Global Pediatric Health
January-December 2015: 1–5

DOI: 10.1177/2333794X15569302
gph.sagepub.com
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# Rapid and Sensitive Identification of Bacterial Infection and Bacteria Gram Types in Pleural Fluid of Children

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**Abstract**

Real-time polymerase chain reaction (RT-PCR) techniques have been increasingly used to detect microbial DNA in clinic for the diagnosis of bacterial infection. This study aims to developing an RT-PCR method to detect bacteria in pleural fluid (PF). We performed a method to simultaneously detect and classify the clinically relevant bacterial pathogens in hydrothorax with Gram probe RT-PCR (GRT-PCR), which targets the conserved region of the 16S rRNA gene. Our results showed this method could specifically and correctly identify 14 clinically important bacterial strains in hydrothorax including 7 gram-positive and 7 gram-negative bacteria. And the sensitivity of this GRT-PCR method in serial dilution can reach 10 CFU/mL. In clinical trial, 180 PF samples from children who were clinically suspected to suffer from bacterial pneumonia and empyema were collected. These samples were detected by GRT-PCR, standard culture, and biochemical routine analysis. The positive rate of the GRT-PCR array was 17.78% (32/180), significantly higher than that of PF culture (11.67%; 21/180; $P = .003$). When PF culture was used as control, the sensitivity of GRT-PCR was 95.24% (95% confidence interval = 74.13-99.75), and the specificity was 92.45% (95% confidence interval = 86.89-95.86). Our study showed that GRT-PCR is a more effective method for rapid, sensitive, and specific diagnosis of bacterial infection in hydrothorax compared with other traditional methods.

**Keywords**

GRT-PCR, bacterial infection, pleural fluid, children

## Introduction

The incidence of pleural infection is rising worldwide. Up to now, about half of the 4 million patients who suffer from pneumonia each year will develop a parapneumonic effusion, which can be secondarily infected by bacteria.[1,2] Identifying the causative organism(s) is important to guide antimicrobial therapy.[3,4] Inappropriate use of antibiotics and delay in their initiation, however, remains common and contributes to morbidity.[5-7] Especially among pediatric patients bacterial pleural infection progresses even faster.[2] A fast and correct diagnosis, followed by rapid treatment, plays an important role in the reduction of mortality in children resulting from bacterial pleural infection.[8-11]

Currently, the gold standard is bacterial culture for diagnosis of the presence of bacterial pathogens in clinical samples.[7] However, samples must be incubated for 5 days or more until they show a positive signal in the continuously monitored culture systems for detection of bacteria. Moreover, the culture may lead to false-negative results when fastidious or slowly growing bacteria are involved or when samples are obtained after antimicrobial therapy has been started.[10,12] Pleural fluid (PF) biochemical routine analysis is needed as an important auxiliary examination method, but the routine analysis lacks specificity to identify concrete etiology.[7,8] So a rapider, more sensitive, and specific method is required.

Polymerase chain reaction (PCR)–based assays have showed the potential to provide an early and accurate diagnostic method for bacterial diseases and have improved the rate of microbial detection.[12,13] In this study, we describe a gram probe real-time PCR (GRT-PCR) system involving the 16S rRNA gene that allows simultaneous detection and discrimination of clinically relevant gram-positive and gram-negative bacteria

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**Table 1.** Representative Bacterial Species Detected by GRT-PCR.

| Genus | Species | GRT-PCR ($C_T$[a]) | | GenBank Accession Number |
|---|---|---|---|---|
| | | $G^+$ Probe, $C_T$ ± SD | $G^-$ Probe, $C_T$ ± SD | |
| Gram-positive bacteria | | | | |
| *Enterococcus* | *E faecalis* | 19.26 ± 1.28 | — | AJ276460 |
| | *E faecium* | 18.37 ± 0.08 | — | AJ874342 |
| *Staphylococcus* | *S aureus* | 20.97 ± 0.45 | — | X68417 |
| | *S epidermidis* | 21.06 ± 0.19 | — | L37605 |
| *Streptococcus* | *S intermadius* | 24.24 ± 0.28 | — | AB023574 |
| | *S pneumoniae* | 21.87 ± 0.45 | — | AF003930 |
| | *S pyogenes* | 17.53 ± 0.54 | — | AB023575 |
| Gram-negative bacteria | | | | |
| *Acinetobacter* | *A baumannii* | — | 22.47 ± 0.66 | DSM30008 |
| *Citrobacter* | *C freundii* | — | 20.45 ± 0.56 | AF025368 |
| | *C koseri* | — | 20.78 ± 0.45 | AM184281 |
| *Escherichia* | *E coli* | — | 17.35 ± 0.42 | AF233451 |
| *Haemophilus* | *H influenzae* | — | 24.38 ± 0.38 | AF224306 |
| *Klebsiella* | *K pneumoniae* | — | 18.05 ± 0.38 | AF130981 |
| Pseudomonas | *P aeruginosa* | — | 22.75 ± 0.41 | AF094720 |

Abbreviation: GRT-PCR, gram probe real-time polymerase chain reaction.
[a]Mean ± standard deviation of 3 independent experiments.

directly from PF samples. A total of 180 PF specimens from children with suspected bacterial pneumonia were evaluated. The system may provide a rapider and more accurate diagnostic method for bacterial pleural infection in sick children and guide early antibiotic treatment.

## Materials and Methods

### Bacterial Strains and Patients

The bacterial strains used in this study and their sources are listed in Table 1. Total human genome, human cytomegalovirus (HCMV) DNA, hepatitis B virus (HBV) DNA, and Epstein–Barr virus (EBV) DNA are used as negative controls. From January 2010 to December 2012, a total of 180 PF samples from different patients who were clinically suspected to have bacterial pneumonia or empyema were evaluated from the microbiology laboratories of Children's Hospital, Zhejiang University, China. The age of the 180 patients (82 females, 98 males) ranged from 7 day to 14 years. All these samples were detected by both PF culture method and the GRT-PCR technique. The study was approved by the Medical Ethics Committee of the Medical College, and informed consent was obtained.

### GRT-PCR

The designed primers and probes were based on regions of identity within the 16S rRNA gene following the sequences of the group's clinical bacterial pathogens, which referenced to our recent research.[14] The sequences of primers were as follows: forward primer: 5′-GCAACGCGAAGAACCTTACC-3′; reverse primer: 5′-CGCTCGTTGCGGGACTTA-3′; Sequences of gram-positive (G+) probe and gram-negative (G−) probe were as follows: G+ probe: FAM-TGACGACAACCATGCACCACCT-BHQ1; G− probe: HEX-ACGACAGCCATGCAGCACCT-BHQ1. The primers and probes were synthesized by Takara (Otsu, Shiga, Japan). The reaction mixtures comprised 400 nM (each) forward and reverse primers, 100 nM (each) gram-positive and gram-negative respective fluorescence-labeled specific probes, 1 U of Taq DNA polymerase (Takara), and 5 μL of template DNA, and water was added to give a final volume of 50 μL for each sample. GRT-PCR was performed on an ABI 7500 real-time PCR system. The cycling parameters of PCR were initial preincubation at 94°C for 2 minutes and then 40 cycles consisting of 94°C for 15 seconds and 60°C for 45 seconds. A positive control with *Escherichia coli* and *Staphylococcus aureus* and a negative control with water were used in every PCR reaction as previously described.[15]

### Bacterial Culture

PF specimens were first inoculated into 20 mL of blood enrichment medium. The culture bottle was incubated at 35.5°C. Subcultures were performed after 3 days of incubation. Samples were removed from each culture

bottle aseptically to a blood agar plate and a chocolate agar plate. The positive growth was recorded at 24 hours, and biochemical tests were carried out to identify the bacteria by use of the VITEK-60 microorganism auto analysis system. The culture bottle and VITEK-60 microorganism auto analysis system were from bio-Mérieux (France).

### *Biochemical Routine Analysis*

Samples were collected into sterile polystyrene tubes and immediately submitted for analysis. Hydrothorax glucose and lactate dehydrogenase were determined using the Roche kit with Hitachi equipment. The total hydrothorax protein was determined by the Hitachi equipment using the benzethonium chloride precipitation technique standardized to the biuret method. Cell counts were determined using a calibrated Fuchs-Rosenthal chamber after samples were stained with toluidine blue. Hydrothorax analysis to distinguish polymorphonuclear leukocytes and lymphocytes was carried out using a Shandon Cytocentrifuge and Pappenheim stain.

### *Data Analysis*

The results were analyzed using the SPSS software (version 11.5). Quantitative data were presented as mean ± SD. McNemar's test with continuity correction was performed to analyze the association between the results of GRT-PCR and standard culture. Two-tailed *P* values less than .05 were considered statistically significant.

## Results

### *Specificity and Sensitivity of GRT-PCR*

The feasibility of GRT-PCR in detecting bacteria DNA was determined for 14 clinically important strains representing 7 gram-positive and 7 gram-negative bacterial species. As shown in Table 1, all gram-positive bacteria DNA strains showed high fluorescence signals, with a range of $C_T$ values from 17.53 to 24.24, and the gram-negative bacteria showed no fluorescence with the G+ probe. While DNA strains from all gram-negative bacteria showed positive signals through G− probe testing, with $C_T$ values between 17.35 and 24.38, and the gram-positive bacteria showed no fluorescence signals. DNAs from the human genome, CMV, HBV, and EBV were also detected with GRT-PCR. Their results were negative (data not shown). In order to evaluate detection range of the real-time PCR established in this study, series dilutions of *S aureus* and *E coli* generated previously were used for sensitivity analysis, each in triplicate. Our results showed that the detection limit reached 10 CFU/mL ($C_T$ *S aureus* = 36.78; $C_T$ *E coli* = 35.06).

**Table 2.** Overall Results Obtained by the GRT-PCR Compared With Bacterial Culture[a].

| | Bacterial Culture | | |
|---|---|---|---|
| Methods | Positive | Negative | Total |
| GRT-PCR | | | |
| Positive | 20 | 12 | 32 |
| Negative | 1 | 147 | 148 |
| Total | 21 | 159 | 180 |

Abbreviation: GRT-PCR, gram probe real-time polymerase chain reaction.
[a]McNemar's test: $P = .003$.

**Table 3.** Comparison of Bacterial Culture, GRT-PCR Results, and PF Routine Analysis, From 33 Patients.

| | | GRT-PCR | | |
|---|---|---|---|---|
| Culture Identification | No. of Samples | Gram+/− | Ranged $C_T$ Value | PF Routine Analysis |
| *S pneumoniae* | 10 | + | 18.64-31.32 | Abnormal |
| *S aureus* | 3 | + | 24.17-31.78 | Abnormal |
| *K pneumoniae* | 2 | − | 19.53-27.98 | Abnormal |
| *S pyogenes* | 1 | + | 24.65 | Abnormal |
| *S intermedius* | 1 | + | 29.51 | Abnormal |
| *E faecium* | 1 | + | 30.34 | Abnormal |
| *E coli* | 1 | − | 23.64 | Abnormal |
| *H influenzae* | 1 | − | 29.86 | Abnormal |
| *S epidermidis* | 1 | / | Negative | Abnormal |
| / | 8 | + | 20.02-34.75 | Abnormal |
| / | 4 | − | 22.38-32.45 | Abnormal |

Abbreviations: GRT-PCR, gram probe real-time polymerase chain reaction; PF, pleural fluid.

### *Results of GRT-PCR and Bacterial Culture in Clinical PF Samples*

A total of 180 PF samples were analyzed by both bacterial culture and GRT-PCR. There were 32 positive results (32/180, 17.78%) with the GRT-PCR and 21 positive results (21/180, 11.67%) with bacterial culture. The positive rate of the GRT-PCR was significantly higher than that of bacterial culture ($P = .003$). When PF culture was used as a control, the sensitivity of GRT-PCR was 95.24% (95% confidence interval (CI) = 74.13-99.75), and the specificity was 92.45% (95% CI = 86.89-95.86; Table 2).

In comparison with the bacterial culture, 20 of the 32 GRT-PCR-positive samples were identified successfully in PF culture. As shown in Table 3, *S pneumoniae* were identified most commonly in PF culture (n = 10). PF routine analysis was also performed on all the 180 clinical samples. The data showed that 20 PF culture-positive/

**Table 4.** Evaluation of the Effect of Antibiotic Before Thoracentesis on 33 Bacterial Culture or GRT-PCR Positive Results.

| | Antibiotic Before Thoracentesis | | |
|---|---|---|---|
| Results | Yes (n = 19) | No (n = 14) | *P* |
| Positive GRT-PCR (%) | 18 (94.7) | 14 (100) | 1.000 |
| Positive culture (%) | 9 (47.4) | 12 (85.7) | .033 |
| *P* values | .003 | .481 | |

Abbreviation: GRT-PCR, gram probe real-time polymerase chain reaction.

GRT-PCR positive samples were all abnormal in PF routine analysis. For 12 PF culture-negative/GRT-PCR-positive samples, PF routine analysis results were abnormal with increase in white blood cells and decrease in glucose, which indicated that the GRT-PCR result is accurate in diagnosis of bacterial pleural effusion.

The use of antibiotics before thoracentesis was evaluated on 33 bacterial culture or GRT-PCR positive results. They did not interfere with detection by GRT-PCR ($P = .003$). On the other hand, there was a statistical significance between treated and untreated groups by standard culture ($P = .033$; Table 4).

## Discussion

Pathogen examination plays an extremely critical role in the diagnosis and antibiotics treatment of bacterial pleural infection.[1-4] The etiologic diagnosis of pneumonia with pleural effusion in infants and children has been widely studied. The early detection and rapid differentiation of bacteria and rapid treatment play an important role in the reduction of children mortality.[1,2]

Up to now, hydrothorax routine analysis and standard culture are usually used in bacterial infection in PF. However, such methods cannot get a rapid, sensitive, and specific result. Because hydrothorax culture is influenced by various factors and can reduce the chance of a positive result, it requires at least 2 to 3 days for incubation and subsequent biochemical tests to identify the bacteria. Hydrothorax routine analysis usually includes white blood cell counts, total protein concentration, and glucose concentration, which vary over a wide range and could not reveal the kinds of pathogens.[7] And there may be no abnormal microbiological and chemical findings in the hydrothorax of some patients whose clinical manifestation are obvious. In these situations, antibiotics have to be prescribed empirically, which may contribute to morbidity.[8-10]

In our previous reports, we used real-time PCR, which targets the 16S rRNA gene, to detect bacterial infection in blood and cerebrospinal fluid.[12-15] In this study, we are the first to describe the GRT-PCR system involving the 16S rRNA gene that allows simultaneous detection and discrimination of clinically relevant gram-positive and gram-negative bacteria directly from PF samples. Our GRT-PCR system was specific for the bacteria tested. It allowed simultaneous detection and discrimination of gram-positive and gram-negative bacteria by means of fluorescence hybridization probes in one PCR tube. No fluorescence was detected, and no cross-reaction was found using DNA extracted from the human genome, CMV, HBV, or EBV. To determine the detection limits of GRT-PCR, *S aureus* and *E coli* were used to establish the standard curve. The standard curve showed that the amplification efficiency of templates with different concentrations of DNA was almost the same and the maximum sensitivity could reach 10 CFU/mL at least.

In these 180 patients, a total of 33 patients demonstrated positive for bacteria in either PF standard cultures or GRT-PCR. Among these 33 patients tested with positive results, 20 patients were detected to be positive by both PF cultures and GRT-PCR. Twelve patients were detected to be positive by GRT-PCR and negative by PF cultures. For the 12 PCR-positive/culture-negative samples, the routine examination of PF also revealed abnormality, and the 12 amplifications were considered as true bacterial positive results based on sequencing and their PF routine analysis. The possible reasons for low sensitivity of the PF culture method may be because of the antibiotics therapy that had been taken by these patients prior to lumbar puncture, or low bacteria DNA copies in these patients. All these results suggested that the GRT-PCR method has a higher sensitivity.

In conclusion, the GRT-PCR technique proved to be a rapid, highly sensitive, and specific molecular assay in clinical practice. We hypothesize that GRT-PCR will be proved as the most effective method to detect bacteria in PF and compensates for the lack of sensitivity of culture, particularly for children receiving antibiotic treatment. It not only can differentiate bacterial from viral or other pathogens but also can classify gram staining with a much shorter turnaround time than the gold standard culture method. It has a promising perspective in clinical practice for rapid and specific diagnosis and treatment.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by Key Projects in the

National Science & Technology Pillar Program (2012BAI04B05) and National Natural Science Foundation of China (81370774).

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