

ORIGINAL RESEARCH

Broad-Range Bacterial Polymerase Chain Reaction in the Microbiologic Diagnosis of Complicated Pneumonia

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BACKGROUND: A bacterial cause is not frequently identified in children with pneumonia complicated by parapneumonic effusion (ie, complicated pneumonia).

OBJECTIVES: To determine the frequency of positive blood and pleural fluid cultures in children with complicated pneumonia and to determine whether broad-range 16S rRNA polymerase chain reaction (PCR) improves identification of a microbiologic cause.

METHODS: This prospective cohort study included children 1–18 years of age hospitalized with complicated pneumonia.

RESULTS: Pleural fluid drainage was performed in 64 (51.6%) of 124 children with complicated pneumonia. A microbiologic cause was identified in 11 of 64 patients (17.2%; 95% confidence interval [CI]: 8.9%–28.7%). Bacteria were isolated from pleural fluid culture in 6 of 64 patients (9.4 %; 95% CI: 3.5%–19.3%) undergoing pleural drainage; the causative bacteria were *Staphylococcus*

aureus (n = 5) and *Streptococcus pneumoniae* (n = 1). Blood culture identified a bacterial cause in 3 of 44 cases (6.8%; 95% CI: 1.4%–18.7%) undergoing pleural fluid drainage; *S. pneumoniae* (n = 1), *Haemophilus influenzae* (n = 1), and *S. aureus* (n = 1) were isolated. Only 3 of the 19 pleural fluid samples (15.8%; 95% CI: 3.4%–39.6%) analyzed with 16S rRNA PCR were positive. *S. pneumoniae* was the only organism detected in all three samples; two of these three had negative pleural fluid cultures and absence of bacteria on Gram stain. *S. aureus* was isolated from pleural fluid culture in one patient with a negative 16S rRNA PCR test.

CONCLUSIONS: Causative bacteria were infrequently identified in children with complicated pneumonia. Broad-range 16S rRNA PCR only modestly improved the microbiologic yield over conventional culture methods. *Journal of Hospital Medicine* 2012;7:8–13. © 2011 Society of Hospital Medicine

Community-acquired pneumonia (CAP), the most common serious bacterial infection in childhood, may be complicated by parapneumonic effusion (ie, complicated pneumonia).^{1,2} The clinical outcome of children with complicated pneumonia is directly influenced by the causative organism, and precise identification of the infectious agent has the potential to improve clinical management.^{3–5} The incidence of complicated pneumonia has recently increased, highlighting the need to better understand the reasons for this changing epidemiology.^{6–8} However, using con-

ventional bacterial culture, bacteria are isolated from clinical samples obtained from children with complicated pneumonia in only 17%–42% of cases.^{1,9,10} The low rate of positive culture results may be related to stringent bacterial growth requirements or to administration of broad-spectrum antibiotics before obtaining blood or pleural fluid samples for culture.

Unlike bacterial culture, polymerase chain reaction (PCR) relies on the detection of bacterial DNA rather than on the recovery of viable bacteria and is therefore less affected by the prior administration of antibiotics. Broad-range or “universal primer” 16S rRNA PCR detects a conserved region of the 16S ribosomal RNA (rRNA) gene and can detect a wide range of bacterial species with a single assay.¹¹ However, few studies have evaluated the role of 16S rRNA PCR in detecting bacteria in the pleural fluid of children with complicated pneumonia.^{12,13} The purpose of this study was to determine the frequency of positive blood and pleural fluid cultures in children with complicated pneumonia, and to determine whether broad-

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range 16S rRNA PCR can increase the proportion of children with an identifiable microbiologic cause of complicated pneumonia.

METHODS

Study Design, Setting, and Participants

This prospective cohort study was conducted at The Children's Hospital of Philadelphia (CHOP), an urban tertiary care children's hospital. The proposal was approved by the CHOP Committees for the Protection of Human Subjects. Patients were eligible for participation if they were ≤ 18 years of age, admitted to the hospital between October 1, 2007 and March 31, 2010, and diagnosed with complicated pneumonia. Parental informed consent was obtained for all patients, and verbal assent was obtained for all children over seven years of age. Patients with chronic medical conditions predisposing them to severe or recurrent pneumonia, such as human immunodeficiency virus, malignancy, and sickle cell disease, were excluded. The study team had no role in the clinical management of study patients. As this test is still considered experimental, pleural fluid 16S rRNA PCR was not performed until the patient was discharged from the hospital; these test results were not shared with the treating physicians.

Study Definition

Complicated pneumonia was defined by a temperature $>38.0^{\circ}\text{C}$, and the presence of lung parenchymal infiltrates and pleural effusions of any size or character on chest radiography or computed tomography.

Microbiologic Methods—Conventional Culture

Pleural fluid or blood (≤ 4 mL) was inoculated onto a single pediatric BacT/Alert FAN bottle (BioMérieux, Durham, NC) which was immediately transported to the laboratory at room temperature. Additional pleural fluid was also submitted to the laboratory for a cytospin Gram stain. Once received in the laboratory, the bottles were immediately loaded into the BacT/Alert instrument. Bottles were automatically checked by the instrument for production of CO_2 every ten minutes, and remained in the instrument for a total of five days. Bottles flagged as positive by the BacT/Alert system were removed from the instrument, subcultured to agar plates, and Gram stained. Bacterial isolates were identified and the antibiotic susceptibility tested by conventional methods following Clinical and Laboratory Standards Institute guidelines.

Microbiological Methods—16S rRNA PCR

The 16S rRNA primers and probe used in this study have been validated previously and have been used extensively for the identification of organisms from the Domain Bacteria.¹⁴ *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 29213) were used as Gram-negative and Gram-positive con-

trols, respectively. DNA extraction was performed on a 300 μl aliquot of pleural fluid using the MagMAX Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA). Samples were disrupted using a Vortex adapter for 15 minutes at the highest setting. Tubes were then centrifuged at 16,000 relative centrifugal force (RCF) for three minutes, and DNA extraction was performed using the KingFisher Flex Automated Purification System (Thermo Scientific, Waltham, MA). All DNA extracts were frozen at -20°C prior to use. Details of the 16S rRNA PCR assay optimization and validation appear in the Appendix.

DNA was amplified on an Applied Biosystems 7500 thermal cycler (ABI, Foster City, CA) using the primers, 16S F: 5'-GCT GTC ATC TCT TGT GGG CTG T-3', 16S R: 5'-AAA CTC ATG GGA GCT GCT GGT T-3', and the fluorescent labeled TaqMan probe, 16s Probe: 5'-Cy5/CCT GTC ATG CCC ACA CAA ATC TCT CC/BHQ-2-3'. Human DNA (0.5 nanograms) was added to each assay as a standardized internal amplification control. The primers, AlbICF: 5'-GCT GTC ATC TCT TGT GGG CTG T-3', AlbICR: 5'-AAA CTC ATG GGA GCT GCT GGT T-3', and the TaqMan probe, AlbICP: 5'-Cy5/CCT GTC ATG CCC ACA CAA ATC TCT CC/BHQ-2-3' detect a region of the human albumin gene. Amplification was performed as follows: initial denaturation of 94°C for 20 seconds, followed by 35 cycles of 94°C for 10 seconds, 61°C for 31 seconds, and 72°C for 5 seconds. Each run included positive (*E. coli* ATCC 25922 DNA) and negative (nuclease free water) controls. A cycle threshold value of 30 or less indicated a positive sample. All positive samples were confirmed using the MicroSeq[®] 500 16S rDNA Bacterial Identification kit (Applied Biosystems).

DNA Sequencing

DNA sequencing was performed directly on PCR products using an ABI 3730 with the BigDye Taq FS Terminator V3.1. Sequencing was completed at the University of Pennsylvania DNA Sequencing Facility, and sequence identification was performed by BLAST at the National Center for Biotechnology Information web site (<http://www.ncbi.nlm.nih.gov>).

Statistical Analysis

Data were analyzed using STATA 10.0 (Stata Corp., College Station, TX). Categorical variables were described using frequencies and percentages, and were compared using the chi-square test. Continuous variables were described using mean, median, intraquartile range (IQR), and range values. Binomial exact 95% confidence intervals (CIs) were calculated for the proportion of positive blood and pleural fluid cultures and pleural fluid 16S rRNA PCR tests.

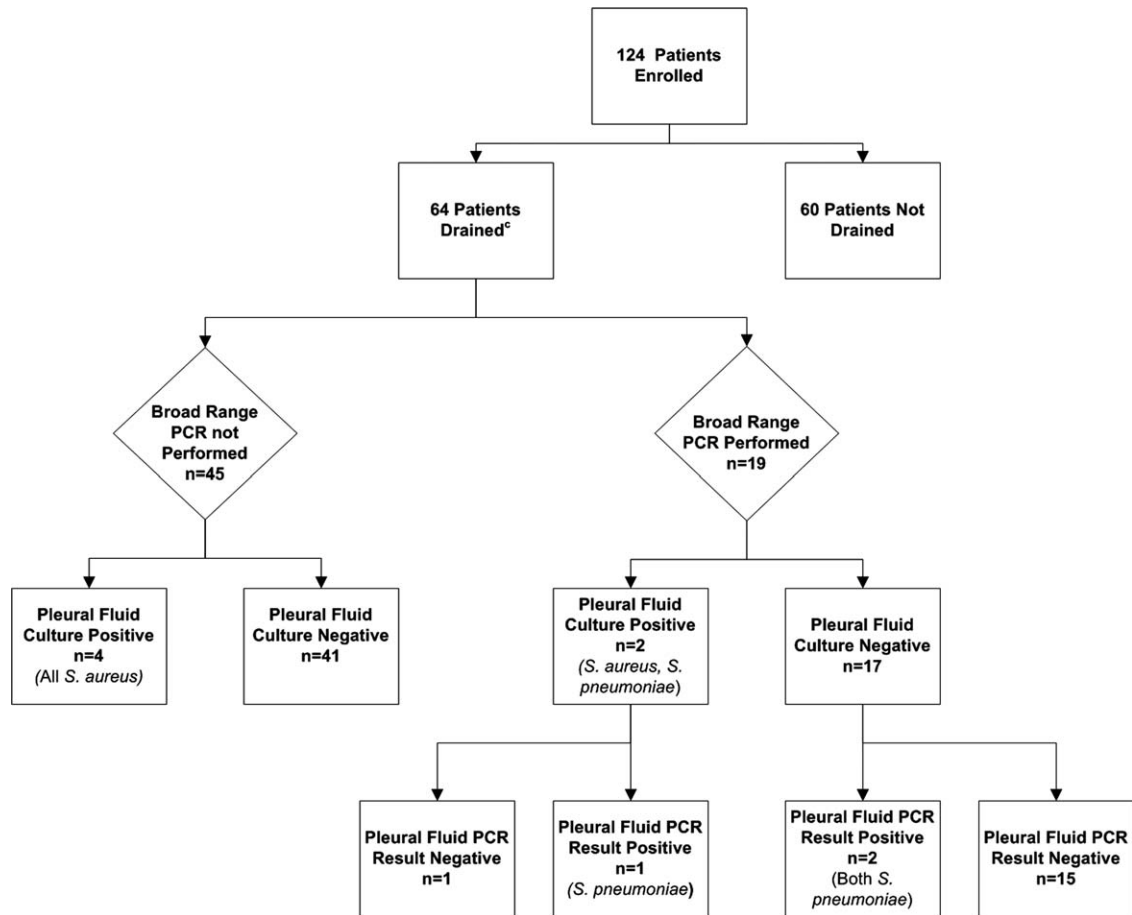


FIG. 1. Diagnostic evaluation of children with complicated pneumonia. **Abbreviation:** PCR, polymerase chain reaction.

RESULTS

During the study period, 124 patients with complicated pneumonia were identified; the median age was 4.9 years (IQR, 2.7–8.8 years) and the ratio of female to male patients was 1:1. The racial distribution was as follows: non-Hispanic white, 62 (50%); non-Hispanic black, 43 (34%); and Hispanic, 7 (6%). The median length of stay was 11 days (range, 2–40 days; IQR, 8–11 days). Forty-one (33.1%) patients were admitted to the intensive care unit, and nine (7.3%) patients required mechanical ventilation. Overall, 5 of the 71 blood cultures performed (7.0%; 95% CI: 2.3%–15.7%) were positive.

Pleural fluid drainage was performed in 64 (51.6%) patients. The median duration of antibiotic treatment before pleural fluid drainage was two days (IQR, 0–4 days). Differences in duration of antibiotic pretreatment between those with and without pleural fluid drainage were not significant. Patients received beta-lactam agents (76.6%), clindamycin (51.6%), and vancomycin (23.4%) before pleural drainage. Blood cultures were performed in 27 (45.0%) of 60 patients not undergoing pleural fluid drainage; 2 of these 27 (7.4%; 95% CI: 0.9%–24.3%) blood cultures were positive, and *Streptococcus pneumoniae* was isolated in both cases.

Using a combination of pleural fluid culture, blood culture, and broad-range PCR, a microbiologic cause of infection was identified in 11 of 64 (17.2%; 95% CI: 8.9%–28.7%) patients undergoing pleural fluid drainage (Figure 1). Patients with an identified bacterial cause were younger than those without an identified bacterial cause (Table 1). However, there were no statistically significant differences between the groups with and without identified bacteria with respect to sex, race, clinical examination findings and laboratory results at admission, duration of antibiotic therapy before pleural fluid drainage, or length of stay (Table 1). The median pleural fluid white blood cell count was 6880 per mm³ (IQR, 1542–27,918 per mm³).

Blood was obtained for culture from 44 (68.8%) of 64 patients that underwent pleural fluid drainage. Blood cultures were positive in three (6.8%; 95% CI: 1.4%–18.7%) of these patients; causative bacteria were *S. pneumoniae* (n = 1), *Haemophilus influenzae* (n = 1), and *Staphylococcus aureus* (n = 1). Pleural fluid cultures were positive from 6 of 64 patients (9.3%; 95% CI: 3.5%–19.3%) that underwent pleural fluid drainage; causative bacteria were *Staphylococcus aureus* (n = 5) and *Streptococcus pneumoniae* (n = 1). Three of the 19 pleural fluid samples (15.8%, CI 3.4%–39.6%) tested by 16S rRNA PCR yielded positive results; *S.*

TABLE 1. Demographics of Patients Who Underwent Pleural Fluid Drainage Stratified According to Those with Identified Causative Organisms and Patients with No Identified Organism

Demographics	Causative Bacteria Identified		P Value
	Yes (n = 11)	No (n = 53)	
Female sex	3 (27.3%)	29 (54.7%)	0.10
Age (years)	2 (1.3–4.50)	4.3 (3.1–8.1)	0.03
Race			0.09
Non-Hispanic white	3 (27.3%)	29 (54.7%)	
Non-Hispanic black	7 (63.6%)	13 (24.5%)	
Hispanic	0 (0.0%)	3 (5.6%)	
Other	1 (9.1%)	8 (15.1%)	
History of asthma	2 (18.2%)	12 (22.6%)	0.75
Duration of antibiotics before drainage (days)	0.5 (0–3)	2 (0–4)	0.43
Intensive care unit admission	7 (63.6%)	25 (47.2%)	0.32
Initial temperature (°C)	37.8 (37.2–39.5)	38.1 (37.6–39.3)	0.76
Initial oxygen saturation (%)	94 (93–97)	95 (93–96)	0.97
Peripheral white blood cell count (per mm ³)	21.4 (14.0–28.4)	16.3 (10.9–22.6)	0.12
Platelet count (per mm ³)	434 (298–546)	402 (312–575)	0.95

NOTE: Data presented as number (percent) or median (interquartile range).

pneumoniae was identified by DNA sequencing in all three of these samples (Figure 1). Of these three specimens, two had both negative Gram stains and negative pleural fluid cultures, while one specimen had Gram-positive cocci in pairs identified on Gram stain along with a pleural fluid culture-positive for *S. pneumoniae*. Of the 16 patients with negative PCR results, one was found to have a pleural fluid culture-positive for *S. aureus*; no bacteria were detected on the pleural fluid Gram stain (Figure 1). Differences in the demographic characteristics between patients in whom broad-range PCR was performed and not performed were not statistically significant (Table 2). Differences in the median length of hospital stay between patients who had PCR performed (9 days; IQR, 7–12 days) and those who did not (11 days; IQR, 9–14 days) were not statistically significant ($P = 0.19$). Antibiotic therapy was simplified from treatment with multiple antibiotics to a single antibiotic in each instance of a positive culture, though the number of positive cultures was too small to lead to meaningful reductions in antibiotic use or spectrum.

DISCUSSION

Identification of the causative organism can improve the treatment of children with complicated pneumonia by enabling clinicians to target the infection with effective, narrow-spectrum antibiotics. However, broad-spectrum antibiotics are typically given before blood or pleural fluid samples are obtained, lowering the yield of conventional bacterial cultures. In our study, pleural fluid and blood cultures were infrequently positive. However, the use of 16S rRNA broad-range PCR and DNA sequencing as ancillary tests only modestly improved our diagnostic yield.

TABLE 2. Comparison of Demographic Characteristics of Patients Analyzed with PCR and Patients Not Analyzed with PCR

Demographics	PCR Performed		P Value
	Yes (N = 19)	No (N = 45)	
Female sex	9 (47.4)	23 (51.1)	0.78
Age (years)	4.9 (2.7–8.8)	4.0 (2.8–8.1)	0.76
Race			0.42
Non-Hispanic white	10 (52.6%)	22 (48.9%)	
Non-Hispanic black	5 (26.3%)	15 (33.3%)	
Hispanic	0 (0.0%)	3 (6.7%)	
Other	4 (21.1%)	5 (11.1%)	
History of asthma	5 (26.32%)	9 (20.0%)	0.58
Duration of antibiotics before drainage (days)	2 (0–3)	1.5 (0–4)	0.65
Intensive care unit admission	8 (42.1%)	24 (53.3%)	0.41
Initial temperature (°C)	38.1 (37.3–39.4)	38.0 (37.6–39.4)	0.86
Initial pulse oximetry (%)	96 (94–97)	94 (92–96)	0.34
Initial white blood cell count (×1000 per mm ³)	16.1 (9.5–27.4)	17.8 (12.6–22.8)	0.93
Platelet count (×1000 per mm ³)	397 (312–575)	431 (303–597)	0.73

NOTE: Data presented as number (percent) or median (intraquartile range).

Abbreviation: PCR, polymerase chain reaction.

Two prior studies have shown that broad-range PCR analysis of pleural fluid can detect pathogenic organisms from pleural fluid even after the administration of antibiotics. Saglani et al.¹² used bacterial culture and broad-range PCR to analyze pleural fluid from 32 children with complicated pneumonia. Although the cohort had received a median of eight days of antibiotic therapy prior to fluid aspiration, a combination of broad-range PCR and DNA sequencing identified organisms, predominantly *S. pneumoniae*, in 17 of 26 (65.4%) pleural fluid samples with negative culture. Five of the six culture-positive samples also had positive PCR results, suggesting that a low proportion of the PCR results were false-negatives. Le Monnier et al.¹³ cultured the pleural fluid from 78 children with complicated pneumonia; 15 samples were excluded from PCR testing because they grew bacteria other than *S. pneumoniae*. Broad-range 16S rRNA PCR detected bacteria in 22 of 40 (55.0%) samples that were culture-negative; subsequent DNA sequencing identified *S. pneumoniae* (n = 17), *S. pyogenes* (n = 3), *S. aureus* (n = 1), and *H. influenzae* (n = 1). *S. pneumoniae* was identified by 16S rRNA PCR followed by DNA sequencing in 20 of the 23 (87.0%) pleural fluid samples that grew *S. pneumoniae* on culture.

PCR with organism-specific primers has also been shown to detect pathogenic bacteria in 35%–70% of pleural fluid samples from patients with complicated pneumonia.^{15,16} These rates of detection are comparable to the results of prior studies that used broad-range PCR, and suggest that 16S rRNA and organism-specific PCR are similarly sensitive tests.

Our study found that the proportion of positive results with broad-range PCR was greater than the

proportion observed with conventional pleural fluid culture. We were also able to identify *S. pneumoniae* by broad-range PCR in two culture-negative pleural fluid samples. The detection of *S. pneumoniae* by PCR in the setting of negative pleural fluid culture is not surprising, as most patients received empiric antibiotic therapy against *S. pneumoniae* before undergoing pleural fluid drainage. This prior antibiotic therapy would be expected to decrease the yield of pleural fluid culture but have a less significant impact on PCR. The reason for the failure to detect *S. aureus* by PCR from our clinical samples is not known. We explored whether this issue could be attributable to test characteristics during validation of our assay (as described in the technical Appendix), and *S. aureus* was consistently identified in pleural fluid samples “spiked” with *S. aureus*. While our diagnostic yield was low compared with prior studies that used broad-range PCR, the distribution of causative bacteria was similar to prior studies of complicated pneumonia.^{9,10} There were too few positive cultures among the specimens available for broad-range PCR testing for us to reliably assess 16S rRNA PCR sensitivity and specificity. Thus, it was not possible to reliably assess the value of 16S rRNA PCR as an ancillary test in culture-negative complicated pneumonia.

Several factors may have contributed to the low yield of broad-range PCR in our study. First, all patients in our study received broad-spectrum antibiotics prior to pleural fluid drainage; most patients received treatment with a beta-lactam agent in combination with either clindamycin or vancomycin. Although PCR is less affected than culture by the prior administration of antibiotics, it is still possible that exposure to antibiotics accelerated degradation of the bacterial genome, thus decreasing the sensitivity of broad-range PCR. The median duration of antibiotic therapy prior to drainage was shorter for patients in whom a bacterial pathogen was identified, compared with those in whom a pathogen was not identified. Though this difference was not statistically significant, this difference emphasizes that early pleural fluid collection may improve bacterial detection. The median duration of prior antibiotic therapy was eight days, in the study by Saglani et al.,¹² however details regarding anti-staphylococcal therapy were not reported. Second, several studies using 16S rRNA PCR in blood or cerebrospinal fluid (CSF) specimens for children with sepsis or meningitis, respectively, have noted decreased sensitivity for detection of Gram-positive bacteria.^{17–20} The cell wall composition makes Gram-positive bacteria particularly difficult to lyse for DNA extraction. Prior studies used a different approach to DNA extraction compared with our study. Saglani et al.¹² used the QIAmp minikit for DNA extraction (Qiagen Ltd, West Sussex, UK) and Le Monnier et al.¹³ used the MagnaPure System (Roche Diagnostics, Indianapolis, IN) for DNA extraction; addi-

tionally Saglani et al.¹² included an additional 15 minutes of incubation at 95°C following Proteinase K digestion to ensure complete lysis of the bacterial cells. We used the MagMAX Total Nucleic Acid Isolation Kit with a bead beating step to degrade the samples prior to lysis. It is unlikely that any of these differences contributed to differences in our study results. Third, human DNA may cause a nonspecific background signal, which could decrease PCR sensitivity. This issue may be particularly important in complicated pneumonia where the pleural fluid white blood cell counts are substantially elevated. Human DNA from white blood cells might be present at much higher concentrations than bacterial DNA, which would create a competitive advantage for binding of human, rather than bacterial, DNA on to the bead matrix.²¹ Saglani et al.¹² did not report pleural fluid white blood cell counts for comparison. However, Le Monnier et al.¹³ reported pleural fluid white blood cell counts slightly higher than those observed in our patients. Thus, differences in pleural fluid white blood cell counts do not necessarily explain the differences in yield across studies. Fourth, while the universal primers used in our study have been validated, including for detection of *S. aureus*, in previous studies,¹⁴ Saglani et al.¹² and Le Monnier et al.¹³ each used a different set of primers. It is unclear whether or not the choice of different primers affected the microbiologic yield.

An important limitation of this study is the relatively small number of patients with pleural fluid available for PCR testing combined with the low PCR yield. This limitation may have caused us to underestimate the true benefit of 16S rRNA PCR as an ancillary diagnostic test, as suggested by the wide confidence intervals around the estimates of PCR yield. Additionally, not all patients underwent pleural drainage, and not all patients undergoing pleural drainage had pleural fluid available for PCR testing. The latter issue would likely have only minimal impact on our study results, as there were no differences in demographics or clinical or laboratory features among those with and without pleural fluid available for PCR testing. However, differences among physicians, and across institutions, may play an important role in the decision to perform pleural drainage. We do not know whether patients undergoing pleural drainage at our institution were more or less likely to have bacteria detected by PCR than patients not undergoing drainage. If they were more likely to have bacteria detected, then our study would underestimate the benefit of PCR as an adjunct diagnostic test. However, there was no difference in the duration of antibiotic pretreatment between these two groups, and the yield from blood culture was similar.

In conclusion, blood and pleural fluid cultures infrequently identify the causative bacteria in children with complicated pneumonia. The use of broad-range PCR increased the microbiologic yield only modestly.

Further refinements to improve the diagnostic accuracy of broad-range PCR testing are needed before this technique can be recommended for widespread use in clinical practice.

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