SUPPLEMENT ARTICLE

Specimen Collection for the Diagnosis of Pediatric Pneumonia

Laura L. Hammitt,[1,2] David R. Murdoch,[3,4] J. Anthony G. Scott,[2,5] Amanda Driscoll,[1] Ruth A. Karron,[6] Orin S. Levine,[1] Katherine L. O'Brien,[1] and the Pneumonia Methods Working Group[a]

[1]International Vaccine Access Center, Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland; [2]KEMRI–Wellcome Trust Research Programme, Centre for Geographic Medicine–Coast, Kilifi, Kenya; [3]Department of Pathology, University of Otago, Christchurch, [4]Canterbury Health Laboratories, Christchurch, New Zealand; [5]Nuffield Department of Clinical Medicine, University of Oxford, United Kingdom; and [6]Center for Immunization Research, Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland

Diagnosing the etiologic agent of pneumonia has an essential role in ensuring the most appropriate and effective therapy for individual patients and is critical to guiding the development of treatment and prevention strategies. However, establishing the etiology of pneumonia remains challenging because of the relative inaccessibility of the infected tissue and the difficulty in obtaining samples without contamination by upper respiratory tract secretions. Here, we review the published and unpublished literature on various specimens available for the diagnosis of pediatric pneumonia. We discuss the advantages and limitations of each specimen, and discuss the rationale for the specimens to be collected for the Pneumonia Etiology Research for Child Health study.

Diagnosing the microbiological etiology of pneumonia is challenging because the site of infection (ie, lung tissue) is not easily accessible for specimen collection. Sterile site specimens are the ''gold standard'' for the diagnosis of invasive disease, but specimens from the respiratory tract are accessed most easily through non-sterile approaches. Development of a more complex gold standard incorporating a number of methods has been suggested [1]. The problem of appropriate specimen collection and testing in episodes of pneumonia among infants and children is magnified in settings in the developing world because of the reduced capacity for clinical procedures and because of limited diagnostic facilities; paradoxically, these settings have the greatest prevalence of severe respiratory illness [2, 3]. Advances in pneumonia diagnostics have made it possible to identify a wide variety of pathogens through directed molecular testing. As a result, when body fluid or tissue is collected, careful consideration must be given to handling it in ways that maximize its use for a wide range of diagnostic assays.

We sought to establish a foundation of evidence on which to base decisions about specimen collection for the purpose of the PERCH study (Pneumonia Etiology Research for Child Health; a multisite case-control study of pneumonia etiology in the developing world), taking into consideration the range of body fluid and tissue specimens from which relevant data might be obtained, the clinical and laboratory resources available in developing country settings, patient safety, the case-control study design, and the aim of future pathogen discovery. We reviewed the published and unpublished literature to formulate a rational approach, aiming to minimize the influence of a priori notions of expected pneumonia etiology. Here, we discuss various body fluid or tissue specimens and the rationale for the PERCH specimen collection algorithm.

SPECIMENS FOR DETERMINING THE ETIOLOGY OF PNEUMONIA

Lung Aspirates

From a diagnostic standpoint, the ideal way to determine the etiology of pneumonia is to obtain a specimen

[a]Pneumonia Methods Working Group members have been listed in the Acknowledgments section.

Correspondence: Laura L. Hammitt, MD, Johns Hopkins Bloomberg School of Public Health, 621 N Washington St, Baltimore, MD 21205 (lhammitt@jhsph.edu).

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directly from the location of the infection (ie, the lung). Lung aspirates, commonly used for the cytological evaluation of suspected malignancy, can also be used to detect infection. In the developed world, the need to identify the etiology of pneumonia is less pressing because mortality due to pneumonia is a rare event; access to care and to broad-spectrum antibiotics have obviated the need for lung aspirates except in the case of recalcitrant infection in immunocompromised hosts. However, in the developing world, pneumonia kills more than a million children every year and there is a real need to determine etiology; in this context, the role of lung aspirates is discussed below.

The general technique used for pediatric lung aspiration is to (1) insert a needle blindly over the top of a rib into the area of consolidation (identified by chest radiograph or maximum physical findings), avoiding the area near the heart, great vessels, or other vital structures; (2) apply suction to the plunger of the syringe, and (3) withdraw the needle, while maintaining constant suction. The procedure is performed under sterile conditions and the aspiration takes 2–3 seconds. The primary risks associated with lung aspiration are pneumothorax and hemoptysis. A review of >2500 children undergoing needle aspiration over the past 8 decades from around the world reported complications in 5% (including pneumothorax in 3.2% and chest tube drainage required in 0.5%) [4]. In >6000 procedures in adults and children, death was temporally, though not necessarily causally, associated with lung aspiration in 6 patients (0.1%) [5]. In more recent years, the occurrence of adverse events following lung aspiration has decreased, presumably because of greater awareness of risks and improvements in technique (eg, use of smaller-gauge needles). In our review of all published pediatric lung aspirate procedures from the past 25 years, transient minor complications were reported in 25 (3.9%) of 690 procedures and pneumothorax requiring chest-tube drainage in 2 (0.3%); there were no deaths related to the procedure (Supplementary Table A). Conditions that predispose to bleeding or pneumothorax (eg, coagulopathy, chest hyperexpansion, chest cysts or bullae, suspected *Pneumocystis jirovecii* infection) are usually considered contraindications to lung aspiration.

The diagnostic yield of lung aspirate culture among pediatric patients with a clinical syndrome and chest radiographic findings of pneumonia varies depending on the technique, setting, and tests performed, but studies from the past 25 years have reported yields of 17%–78% [4, 6, 7]. A normal, healthy lung rarely contains sufficient organisms to produce a positive culture in the aspirate specimen; hence, the specificity of the technique is very high. Negative results are not uncommon when relying on culture, but the use of molecular techniques improves the yield considerably [8, 9]. Identification of an etiologic agent has been reported to be similar among patients with lobar pneumonia (50%) or bronchopneumonia (55%) [4]; however, in recent studies, the procedure has been performed only in children with a distinct peripheral consolidation [6–8].

Although there have been no randomized studies of the clinical benefit of lung aspiration, a comparison of outcomes among children in Papua, New Guinea, undergoing lung aspiration with children from the same ward in the previous year without lung aspiration suggested that mortality from pneumonia was lower during the year that lung aspirates were utilized [10]. Although this comparison reportedly involved children with pneumonia of equal severity, it is possible that selection bias may have contributed to this finding. Nonetheless, outcomes may be improved in children undergoing lung aspiration because of the ability to provide pathogen-directed antimicrobial therapy. In many cases, the pathogen identified on lung aspirate culture is not susceptible to World Health Organization–recommended empiric antibiotics and treatment regimens are altered accordingly [7, 11].

Children in whom lung aspirates are performed are not representative of all children hospitalized with pneumonia because of the application of selection criteria for the procedure. In addition, some centers may be unable to perform lung aspirates because of practical restrictions (eg, the limited availability of a radiographer or radiographic equipment). Although this will bias a group of patients toward a subpopulation of radiographically evident cases that are sometimes less severe, the information gained from lung aspirates is valuable for individual patient management and remains the most conclusive information available on the etiology of pneumonia. The technique can be used in settings that have the capacity for careful monitoring (eg, nursing observations, pulse oximetry, and chest radiography) and managing complications effectively (eg, equipment and expertise in chest-tube placement).

Lower Respiratory Tract Secretions

Secretions from the lower respiratory tract (LRT) of children with pneumonia are of diagnostic importance because this specimen comes from the site of infection and can be collected in a noninvasive fashion from the vast majority of cases. Children have difficulty expectorating sputum, primarily because they swallow it, so it is necessary to use bronchoalveolar lavage (BAL) or sputum induction to collect an LRT specimen.

Several studies have documented the diagnostic utility of BAL (bronchoscopic or nonbronchoscopic) in intensive care unit settings, particularly for the diagnosis and management of ventilator-associated pneumonia [12–15]. However, because of the need for mechanical ventilation, the possible need to

anaesthetize or sedate small children prior to the procedure, and the degree of clinical training and support to assure the safety of patients, BAL is not ideal for a study of community-acquired pneumonia among infants and children in resource-poor settings.

Sputum induction is most often used to diagnose pneumonia in settings with high tuberculosis prevalence [16] and among children with cystic fibrosis [17, 18]. However, it has also been demonstrated to be useful in children hospitalized with community-acquired pneumonia [19, 20]. The methodology, risks, benefits, and diagnostic yield of induced sputum sampling are reviewed by Grant et al [21]. In brief, the most common method for sputum induction is administration of hypertonic saline via nebulizer, followed by percussion of the chest wall to mobilize secretions. The sputum may be expectorated directly or collected using a suction catheter inserted into the pharynx. The procedure is well tolerated, although minor side effects of coughing, vomiting, and wheezing may occur. To minimize contamination of the sputum specimen by secretions from the oronasopharynx, suction is applied to the catheter only after it has been inserted into the pharynx and is discontinued before it is withdrawn.

Even with meticulous technique, contamination from the pharynx commonly occurs and bacterial culture results and nucleic acid detection tests of induced sputum must be interpreted carefully to determine whether detection of a potential pathogen represents contamination from the upper respiratory tract or disease in the LRT. The availability of paired induced sputum and lung aspirate specimens from the PERCH study will test the validity of induced sputum diagnostic testing.

Pleural Fluid

Diagnostic testing on pleural fluid can be useful among the subset of children who have pneumonia complicated by pleural effusion. The technique for specimen collection is well established and routinely used in clinical medicine. Standard tests include Gram stain for bacterial culture and Ziehl-Neelsen stain for mycobacterial culture, but antigen testing and polymerase chain reaction (PCR) increase diagnostic yield substantially [22–24].

Upper Respiratory Tract Specimens

The oropharynx (OP) and nasopharynx (NP) are 2 of the most common portals for the introduction of microorganisms into the respiratory tract. However, the detection of a pathogen in the upper respiratory tract (URT) is neither necessary nor sufficient evidence of the cause of pneumonia. The etiological significance of detecting microorganisms in the naso-oropharynx during an acute episode of pneumonia can be difficult to interpret against a background of asymptomatic colonization, replication, or persistence of genetic material beyond the period of acute infection. Nevertheless, for many infections, identification of the organism in the URT provides circumstantial evidence of causality.

We considered 4 sampling methods for PERCH: nasopharyngeal swabs, nasal aspirates, nasal washes, and throat swabs (Supplementary Table B). A fifth sampling method, collecting nasal discharge by wiping the patient's nose on tissue paper, shows promise as a less uncomfortable alternative to NP swab sampling among children with coryza [25]. We did not consider this technique for PERCH because many case and control subjects are unlikely to have sufficient nasal discharge.

Any of these URT specimens may be assayed by a variety of methods to detect a variety of pathogens (see reviews by Bhat et al and Murdoch et al [26, 27]). The ideal specimen to detect viruses depends on the type of assay being performed. In studies using direct fluorescent-antibody assay testing or reverse transcription PCR, the sensitivity of NP swabs (particularly flocked swabs) for detection of respiratory viruses is comparable to nasal wash or aspirate specimens [28–30]. Compared to NP swabs, nasal aspirates and nasal washes are more technically challenging, and because of aspiration risk, are not practical in very severely ill children in resource-poor settings. In addition, nasal aspirates and washes are likely to be less acceptable to healthy control children than an NP swab. For these reasons, we considered the NP swab as the preferred method of URT sampling for detection of viruses in PERCH.

OP swab specimens have been found to be consistently less sensitive than NP specimens for a variety of viruses; however, maximum sensitivity is attained by using multiple types of specimens [31–33]. An OP swab can increase the molecular detection of viral pathogens by 15% over an NP swab alone [34] and has been found to be more sensitive for the detection of certain viruses [35]. It is not known whether the increase in detection is related to pathogen tropism for different anatomical sites (eg, 2009 pandemic influenza A for the oropharynx) or simply a result of testing additional sample material; however, collection of an OP swab is quick, simple, involves minimal risk, and is likely to be acceptable to healthy controls. The cost of consumables can be reduced by placing the OP swab and NP swab into the same vial for transport and testing. The impact of OP swab composition on test performance is unknown.

For bacteria that are not commonly found in the upper airways (eg, *Bordetella pertussis* and *Mycoplasma pneumoniae*), detecting them in NP or OP specimens by PCR and/or culture provides useful diagnostic information. However, for most bacterial pathogens, as with most viruses, it is unclear

whether detection in the NP has any predictive value in defining the etiology of pneumonia. This is particularly true for pathogens that are frequently detected in the upper airways of children (eg, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*). For some pneumococcal serotypes that are rarely found in the NP but are well-recognized causes of invasive disease (eg, serotype 1 [36]), identification in the NP at the time of pneumonia may be highly predictive of pneumococcal pneumonia. Similarly, the absence of a pathogen in the NP at the time of pneumonia might suggest that it is not the etiological agent (ie, high negative predictive value). Quantification of bacterial load in NP specimens may help differentiate colonization from disease in the context of pneumonia, but only very limited information is available at present [37, 38].

For the culture of pneumococci from NP swabs, there is some evidence that rayon swabs perform better than Dacron swabs [39]. It is recommended that swabs be transported and stored in skim milk-tryptone-glucose-glycerin (STGG) prior to bacterial culture [40]; however, swabs targeted for molecular testing are typically transported in viral or universal transport media, meaning that at least 2 URT specimens are required for both culture and PCR. Recently, it has been shown that the sensitivity of PCR for viral respiratory pathogens (respiratory syncytial virus, influenza A, influenza B, and adenovirus) in swabs stored in STGG is 87% (95% confidence interval, 79.4–93.1) compared with swabs stored in universal transport media, suggesting that it may be possible to do both PCR and culture on a single swab [41].

URT specimens can be collected on all children with pneumonia so there is no sampling bias. A case-control design in which URT samples are collected from cases and controls using identical materials and techniques will facilitate statistical testing of the association between pathogen detection and pneumonia.

Blood Specimens

A variety of tests can be performed on blood to diagnose pneumonia. A comprehensive review of this subject is provided by Murdoch et al [27]. Given that a limited amount of blood can be obtained for clinical and research purposes, it is important to consider the most efficient uses of this valuable specimen.

Although positive blood cultures are found in only a small minority of children hospitalized with pneumonia, organisms identified by blood culture are widely accepted to be indicative of etiology of pneumonia, and antibiotic susceptibility results from these pathogens are used to guide therapy [6, 15]. The yield of blood cultures can be improved with careful attention to the volume of blood inoculated, the ratio of specimen volume to media, the minimization of specimen contamination, the optimization of storage, transport, and incubation conditions, and the ensuring of adequate microbiological capacity to evaluate positive culture bottles. Additional diagnostic information may be gained by doing PCR on blood culture specimens that flag positive on an automated culture instrument but are negative on subculture [42, 43]. It is expected that blood cultures from well children in the community would rarely, if ever, be positive for a pathogen [44] and that significant costs could be incurred solely from the evaluation of contaminated cultures; therefore, blood cultures from control subjects are not recommended.

Serological testing of acute or paired acute/convalescent samples was one of the earliest techniques developed for the diagnosis of pneumonia etiology and continues to be used today [45–47]. Serology may be useful in detecting fastidious pathogens, and it may provide supportive evidence for an association between detection of a pathogen in the URT and pneumonia. This type of association analysis may be particularly useful for pathogens that are known to have prolonged shedding in the nasopharynx or are highly prevalent in a control population.

Additional blood tests that provide information for the diagnosis of pneumonia include assessments of risk factors (eg, malaria, hemoglobinopathy, human immunodeficiency virus [HIV] infection) and biomarkers (eg, C-reactive protein, procalcitonin). Correct interpretation of these results necessitates that the tests be performed in both case and control subjects.

The collection of a small volume of blood is considered to be a minimal risk activity for patients and for control subjects. Many of the PERCH sites have had experience in the collection of blood from control subjects; the results of certain tests (eg, malaria, hemoglobin, HIV) can be reported and treatment provided if indicated. Although there are no universal guidelines on acceptable volumes of blood that may be safely collected from children, a recommendation has been published based on a review of the literature [48]. Among sick children, a maximum of 3 mL/kg over 24 hours is suggested as a reasonable guideline, although greater caution may be needed in children with anemia or blood volume depletion.

Urine Specimens

Several infectious causes of pneumonia can be detected by urinary antigen tests. Although urinary antigen testing can be used to diagnose pneumococcal pneumonia in adults, the test lacks specificity in children as a result of the high prevalence of pneumococcal colonization during childhood [49]. Detection of *Legionella* antigenuria is both sensitive and specific; however, this is a rare cause of pneumonia in children.

Recent studies suggest that analysis of the metabolic profile of urine specimens may be a useful tool in differentiating

Table 1. PERCH Algorithm for Specimen Collection and Laboratory Testing in Case Subjects

Specimen	Subjects[a]	Assay
Acute blood	All (>95%)	Blood culture
		Pneumococcal antigen testing on blood culture alarm (+) culture (−) specimens
		Complete blood count with differential
		Pneumococcus PCR
		HIV test
		Hemoglobinopathy testing (selected sites)
		Malaria antigen testing or microscopy (selected sites)
		Serologic testing
		C-reactive protein, other biomarkers
		Host genetic studies
Convalescent serum	All (>90%)	Serologic testing
Convalescent plasma	Select cases (site specific)	CD4 testing for HIV^+ cases in selected sites
Urine	All (>95%)	Storage for future antigen testing, biomarkers
NP flocked swab	All (>95%)	PCR for respiratory pathogens
NP rayon swab	All (>95%)	Bacterial culture and serotyping for pneumococcus
Throat rayon swab	All (>95%)	PCR for respiratory pathogens
Induced sputum	All, except when contraindicated (>90%)	Microscopy, bacterial culture and AST
		Mycobacterium tuberculosis microscopy, culture
		PCR for respiratory pathogens
Lung aspirate (select sites)	Select cases (<10%)	Microscopy, bacterial culture and AST
		M. tuberculosis microscopy, culture
		PCR for respiratory pathogens
Gastric aspirate	Select cases (<5%)	*M. tuberculosis* microscopy, culture
Pleural fluid	Select cases (<5%)	Microscopy, bacterial culture and AST
		Cell count, protein, glucose
		M. tuberculosis microscopy, culture
		Antigen detection (pneumococcus)
		PCR for respiratory pathogens
Lung tissue (select sites)	Postmortem cases (<2%)	Histology and immunohistochemistry
		Gram stain, bacterial culture and AST, mycobacterial culture
		Multiplex PCR and 16S RNA typing

Abbreviations: AST, antibiotic susceptibility testing; HIV, human immunodeficiency virus; NP, nasopharyngeal; PCR, polymerase chain reaction; PERCH, Pneumonia Etiology Research for Child Health; STGG, skim milk-tryptone-glucose-glycerin; VTM, viral transport media.

[a] Shown with the proportion of cases expected to have a specimen available.

pneumonia from other febrile illnesses and in identifying children in whom detection of pathogens represents an ''innocent bystander'' state, rather than disease [50]. Mouse models also suggest a possible role for metabolomics in identifying the actual infection causing pneumonia [51–53].

Collection of a bagged urine specimen is a minimal risk procedure and the specimen can be collected from all children with pneumonia and from control subjects, so there is no sampling bias. The biggest challenge in collecting this specimen is the inability to obtain a specimen ''on demand,'' especially in patients who are ill and may be dehydrated or anuric.

Postmortem Lung Tissue Specimens

Identification of the cause of fatal pneumonia is critical to understanding and preventing pneumonia deaths; however, there are considerable cultural and social constraints on postmortem examination in many countries. Immediate postmortem percutaneous lung biopsy offers a potentially simpler and less invasive approach to obtain lung tissue. A comprehensive discussion of the risks, benefits, and methodology of postmortem lung tissue sampling is provided by Turner et al [54]. In brief, microbiological testing of lung tissue can be used to compare postmortem and premortem specimens on the same patient to validate

Table 2. PERCH Algorithm for Specimen Collection and Laboratory Testing in Control Subjects

Specimen[a]	Assay
Acute blood	Pneumococcus PCR
	HIV test
	CD4 testing (HIV^+ controls in selected sites)
	Hemoglobinopathy testing (selected sites)
	Malaria antigen testing or microscopy (selected sites)
	Biomarkers
	Storage for future serologic testing
	Host genetic studies
NP flocked swab	PCR for respiratory pathogens
NP rayon swab	Bacterial culture and serotyping for pneumococcus
Throat rayon swab	PCR for respiratory pathogens
Urine	Storage for future antigen testing

Abbreviations: HIV, human immunodeficiency virus; NP, nasopharyngeal; PCR, polymerase chain reaction; PERCH, Pneumonia Etiology Research for Child Health.

[a] Each specimen type will be collected from each control subject.

premortem diagnostics, and importantly, can help to establish a diagnosis where one is lacking, particularly for children who arrive at a hospital in extremis and die shortly after arrival, before investigations can be initiated.

Exhaled Breath Specimens

There is a growing body of literature on the use of exhaled breath and exhaled breath condensate (EBC) to investigate occupational lung diseases and atopic lung disease. Biomarkers in EBC samples have also been correlated with infection in several small pilot studies [55–57]. Although this is a relatively simple specimen to collect and comes directly from the infected site, the technique is not fully standardized and the specificity of findings for pneumonia versus other types of illness or infection has not been established. Considerable validation studies would be required to establish normal values for biomarkers in EBC in children with pneumonia and to ascertain specific associations between EBC values and lung pathology. For these reasons, this novel technique was not recommended for collection in PERCH subjects.

SPECIMEN STORAGE AND TRANSPORT

Assuring the quality and standardization of specimen transport, storage and laboratory testing across PERCH study sites is a fundamental activity for the success of the project. Specimen transport and storage are subject to a standard operating procedure so that the conditions under which these activities take place are standardized across all sites (Supplementary Table C). In addition, a Lab Quality Plan establishes guidelines for quality assurance/quality control activities at each PERCH site, including a system for external quality assessment.

CONCLUSIONS

The PERCH specimen collection algorithm, summarized in Table 1 for cases and Table 2 for controls, focuses on tests of inherently high specificity (lung aspirates, pleural effusion) but also includes induced sputum and upper respiratory tract sampling to provide a minimum of information on the vast majority of pneumonia patients. PERCH will rely on epidemiological and statistical approaches to interpret results (especially in children with positive assays for multiple pathogens or discordant results from different specimens) and attribute causality [58].

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://www.oxfordjournals.org/our_journals/cid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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