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Molecular Analysis Improves Pathogen Identification and Epidemiologic Study of Pediatric Parapneumonic Empyema

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Introduction

Parapneumonic empyema (PPE) is a serious complication of bacterial pneumonia that is becoming more common.[1] Recently published reports implicate *Streptococcus pneumoniae* serotypes not contained within the 7-valent pneumococcal conjugate vaccine (PCV-7; Wyeth, Madison, NJ), and *Staphylococcus aureus*, particularly MRSA as important pathogens. [2, 3] For the most part these data rely on culture-based pathogen identification. Unfortunately, in most children with PPE the bacterial etiology is unknown.[3–9]

The low rate of pathogen detection complicates clinical care and selection of appropriate antibiotics. There are several factors that contribute to difficulties in isolating bacterial pathogens in patients with PPE. The most significant factor is likely the pre-treatment of children with antibiotics prior to obtaining blood or pleural fluid cultures. In addition, recovery of *S. pneumoniae* in culture is inherently difficult, due both to its sensitivity to

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transport conditions and its propensity for autolysis.[10] The combination of these factors leads to a high rate of culture-negative disease (up to 60% of cases).[3, 4] Thus the reliance on culture-based methodologies may be an impediment to accurately understanding of the epidemiology of PPE.

Our institution has had an increasing rate of pediatric PPE for more than a decade. The incidence increased more than 10-fold between 1993 and 2007, from 1/100,000 to 12.5/100,000. [4,11] Previous studies have defined the epidemiology of culture-positive PPE in Utah children,[11, 12] however the etiology of culture-negative disease has remained elusive. Recent studies from other centers have shown promising results using culture-independent techniques to determine the etiologic diagnosis of PPE.[5, 7, 13–15]

We have developed PCR-based assays that can detect and identify the major bacteria thought to be responsible for PPE. Additionally, our collaborators in Spain have developed PCR-based serotypying assays for pneumococci.[16] Using these molecular assays we sought to 1) evaluate pleural fluid specimens from children with PPE to better define the etiology and epidemiology of disease 2) compare PCR-based detection of pathogens to culture-based detection and 3) compare data from patients culture-positive for a bacterial pathogen in blood or pleural fluid to data from patients in whom cultures were negative in an effort to identify whether there were differences in pathogens, pneumococcal serotypes, clinical presentation and/or outcomes between culture-positive and culture negative PPE.

Methods

Protection of Human Subjects

The Institutional Review Boards of the University of Utah and Primary Children's Medical Center approved this study. Informed consent was waived.

Setting and Study Population

Children hospitalized at Primary Children's Medical Center (PCMC), Salt Lake City, Utah, between January 1 and December 31, 2009 for treatment of pneumonia complicated by PPE were included. PCMC is a 278-bed children's hospital that serves both as a community pediatric hospital for Salt Lake County, Utah, and as a tertiary referral center for 5 states (Utah, Idaho, Wyoming, Nevada, and Montana).

Identification of Children with Parapneumonic Empyema

Since 2003 the PCMC microbiology laboratory has routinely archived at −70 C all available residual pleural fluid samples cultured for bacteria. For this study we used molecular testing to evaluate all pleural fluid specimens archived in 2009 from patients classified as having pneumonia with PPE. To identify patients with PPE, we queried a computerized data record system for children < 18 years of age, hospitalized at PCMC between 1/1/2009 and 12/31/2009 with ICD-9 discharge diagnosis codes specific for pneumonia (481) and parapneumonic empyema (510), combined with procedure codes for chest tube placement (34.04. 34.06) and/or video-assisted thoracoscopic surgery (34.51, 34.52). We excluded all children with ICD-9 coded parapneumonic empyema following thoracic surgery. One investigator (KA) reviewed all medical records to confirm the diagnosis of PPE based on pleural fluid characteristics. [17] Demographic and clinical data as well as conventional bacterial culture results were reviewed and abstracted.

Definitions

Children were included in this study if they had confirmed PPE, bacterial cultures were performed and a residual pleural fluid specimen was available. Culture-positive PPE was

defined as an episode in which *either* a blood or pleural fluid specimen was positive for a bacterial pathogen thought to be a causative agent of pneumonia. Culture-negative PPE was defined as an episode of PPE in which a causative bacterial pathogen was not identified by culture of blood or pleural fluid.

Pleural Fluid Testing by PCR

Identification of Pathogens—Pleural fluid analysis for bacterial detection by nested PCR was performed as previously described [18] with the addition of PCR for *Mycoplasma pneumoniae* using the toxin (*tox*) gene. Other bacterial targets included: *S. pneumoniae* (*ply*, *lytA* and *rpoB*), *S. aureus* (*nuc* and *mecA*), *S. pyogenes* (*rpoB*) and *Haemophilus influenzae* (*rpoB*). All pleural fluid specimens underwent testing with all assays. Because *ply* is found in other streptococcal species, we required amplification of *lytA* to consider a specimen positive for *S. pneumoniae.*

Molecular Serotyping—Serotyping was performed on residual nucleic acid samples after identification of *S. pneumoniae.* Serotyping was performed using real-time PCR targeting different capsular locus genes. These assays have been previously described and validated.[16]

Statistical Analyses

Fisher's exact test was used to calculate differences in categorical data. Continuous data such as patient age, duration of illness and length of stay were generally not normally distributed and thus were compared using Mann-Whitney Wilcoxon tests. For both categorical and continuous data a p-value of 0.05 was considered the cut-off for significance.

Results

Clinical Findings Among Patients With Parapneumonic Empyema

In 2009, 85 children were hospitalized at PCMC who met the case definition of pneumonia with PPE. Pleural fluid specimens from 63 (74%) of these children were available for PCR testing. The median age of the 63 children tested was 56 months (range 11–207). Only 19% had a pre-existing medical condition. Patients had median length of illness prior to presentation of 6 days (range 1–16 days) and 86% had been pre-treated with antibiotics when their pleural fluid was sampled. Children with PPE had a median length of hospital stay of 9 days (range 4–49 days) and 32% required care in the ICU. No child died of their infection. There were no significant differences in demographic or clinical data when all children with PPE during 2009 were compared with the subgroup with pleural fluid available.

Culture-Based Pathogen Identification

Using culture, pathogens were isolated from the blood and/or pleural fluid of 22 children (35% of the 63 children evaluated). *S. pneumoniae* was isolated in 15 (24%), *S. pyogenes* in 3 (5%) and *S. aureus* (all MRSA) in 4 (6%) (see Table, Supplemental Digital Content 1, http://links.lww.com/INF/A651). Pleural fluid culture alone identified a pathogen in only 15/63 (24%) of patients. Blood cultures were positive in 11 patients (10 *S. pneumoniae*, 1 MRSA). In 7/22 (32%) patients in whom a pathogen was identified by culture, blood was the only positive specimen.

PCR-based Identification of Pathogens

By PCR, 59 pathogens were detected in the pleural fluid of 53 children (84%). *Streptococcus pneumoniae* was detected in 45/63 (71%), *S. pyogenes* in 7 (11%), *S. aureus*

in 5 (8%; MSSA-2, MRSA-3), *Haemophilus influenzae* in 1 (2%) and *Mycoplasma pneumoniae* in 1 (2%)(see Table, Supplemental Digital Content 1, http://links.lww.com/INF/A651). Ten pleural fluid samples (16%) were negative by PCR.

In all cases in which a pathogen was identified by culture of pleural fluid (n=15), that pathogen was also identified by PCR. Seven patients had positive cultures from blood and negative cultures of pleural fluid. In 6/7 of these cases, the pathogen identified in blood was identified in pleural fluid by PCR. In one patient with a negative pleural fluid culture, blood culture alone was positive for MRSA. PCR of pleural fluid was negative. Of note, the pleural fluid had been sampled 72 hours later than blood, with the patient on antimicrobial therapy directed against MRSA.

In six samples (10%), two bacterial pathogens were detected: *S. pneumoniae* and *S. pyogenes* (2); *S. pneumoniae* and *S. aureus* (MSSA; 2); *S. pneumoniae* and *M. pneumoniae* (1); *S. aureus* (MRSA) and *S. pyogenes* (1). In four of these, one of the pathogens was identified by culture of blood or pleural fluid [*S. pneumoniae* (2), *S. pyogenes* (1) and MRSA (1)].

Overall, PCR testing of pleural fluid significantly increased pathogen identification compared to culture of blood and pleural fluid (35% vs. 84%; $p < 0.001$), in particular detection of *S. pneumoniae* (24% vs. 71%; $p < 0.001$).

PCR-based Serotyping

Of 45 samples positive for *S. pneumoniae* by culture and/or PCR, 35 were typeable using molecular methods. Serotype distribution is shown in Table 1. Four serotypes were detected: 1, 3, 7F and 19A. Serotype 7F was the most frequently detected serotype in both culture-positive and culture-negative disease, representing 8/15 (53%) cultured isolates and 21/45 (47%) of total pneumococci. Inability to type specimens was most often due to limited residual sample volume.

Comparison of Culture-Positive and Culture-Negative PPE

Forty-one patients (65%) with PPE had no pathogen identified by culture of either pleural fluid or blood. Of these 41 culture-negative patients, 30 (73%) had a pathogen identified by PCR of pleural fluid. Most patients with culture-negative PPE were positive for *S. pneumoniae* [27/41 (66%)]. Three patients with culture-negative PPE (7%) had *S. pyogenes* identified by PCR (including one dual *S. pneumoniae/S. pyogenes* positive sample). Only one patient with culture-negative pleural fluid (2%) had *S. aureus* (MSSA) detected. MSSA was detected by PCR in one other patient who was culture positive for *S. pneumoniae*. MRSA was not detected in any patient with culture-negative PPE.

To determine if patients with pathogens detected by culture were representative of the spectrum of PPE, we compared the demographics and clinical characteristics, bacterial etiology, and outcomes for patients with culture-positive and culture-negative PPE. (Table 2) Culture-positive patients had been ill for a significantly shorter period of time on admission than those with negative cultures (median of 4.5 vs. 7.0 days; $p = 0.002$), although the proportion treated with antibiotics before admission was similar (77% vs. 90%).

There were apparent differences in the likelihood of detecting specific pathogens by culture. Pneumococcal serotype 19A was more likely to be detected by culture than other pneumococcal serotypes. Five of 6 (83%) infections with serotype 19A were detected by culture compared to 10 of 39 for serotypes 1, 3 and 7F combined (26%; P=0.012). Four of six *S. aureus* infections and 4 of 4 infections with MRSA were detected by culture.

Finally, patients with positive cultures were significantly more likely to require ICU care than those with negative cultures (50% vs. 17%; p = 0.009). This may have been related to the pathogen causing disease. When analyzed by pathogen, patients with *S. pneumoniae* serotype 19A and MRSA were more likely to be admitted to ICU and those with MRSA had longer hospital stay. Due to the small number of patients in each group, these differences were not statistically significant (Table 3).

Discussion

Parapneumonic empyema is a serious and increasingly common complication of bacterial pneumonia.[1] The bacterial etiology of PPE is often unknown due to sterile bacterial cultures.[3–9] Our study used a combination of multi-pathogen molecular detection and molecular serotyping of *S. pneumoniae* to perform a detailed analysis of the etiology of both culture-positive and culture-negative disease. There are several important findings from this work. First, we demonstrated that molecular diagnostic testing significantly increased the identification of pathogens in PPE. Second, dual infections may be more common than previously appreciated. They often involve *S. pneumoniae* and may occur in patients with cultures positive for a single pathogen. Third, *S. pneumoniae* is the most common cause of culture-negative PPE. Importantly, MRSA was never detected in our culture-negative specimens. Fourth, there are significant differences between culture-negative and culture-positive PPE in duration of illness before presentation, pathogen profile and severity of disease.

A number of studies have described the utility of molecular or antigen-based detection strategies for identification of pathogens in PPE.[5, 7, 13–15] In most of these studies, *S. pneumoniae*-specific testing was performed, either pneumococcal antigen testing (i.e. BinaxNOW, Inverness Medical, Princeton, NJ)[5, 15] or PCR with specific primers for pneumococcal virulence factors.[13, 14] In general, these culture-independent diagnostic technologies significantly improved detection of *S. pneumoniae*, demonstrating this pathogen in up to 70% of PPE patients.[14, 15] Our study was similar, with 71% of pleural fluid samples positive for *S. pneumoniae*.

Only a small number of previous studies have used molecular testing to evaluate pleural fluid for other pathogens.[5, 7] Some studies have used broad-range 16S rRNA PCR of pleural fluid for evaluation, and have identified other bacteria as contributory, including *S. pyogenes, H. influenzae* and *M. pneumoniae*. Our results are similar. Others have used multiplex PCR approaches for pneumonia diagnostics, such as combining PCR for *S. pneumoniae, H. influenzae, S. pyogenes, M. pneumoniae, C. pneumoniae* and *L. pneumophila*,[20, 21] however they have not examined patients with empyema and complicated pneumonia specifically. This is the first study, to our knowledge, to use multiple species-specific assays to evaluate pleural fluid from children with PPE.

There were several advantages to our use of multi-species detection. While previous studies[4, 12] have demonstrated the significant contribution of pneumococci to culture-positive PPE the contribution of *S. pneumoniae* and other pathogens to culture-negative disease has been difficult to elucidate. In this study while the majority of patients (71%) with both culture-positive and culture-negative disease were infected with *S. pneumoniae*, the addition of 4 other bacteria to our diagnostic testing panel further increased our rate of pathogen detection to 86%. Surprisingly, we detected dual infection in 6/63 (10%) patients. *S. pneumoniae* was frequently involved as one of the pathogens, present in 5/6 dual infections. *S. pneumoniae* was detected by culture as well as PCR in 2 cases, and by PCR alone in 3. Culture alone did not detect any dual infections, however 4/6 patients were culture-positive for one of the pathogens. At least one other study has demonstrated similar

findings.[5] Further studies are needed to determine the clinical importance of polymicrobial infection.

We used molecular serotyping directly from clinical samples to investigate the epidemiology of pneumococcal PPE. Our institution has had a high incidence of invasive pneumococcal disease for many years, particularly PPE, , [5–7] due to non-vaccine serotypes. Serotype 1 was a predominant strain before the introduction of PCV7, and more recently serotypes 3, 7F and 19A have emerged.[11, 12] These serotypes have been major contributors to PPE in other studies. [6, 8, 22]

During 2009 serotype 7F was predominant in our patients with PPE, accounting for 47% of total pneumococcal infections. Serotypes 3 and 19A accounted for another 26% of disease, but serotype 1 was seen in only 4% of specimens. The reasons for this are unclear and outside the scope of this study. Of note, in 22% of cases in which *S. pneumoniae* was identified, serotyping could not be performed. In most cases this was due to limited residual sample volume following molecular detection. It is possible that had we been able to serotype these samples, the results would alter our conclusions regarding serotypic frequencies.

A particular strength of this study is the elucidation of pathogens responsible for culture negative PPE. As expected based on prior studies [13, 14] *S. pneumoniae* was the most common cause of culture-negative disease (66% of all culture-negative specimens). Interestingly, while *S. pyogenes* and *Haemophilus influenzae* were found in culture negative specimens, MRSA was not. We did detect *S. aureus* (including MRSA) by PCR in all cases of staphylococcal PPE in which the pleural fluid culture was positive. Patients with MRSA PPE may be more likely to have positive bacterial cultures on presentation due to the resistance of MRSA to common antibiotic regimens for pneumonia. Our data provide some reassurance that MRSA is less likely to be present in patients with PPE but negative cultures. This finding needs to be confirmed using molecular assays in areas with high MRSA prevalence. [9, 23, 24]

The ability to perform pneumococcal serotyping for culture-negative specimens provides a broader understanding of pneumococcal epidemiology. For example, using culture alone *S. pneumoniae* serotype 19A accounted for 33% of pneumococcal isolates. However, when specimens positive for *S. pneumoniae* by any method were combined, only 13% of pneumococcal disease was due to 19A.

A number of culture-based epidemiologic studies have reported a preponderance of serotype 19A in IPD.[25–27] Serotype 19A has a high prevalence of antibiotic resistance[28, 29] and this may contribute to its recovery despite of the frequency of antibiotic pre-treatment among children with PPE. An additional explanation may be the severity of 19A disease. In our study disease caused by serotype 19A was more severe than 7F based by several measures.

Overall, serotype 7F predominated in our patients, and accounted for 47% of pneumococcal empyema. Serotypes 7F, 3, and 1 in Utah are almost all susceptible to beta-lactam and macrolide antibiotics, likely increasing the rate of false-negative cultures in children pre-treated with antibiotics. Our collaborators have previously published similar molecular serotyping results from pleural fluid in Spain[6] and demonstrated frequent identification of serotype 7F, 1 and 3 in culture-negative specimens. These data suggest a potential for bias in epidemiologic studies that rely exclusively on culture.

This study provides an expanded view of the epidemiology of PPE just before the introduction of PCV-13. The four predominant pneumococcal serotypes found in Utah children with PPE are all contained in the PCV-13 vaccine and it will be essential to follow

the epidemiology of PPE over the next several years. Recent data from across the country demonstrate replacement disease due to non-vaccine serotypes which are predominantly those in PCV-13,[11, 12, 25] but include some, such as serotypes 22F, 15 and 35 that are not.[30, 31](KA, manuscript submitted). Detailed understanding of disease that emerges as PCV-13 use becomes widespread will be crucial for developing future vaccine strategies. Molecular detection and serotyping could enhance surveillance.

There were several limitations to our study. First, this study was performed at a single center with a high rate of pneumococcal PPE. However, clinical and demographic characteristics of patients with PPE in our population were similar to those reported in other studies.[1, 5, 8] The overall incidence of MRSA in our population, and rates of MRSA PPE in our center are low. Therefore our data may not be applicable at centers with a high incidence of MRSA infection. This study was performed over a single year and does not specifically evaluate changes over time. 2009 was marked by 2009 H1N1 pandemic influenza, and our findings may not represent the epidemiology of PPE in inter-pandemic periods. Our samples were analyzed retrospectively and not all patients in 2009 had sufficient residual pleural fluid for molecular testing. Finally, by doing multi-pathogen rather than broad-range PCR we were limited in our findings to the 5 species that were evaluated. While these species were chosen based on the culture-based epidemiology of PPE as well as literature describing the most common causes of complicated pneumonia, fourteen percent of pleural fluids remained negative by PCR. This could be due to pathogens not included in our PCR primer set or perhaps by inadequate sensitivity of the assays. Addition of more assays to the analysis might identify previously unrecognized pathogens contributing to culture-negative PPE.

Despite these limitations this study contributes to our understanding of the epidemiology of PPE, particularly culture-negative disease. It also demonstrates the utility of molecular diagnostics in the evaluation of PPE. Studies such as this demonstrate that molecular testing can provide detailed information regarding the etiology and epidemiology of PPE and other serious infections, and can inform better clinical care.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Serotype Distribution Among 45 Specimens Positive for *S. pneumoniae*

***S. pneumoniae* Serotype**	**Number (%) Positive**
19A	6 (13%)
7F	21 (47%)
3	6 (13%)
1	2 (4%)
Untyped	10 (22%)

Table 2

Culture-Positive vs. Culture-Negative PPE

Variable	All PPE (n=63)	Culture-Positive (n=22)	Culture-Negative (n=41)	p-value[1]
Demographic Characteristics				
Male sex	38 (60%)	16 (73%)	22 (54%)	0.181
Age in months, median (IQR)	50 (27–113)	39.5 (27–138)	58 (27–100)	0.547
Preexisting condition	12 (19%)	4 (18%)	8 (20%)	1
Admission Characteristics				
Illness days, median (IQR)	6 (4–8)	4.5 (3–6)	7 (5–10)	**0.002****
Antibiotic Pretreatment	54 (86%)	17 (77%)	37 (90%)	0.256
Pathogen				
Streptococcus pneumoniae[2]	45 (71%)	15 (68%)	29 (70%)	1
Serotype 19A	6 (10%)	5 (23%)	1 (2%)	**0.017***
Serotype 7F	21 (33%)	8 (36%)	13 (32%)	0.782
Serotype 3	6 (10%)	1 (5%)	5 (12%)	0.652
Serotype 1	2 (3%)	1 (5%)	1 (2%)	1
Staphylococcus aureus	6 (10%)	4 (18%)	1 (2%)	**0.046***
MRSA	4 (6%)	4 (18%)	0	**0.012***
MSSA[2]	2 (3%)	0	1 (2%)	
Streptococcus pyogenes[2]	7 (11%)	3 (14%)	3 (7%)	0.413
Other[2,3]	2 (3%)	0	1 (2%)	1
Outcome				
LOS days, median (IQR)	9 (7–12)	11.5 (9.5–15)	9 (6–10)	**0.002****
Hospitalization in ICU	20 (32%)	11 (50%)	7 (17%)	**0.009****
Dual Infection	6 (10%)	4 (18%)	2 (5%)	0.171
Death	0	0	0	1

IQR = Intraquartile range (Q1–Q3)

[1] Culture-positive disease was compared to culture-negative disease for all variables

[2] There were 4 instances of dual infection in culture-positive patients. In each, one pathogen was detected by culture, the second by PCR. The uncultured pathogens [*S. pneumoniae* (1), MSSA (1), *S. pyogenes* (1) and *Mycoplasma pneumoniae* (1)] detected by PCR in culture-positive patients are not included in the pathogen count in either the culture-positive or culture negative columns.

[3] Other pathogens detected included *Haemophilus influenzae* and *Mycoplasma pneumoniae*

* $p < 0.05$

** $p < 0.01$

Table 3

Demographic and Clinical Comparisons by Pathogen

Variable	*S. pneumoniae* serotype			
	7F (n=21)	**19A (n=6)**	**MRSA (n=4)**	***S.pyogenes* (n=7)**
Demographic Characteristics				
Male sex	10 (48%)	4 (67%)	4 (100%)	5 (71%)
Age months, median (range)	43 (17–204)	28 (17–43)	182 (154–198)	90 (24–168)
Preexisting condition	4 (19%)	1 (17%)	2 (50%)	0 (0%)
Admission Characteristics				
Illness days, median (range)	7 (3–16)	4.5 (2–7)	4 (2–5)	5 (3–10)
Antibiotic Pretreatment	17 (81%)	5 (83%)	3 (75%)	5 (71%)
Positive Blood or PF culture	8 (38%)	5 (83%)	4 (100%)	3 (43%)
Outcome				
LOS days, median (range)	10 (6–49)	11.5 (9–16)	21 (11–32)	11 (4–30)
Hospitalization in ICU	5 (24%)	5 (83%)	2 (50%)	4 (57%)
Dual Infection[1]	3 (14%)	0 (0%)	1 (25%)	3 (43%)
Death	0 (0%)	0 (0%)	0 (0%)	0 (0%)

[1] Dual Infections included: *S. pneumoniae* and *S. pyogenes* (2); *S. pneumoniae* and *S. aureus* (MSSA; 2); *S. pneumoniae* and *M. pneumoniae* (1); *S. aureus* (MRSA) and *S. pyogenes* (1)