. From "Infected simple renal cyst due to Streptococcus pneumoniae rapidly diagnosed by the melting temperature mapping method: a case report," by Uejima Y, Niimi H, Kato R, Furuichi M, Sato S, Kitajima I, Kawano Y, Oh-Ishi T, Kawashima H, Suganuma E, 2021, BMC Pediatr, 21(1), Figure 1 and 2 (https://doi: 10.1186/s12887-021-02736-7). CC BY 4.0

Table 1: Relationship between difference value and matches at bacterial count above the minimum detection sensitivity using diluted bacterial samples

Difference value (D)	No. of samples	No. of matches	No. of broad matches	No. of mismatches
$0 < D \leq 0.26$	37	37	0	0
$0.26 < D \leq 0.53$	35	34	1[a]	0
$0.53 < D$	4	2	1	1

[a]The number of matches at the genus level.

Table 2: Clinical characteristics of patients in this study

	Patients (n = 156)
Age (Years) (median)	2 (0–17)
Male	94
Clinical specimens (N = 256)	
Blood	165
Specimens other than blood	91
Cerebrospinal fluid	41
Abscess	16
Synovial fluid	8
Urine	7
Ascites	7
Bone tissue	4
Pericardial effusion	4
Pleural effusion	3
Peritoneal dialysis fluid	1

Contaminated samples (eight blood, one urine) from six patients were excluded from the analysis.

Table 3 Comparison of the pathogenic organism detection capabilities of the Tm mapping and conventional culture methods. (A) Results from blood samples. (B) Results from clinical specimens other than blood.

(A)

Bacterial isolates	Tm mapping method			
	Detection	+	−	Total
Conventional culture method	+	16[a] (I = 16)	2[c]	18
	−	37[b] (I = 21, NS = 16)	110[d]	147
	Total	53 (I = 37, NS = 16)	112	165

$P < 0.01$, McNemar's test; Tm, melting temperature.

(B)

Bacterial isolates	Tm mapping method			
	Detection	+	−	Total
Conventional culture method	+	24[e] (I = 19, NS = 5)	0[g]	24
	−	19[f] (I = 11, NS = 8)	48[h]	67
	Total	43 (I = 30, NS = 13)	48	91

$P < 0.01$, McNemar's test; Tm, melting temperature.

[a,d,e,h] Tm mapping identifications matched the culture results.

[b,c,f,g] Tm mapping identifications did not match the culture results.

I: Identified using the Tm mapping method (difference value ≤ 0.53).

NS: Bacteria were detected rather than suitable for identification using the Tm mapping method (difference value > 0.53).

Table 4

Reference number	Specimens	Clinical diagnosis	Organisms detected using the Tm mapping method	Antibiotics administered before the collection of samples	Specimens that tested positive by another method (time)[a]
"true" pathogen					
(22)	Abscess	Sepsis, Infected simple renal cyst	*Streptococcus pneumoniae*	PIPC/TAZ	The same pathogen in blood by

					culture (d23－)
(49)	Urine	UTI	*Escherichia coli*	CTX	The same pathogen in urine by culture (d1－)
(65)	Blood	Sepsis	*Enterococcus faecalis*	ABPC, GM	The same pathogen in blood by culture (d13－)

(82)	Blood	Sepsis	*Streptococcus pyogenes*	ABPC, CTX	The same pathogen in blood by culture (d1－)
(84)	Blood	Sepsis	*Streptococcus pyogenes*	ABPC, ABK, CLDM	The same pathogen in blood by culture (d8－)
(102)	Ascites	Peritonitis	*Pseudomonas aeruginosa*	CFPM, VCM	The same pathogen in the drainage

					tube by culture
(120)	Blood	Sepsis, Early-onset GBS infection	*Streptococcus agalactiae*	PIPC (Maternal antibiotic exposure)	The same pathogen in skin and stool by culture
(137)	CSF	Meningitis	*Staphylococcus capitis* subsp. *ureolyticus*	VCM	The same pathogen in CSF by culture (d7－)

(138)	CSF	Meningitis	*Staphylococcus capitis* subsp. *ureolyticus*	VCM	The same pathogen in CSF by culture (d17 −)
(150)	Blood	Sepsis, Septic arthritis	*Staphylococcus aureus*	None	The same pathogen in the hip joint by culture (d1+)
(175)	Blood	Meningitis	*Streptococcus agalactiae*	ABPC, CTX	The same pathogen in

					CSF by culture (d3－)
(186)	Blood	Meningitis, Sepsis	*Streptococcus agalactiae*	ABPC, CTX	The same pathogen in blood by culture (d1－)
(204)	Blood	Sepsis	*Streptococcus agalactiae*	ABPC	The same pathogen in the skin, gastric juice, and the pharyngeal

					mucus by culture
"possible" pathogen					
(9)	Blood	Sepsis	*Staphylococcus warneri*	SBT/ABPC	None
(70)	Blood	Sepsis	*Cutibacterium acnes*	MEPM	None
(75)	Abscess	Pyriform sinus fistula-associated infections	*Bacillus cereus*	ABPC, CTX	None

(116)	Blood	Bacteremia, CVID	*Cutibacterium acnes*	CTRX	None
(118)	Blood	Bacteremia, CVID	*Cutibacterium acnes*	CTRX	None
(115)	Blood	Bacteremia, CVID	*Cutibacterium acnes*	CTRX	None
(134)	Blood	Sepsis, Necrotizing fasciitis	*Corynebacterium xerosis*	ABPC, CTX, TEIC	None
(135)	Blood	Necrotizing enterocolitis	*Clostridium butyricum*	VCM, MEPM	None

(162)	Blood	Sepsis	*Staphylococcus haemolyticus*	ABPC, AMK	None
(164)	Synovial fluid	Septic arthritis	*Streptococcus pneumoniae*	CEZ	None
(168)	Ascites	Perforated appendicitis	*Acinetobacter baumannii*	PAPM/BP	None
(177)	Urine	UTI	*Finegoldia magna*	ABPC, CTX	None
(197)	Blood	MAS, Sepsis	*Fusobacterium nucleatum*	ABPC, CTX	The same pathogen in blood by 16S

					rDNA sequence
(217)	Abscess	Subdural empyema	*Streptococcus intermedius*	CTRX, VCM	The same pathogen in abscess by 16S rDNA sequence
(230)	Blood	Bacteremia	*Cutibacterium acnes*	SBT/ABPC	None
(254)	Abscess	Lymphadenitis	*Staphylococcus aureus*	SBT/ABPC	None

"indeterminate" pathogen					
(103)	Blood	Cellulitis	*Clostridium perfringens*	CEZ	*Kocuria spp.* in blood by 16S rDNA
(256)	Blood	CRMO	*Cutibacterium acnes*	LVFX, CAM	None
(257)	Blood	CRMO	*Cutibacterium acnes*	None	None

AMK, amikacin; ABPC, ampicillin; ABK, arbekacin; CRBSI, catheter-related bloodstream infection; CEZ, cefazolin; CTX, cefotaxime; CTRX, ceftriaxone; CSF, cerebrospinal fluid; CRMO, chronic recurrent multifocal osteomyelitis; CVID, common variable

immunodeficiency; CAM, clarithromycin; CLDM, clindamycin; GEM, gentamicin; GBS, group B *Streptococcus*; LVFX, levofloxacin; MAS, Meconium aspiration syndrome; MEPM, meropenem; PAPM/BP, panipenem/betamipron; PIPC/TAZ, piperacillin/tazobactam; SBT/ABPC, sulbactam/ampicillin; TEIC, teicoplanin; UTI, urinary tract infection; VCM, vancomycin[a] Timepoint of pathogen detection. d, days; －, pathogen detection before sampling; +, pathogen detection after sampling.