# Utility of Multiplex PCR in Detecting the Causative Pathogens for Pediatric Febrile Neutropenia

Mitsuda, Yoshihiro

(Degree)
博士（医学）

(Date of Degree)
2011-09-25

(Date of Publication)
2011-10-17

(Resource Type)
doctoral thesis

(Report Number)
甲5324

(URL)
https://hdl.handle.net/20.500.14094/D1005324

Kobe J. Med. Sci., Vol. 57, No. 2, pp. E32-E37, 2011

# Utility of Multiplex PCR in Detecting the Causative Pathogens for Pediatric Febrile Neutropenia

YOSHIHIRO MITSUDA, YASUHIRO TAKESHIMA, TAKESHI MORI, TOMOKO YANAI, AKIRA HAYAKAWA, and MASAFUMI MATSUO

*Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan.*
*7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan*

Received 9 March 2011/ Accepted 15 March 2011

**Key Words:** febrile neutropenia, multiplex polymerase chain reaction, blood culture

## ABSTRACT

**Febrile neutropenia (FN) is a life-threatening complication, and the primary cause of FN is considered to be microbial infection. Therefore, prompt and appropriate antimicrobial therapy is crucial. Clinicians usually prescribe antimicrobial therapy on the basis of presumptive and empirical data. This is because the causative pathogen for FN in blood culture (BC) analysis is detected several days after sampling. Polymerase chain reaction (PCR) analysis has been used for detecting the causative bacteria of infections. Here, we examined whether multiplex PCR is useful for detecting the causative pathogens for FN patients.**

**We extracted DNA from the patients' whole blood and performed multiplex PCR. In total, 128 samples of 40 patients clinically diagnosed with FN were used in this study. Multiplex PCR analysis revealed the causative pathogen in 3 patients with FN; the DNA fragments amplified were those of *Pseudomonas aeruginosa* in 2 cases and *Psedomonas putida* in 1 case. These patients could be started on appropriate antimicrobial therapy a few hours after sampling. However, the DNA fragment of the causative pathogen could not be amplified by PCR in 2 patients, although BC analysis did detect the causative bacteria.**

**Thus, we conclude that multiplex PCR is serviceable in case of FN because of its rapidness. However, BC is also indispensable to treating FN owing to its high sensitivity.**

## INTRODUCTION

Febrile neutropenia (FN) is a life-threatening complication in patients undergoing chemotherapy for malignancy. The cause and focus of fever is unknown in about half the FN cases despite the presence of fever and/or inflammatory responses (e.g., rise in C-reactive protein level). Clinically, the cause of FN is considered to be bacterial infection (4); this is because the symptoms of FN are improved by antibiotic therapy in most cases. Therefore, prompt administration of causative bacteria-targeted antibiotics is crucial. Blood culture (BC) analysis is the current standard in the diagnosis of bacterial bloodstream infections; however, the results of BC become clear several days after sampling. Because of this reason, antimicrobial therapy has to be started on the basis of presumptive and empirical data.

Several studies have reported the usefulness of polymerase chain reaction (PCR) in detecting the causative bacteria of bloodstream infections (2,5,6,10-12). PCR analysis

Phone:+81-799-22-1200 Fax:+81-799-24-5704 E-mail:mitsuday@awaji-hosp.sumoto.hyogo.jp

enables detection of the causative agent within a few hours of sampling, facilitating administration of prompt and proper antimicrobial therapy. Owing to this rapid detection, PCR is more useful than BC in cases of FN with bloodstream infections. Moreover, the use of multiplex PCR, which can amplify multiple products simultaneously, would enable early and clear detection of the causative bacteria as compared to single PCR. Thus, the aim of this study was to examine the utility of multiplex PCR in treating FN.

## PATIENTS AND METHODS

### Patients

In total, 128 samples were obtained from 40 malignancy patients who were admitted to the pediatric ward of Kobe University Hospital and were diagnosed with FN during hospitalization from July 2005 to August 2009. FN was defined as a single axillary temperature of ≥ 37.5°C and a neutrophil count of <1000 cells/μL with a predicted decline to <500 cells/μL (8).

### Sample preparation

Patients who were admitted to the hospital underwent central venous catheter insertion before chemotherapy. Their blood samples were obtained from the catheter and were divided in 2 parts for performing multiplex PCR and BC. Genomic DNA was extracted from these samples automatically using a BioRobot EZ1 DNA Blood 350μL Kit (QIAGEN, Tokyo, Japan).

### Multiplex PCR analysis

Multiplex PCR was performed during 2 time periods: from July 2005 to June 2007 and from August 2007 to August 2009. For PCR analysis, the primers used in the first period were different from those used in the second period. In the first period (July 2005 to June 2007), we performed multiplex PCR using the primers that were specific to the 8 chosen pathogens, shown in table I (3). Because these primers included only a part of causative pathogens for FN (4), the set of primers was changed; we performed multiplex PCR using new primers in the second period (August 2007 to August 2009) (9). These new primers were targeted at the characteristic 16s or 23s rRNA gene sequences of bacteria (table II).

PCR was performed with an initial denaturing step at 96°C for 5 min, followed by 35 amplification cycles at 96°C, 60°C, and 72°C for 30 s each, and a final elongation step at 72°C for 2 min. The PCR products were visualized on 3% agarose gels with a positive control ladder. Multiplex PCR took about 3 hours to complete.

**Table I.** The 8 pathogens for multiplex PCR examination in the first period

| Gram-positive | Gram-negative | Viruses | Fungus |
|---|---|---|---|
| *Group B streptococcus* | *Esherichia coli* | *Herpes simplex virus* | *Candida albicans* |
| *Methicillin-resistant Staphylococcus aureus* | *Pseudomonas aeruginosa* | *Cytomegalovirus* | |
| | *Ureaplasma urealyticum* | | |

**Table II.** Genera of bacteria detected by the multiplex PCR based on 16S or 23S rRNA in the second period

| Gram-positive | Gram-negative |
|---|---|
| *Enterococcus 16S rRNA* | *Enterobacteriaceae 23S rRNA* |
| *Staphylococcus 16S rRNA* | *Pseudomonas 16S rRNA* |
| *Clostridium perfringens 16S rRNA* | |

**Comparison of PCR and BC results**

The blood samples obtained from the catheter were used to perform BC analysis.The results of multiplex PCR were compared with those of BC in order to evaluate the accuracy of multiplex PCR results.

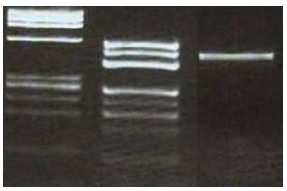
## RESULTS

**PCR-positive cases**

Of the 128 samples, 74 were analyzed in the first period and 54 in the second period.

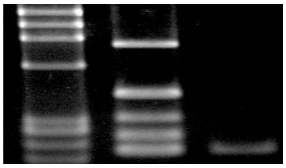
Multiplex PCR successfully detected the causative agents in 3 patients; 1 in the first period (case 1) and 2 in the second period (cases 2 and 3).

Case 1 was a 16-year-old boy hospitalized for the treatment of rhabdomyosarcoma. He had undergone chemotherapy and extirpation of the focus (right elbow). He had high-grade fever during the high-dose chemotherapy administered before auto-peripheral blood stem cell transplantation. On that day, we performed BC and multiplex PCR. In the multiplex PCR analysis, the DNA fragment corresponding to *Pseudomonas aeruginosa* was amplified; this result was obtained on the same day (figure 1). On the other hand, BC results were obtained 2 days later. On obtaining these results, we could start the patient on antibiotics for *P. aeruginosa*; his symptoms improved in 2 days. Case 2 was a 3-year-old boy hospitalized for the treatment of ALL. He had high-grade fever during chemotherapy. Multiplex PCR results were obtained on the same day (figure 2), and appropriate antibiotic therapy was administered. The BC results ascertained the causative pathogen to be *P. aeruginosa* the next day. Case 3 was a 2-year-old girl who was also hospitalized for the treatment of ALL. She too had high-grade fever during chemotherapy. Here again, multiplex PCR results were obtained much before those of BC; the causative agent was ascertained to be *P. putida*. In all these 3 cases, multiplex PCR analysis revealed the causative pathogen rapidly, and proper antimicrobial treatment was initiated within a few hours after the onset of FN.



Mk PC Pt

**Figure1.** The result of electrophoretic analysis by multiplex PCR of case1. (Mk) size marker, X174 *Hae III digest.* (PC) positive control ladders. Each ladder indicates *E.coli, P. aeruginosa, U. urealyticum,Group B streptococcus, Methicillin-resistant S. aureus, Herpes simplex virus,Cytomegalovirus and C. albicans from top to bottom.* (Pt) sample of the patient



Mk PC Pt

**Figure2.** The result of electrophoretic analysis by multiplex PCR of case2. (Mk) size marker, X174 *Hae III digest.*(PC) positive control ladders. Each ladder indicates *Clostridium perfringens 16S rRNA,Enterobacteriaceae 23S rRNA*, *Enterococcus 16S rRNA*, *Staphylococcus 16S rRNA* and *Pseudomonas 16S rRNA from top to bottom.* (Pt) sample of the patient.

**Diagnostic utility of multiplex PCR and BC**

The results of PCR and BC in the first and second period are shown in tables III A and III B, respectively. In 3 cases mentioned above ; 1 in the first period (tableIII A) and 2 in the second period (tableIII B) , the BC results were positive and the causative pathogens could be detected by multiplex PCR. The PCR results were ascertained within about 3 hours, and we started antibiotic therapy not on the basis of BC results but PCR results.

In 20 cases ; 17 in the first period and 3 in the second period, however, the causative pathogens were revealed by BC, but not multiplex PCR analysis. Among these, 18 BC isolates were not included in our PCR primer lists; this could be the reason why multiplex PCR failed to amplify the DNA fragments of these pathogens. Unfortunately, the remaining 2 BC isolates (*Escherichia coli* and *Staphylococcus epidermidis* ; tables III A and III B, respectively) were not detected by multiplex PCR analysis even though the appropriate primer sets were included, as shown in tables I and II. The results of isolates by blood culture are shown in tableIV. In total, 9 and 4 types of bacteria were isolated in the first and second period, respectively .

**Table III.** The results of PCR and blood culture.

A

| | | Multiplex PCR | | |
|---|---|---|---|---|
| | | positive | negative | total |
| BC | positive | 1 | 17(1)* | 18 |
| | negative | 0 | 56 | 56 |
| | total | 1 | 73 | 74 |

B

| | | Multiplex PCR | | |
|---|---|---|---|---|
| | | positive | negative | total |
| BC | positive | 2 | 3(1)** | 5 |
| | negative | 0 | 49 | 49 |
| | total | 2 | 52 | 54 |

TableIII A shows the results of PCR and blood culture in the first period and III B shows the results of PCR and blood culture in the second period. A number in parenthesis means the number of sample in which DNA fragment was not amplified by PCR in which DNA fragment was not amplified by PCR though its appropriate primer set was included.**Escherichia coli* was isolated by BC in the index case. ***Staphylococcus epidermidis* was isolated by BC in the index case.

**Table IV.** The results of isolates by blood culture

| First period | | Second period | |
|---|---|---|---|
| Bacteria | Number of cases | Bacteria | Number of cases |
| *Pseudomonas aeruginosa** | 1 | *Pseudomonas aeruginosa** | 2 |
| *Esherichia coli** | 1 | *Pseudomonas putida** | 1 |
| *Bacillus sp.* | 7 | *Staphylococcus epidermidis** | 1 |
| *Staphylococcus epidermidis* | 3 | *Bacillus sp.* | 2 |
| *Streptococcus haemolyticus* | 2 | | |
| *Serratia sp.* | 1 | | |
| *Enterococcus faecium* | 1 | | |
| *Staphylococcus aureus* | 1 | | |
| *Enterobacter cloacae* | 1 | | |

Asterisks mean bacteria included in our PCR primer lists.

## DISCUSSION

The purpose of this study was to examine of the utility of multiplex PCR in detecting the causative pathogen in FN patients, and to compare those results with BC results.

In 3 FN cases, multiplex PCR analysis successfully revealed the causative pathogen. In these cases, the PCR results were obtained much earlier than the BC results, facilitating prompt and proper administration of antimicrobial therapy. Therefore, multiplex PCR was clinically serviceable. However, unfortunately, multiplex PCR could not detect the causative pathogens in 2 FN cases for which BC analysis could successfully reveal the causative bacteria.

There was 1 limitation to our study. The primers that we used in the second period targeted the genera of bacteria. That is to say, the species of bacteria could not be identified by the primers that we used in the second period. Nevertheless, this study is significant because we could start antimicrobial therapy for some patients on the basis of reliable evidence, and not presumptive and empirical data.

Previous studies have reported FN cases in which the DNA fragments of the causative pathogens could not be amplified by PCR, but their isolates gave positive BC results (7, 11, 12). In this study, there were 2 such cases (2 of 5, 40.0%) as compared with those of previous reports (9 of 43, 20.9% (7); 18 of 119, 15.1% (11); and 18 of 74, 24.3% (12)). These results indicate that PCR is not more sensitive than BC, and BC is still indispensable.

Louie et al. reported that variability of the DNA target site can affect binding of the primers and probes (7). In their study, *Enterococcus faecalis* was not detected by PCR in 5 BC-confirmed cases of *E. faecalis* infection. They postulated that geographic and/or community-distinct strains of organisms might contribute to the lack of detection. They also suggested that excess total DNA in the sample can saturate the enzyme to interfere with PCR amplification. Westh et al. and Burkardt also mentioned that PCR-negative and BC-positive cases were due to either inhibition of the PCR reaction or inappropriate sample preparation (1, 12). Lamoth et al. thought that the smaller blood volumes required for PCR as compared to that required for BC might have contributed to the failure to detect pathogens (5).

The other reason might be the quantity of bacteria present in the isolate (12). In the 2 out of 3 cases wherein multiplex PCR successfully revealed the causative pathogens (cases 1 and 2), it is speculated that the quantity of bacteria present was large. In case 1, the serum endotoxin level was 51,320 pg/mL on the day when we performed PCR; this indicates that a large amount of bacteria already existed in the sample. In case 2, the BC results were ascertained the next day, although BC results are usually obtained after a few days; this again indicates the presence of bacteria in large amounts.

The primers used in the first period were different from those used in the second period. We changed the set of primers because that in the first period targeted only a part of causative pathogens for FN and multiplex PCR analysis could revealed the causative pathogen in only 1 among 74 samples in the first period. However, unexpectedly, the sensitivity of multiplex PCR was not improved in the second period. In this study, only *Pseudomonas* was detected by multiplex PCR throughout the periods. Therefore, we could not conclude which set of primers was better.

Although the cause of FN is considered to be bacterial infections, the number of BC-positive cases was only 23 of 128 samples (18.0%) in our study. Previous studies using PCR for the identification of infections reported that the number of BC-positive cases was 43 of 200 (21.5%) (7), 46 of 119 (38.7%) (11), and 74 of 613 samples (12.1%) (12). The reason why pathogens could not be detected by BC analysis in some FN patients was not clearly

understood; the symptoms of FN might be triggered by small amounts of bacteria, making it difficult to detect the causative pathogen by BC analysis.

In conclusion, we examined the utility of multiplex PCR for detecting pathogens causing FN. Multiplex PCR revealed the causative pathogen for FN more rapidly than did BC analysis, whereas BC was more sensitive. BC is still indispensable for treating FN owing to its high sensitivity, and multiplex PCR analysis combined with BC provides clinically relevant information for appropriate antibiotics treatment of FN patients.

## REFERENCES


1. **Burkardt H.J.** 2000. Standardization and quality control of PCR analyses. Clin Chem Lab Med **38:** 87-91.
2. **Cheng J.C., Huang C.L., and Lin C.C.** 2006. Rapid detection and identification of clinically important bacteria by high-resolution melting analysis after broad-range ribosomal RNA real-time PCR. Clin Chem **52:** 1997-2004.
3. **Enomoto M., Morioka I., Morisawa T., Yokoyama N., and Matsuo M.** 2009. A novel diagnostic tool for detecting neonatal infections using multiplex polymerase chain reaction. Neonatology **96:** 102-108.
4. **Kanamaru A. and Tatsumi Y.** 2004. Microbiological data for patients with febrile neutropenia. Clinical Infectious Diseases **39:** S7-10.
5. **Lamoth F., Jaton K., Prod'hom G., Senn L., Bille J., Calandra T., and Marchetti O.** 2010. Multiplex blood PCR in combination with blood cultures for improvement of microbiological documentation of infection in febrile neutropenia. J Clin Microbiol **48:** 3510-3516.
6. **Lehmann L.E., Hunfeld K., Emrich T., Haberhausen G., Wissing H., Hoeft A., and Stüber F.** 2008. A multiplex real-time PCR assay for rapid detection and detection and differentiation of 25 bacterial and fungal pathogens from whole blood samples. Med Microbiol Immunol **197:** 313-324.
7. **Louie R. F., Tang Z., Albertson T.E., Cohen S., Tran N.K., and Kost G.J.** 2008. Multiplex polymerase chain reaction detection enhancement of bacteremia and fungemia. Crit Care Med **36:** 1487-1492.
8. **Masaoka T.** 2004. Evidence-based recommendations for antimicrobial use in febrile neutropenia in Japan: Executive summary. Clinical Infectious Diseases **39:** S49-52.
9. **Matsuda K., Tsuji H., Asahara T., Kado Y., and Nomoto K.** 2007. Sensitive quantitative detection of commensal bacteria by rRNA-targeted reverse transcription-PCR. Applied and Environmental Microbiology **73:** 32-39.
10. **Skow A., Mangold K.A., and Tajuddin M.** 2005. Species-level identification of Staphylococcal isolates by real-time PCR and melt curve analysis. J Clin Microbiol **43:** 2876-2880.
11. **von Lilienfeld-Toal M., Lehmann L.E., Raadts A.D., Hahn-Ast C., Orlopp K.S., Marklein G., Purr I., Cook G., Hoeft A., Glasmacher A., and Stuber F.** 2009. Utility of a commercially available multiplex real-time PCR assay to detect bacterial and fungal pathogens in febrile neutropenia. J Clin Microbiol **47:** 2405-2410.
12. **Westh H., Lisby G., Breysse F., Boddinghaus B., Chomarat M., Gant V., Goglio A., Raglio A., Schuster H., Stuber F., Wissing H., and Hoeft A.** 2009. Multiplex real-time PCR and blood culture for identification of bloodstream pathogens in patients with suspected sepsis. Clin Microbiol Infect **14:** 544-551.