SUPPLEMENT ARTICLE

The Role of Postmortem Studies in Pneumonia Etiology Research

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The diagnosis of etiology in severe pneumonia remains a challenging area. Postmortem lung tissue potentially increases the sensitivity of investigations for identification of causative pathogens in fatal cases of pneumonia and can confirm antemortem microbiological diagnoses. Tissue sampling allows assessment of histological patterns of disease and ancillary immunohistochemical or molecular diagnostic techniques. It may also enhance the recognition of noninfectious conditions that clinically simulate acute pneumonia. Biobanking of lung tissue or postmortem culture isolates offers opportunities for new pathogen discovery and research into host-pathogen interactions. The Pneumonia Etiology Research for Child Health study proposes a percutaneous needle biopsy approach to obtain postmortem samples, rather than a full open autopsy. This has the advantage of greater acceptability to relatives, but risks greater sampling error. Both approaches may be susceptible to microbiological contamination or pathogen degradation. However, previous autopsy studies have confirmed the value of histological examination in revealing unsuspected pathogens and influencing clinical guidelines for the diagnosis and treatment of future pneumonia cases.

Pneumonia is the largest global infectious cause of childhood mortality [1]. Our current understanding of the causes of pneumonia is based on studies in the 1980s that predated the global epidemic of human immunodeficiency virus (HIV)/AIDS and the introduction of vaccines against two major causes of pyogenic bacterial pneumonia, *Haemophilus influenzae* type b and *Streptococcus pneumoniae*. These two organisms are estimated to cause up to 50% of severe pneumonia in Africa, so effective vaccination programs would lead to a higher proportion of pneumonia caused by other pathogens, with implications for optimal treatment strategies [2].

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The Pneumonia Etiology Research for Child Health (PERCH) project is a large prospective multicentre case control study that aims to address the changing etiology of severe pneumonia in children. It also provides an opportunity to study the pathology of fatal pneumonia. Although postmortem diagnostics will not reveal the causes of fatal pneumonia in all cases, the relevant information obtained will help to prevent deaths from pneumonia in the future by identifying pathogens found in children with fatal illness.

The identification of pathogens that cause pneumonia during life is limited by several factors [3]. First, it is difficult to obtain tissue specimens from the infection site in the lung. At present, pneumonia diagnosis relies on the assessment of clinical samples such as blood or sputum using standard culture-based microbiological techniques. Additional samples are likely to be more representative of the diseased area, such as induced sputum, bronchoscopic lower respiratory tract washings or direct lung needle aspirates. Frequently, none of

these antemortem specimens are able to be collected from cases of very severe pneumonia in which the patients die during or soon after admission, leaving an important gap in our knowledge of the causes of fatal pneumonia. A second factor that introduces complications into the microbiological workup of pneumonia is the large number of organisms (which may or may not be pathogenic) inhabiting the upper respiratory tract, which is often the site of sampling in children with pneumonia. For example, *S. pneumoniae*, the most common global cause of bacterial pneumonia in children, is also carried in the nasopharynx of the majority of children >5 years of age [4]. This lowers the specificity of PCR tests or other tests that are conducted on samples such as sputum or nasopharyngeal culture that originate from or pass through the upper respiratory tract.

Standard clinical and laboratory diagnostics will therefore fail to identify a causative pathogen in a significant proportion of cases of pneumonia. Postmortem examination of fatal pneumonia cases can increase the overall proportion of cases with a definitive diagnosis, and importantly, provide information that increases our understanding of the causes of fatal pneumonia. Autopsy data are usually difficult to obtain in research studies because of acceptability to relatives, approval by ethical review boards and logistics. The PERCH project offers a rare and valuable opportunity to obtain postmortem data to match comprehensive antemortem microbiological testing in the same patients. The investigators have therefore planned to undertake such a study, taking into account previously published data, the need to maximize recruitment in order to collect useful information, and the problems of autopsy studies in general. The aims of this study are summarized in Supplementary Table 1.

THE AIMS OF AUTOPSY STUDIES IN FATAL PNEUMONIA

Establishing a Diagnosis

The etiology of severe pneumonia in patients who arrive at the hospital *in extremis* and die shortly after arrival may remain unknown, as there may be no time to take appropriate samples. Where antemortem microbiology fails or is unavailable, an autopsy may allow a definitive diagnosis of the causative pathogen either through culture or histology. For example, retrospective autopsy studies have been used to identify both adenovirus and *Mycoplasma pneumoniae* as important pathogens causing pediatric fatal pneumonia in China [5, 6].

The most comprehensive postmortem study of the etiology of respiratory illness was conducted in 264 fatal pediatric cases in Zambia [7]. The most common causes of death were pyogenic bacterial pneumonia (44%), *Pneumocystis jirovecii* pneumonia (PCP, 22%), cytomegalovirus pneumonia (16%) and pulmonary tuberculosis (20%), and there was a high prevalence of dual infections (48% in HIV-positive cases and 33% in HIV-negative cases). These diagnoses were made for both HIV-positive and HIV-negative groups, but surprising findings included the high prevalence of PCP and pulmonary tuberculosis in HIV-negative patients, and the substantial proportion (18%) of HIV-negative patients with lymphocytic pneumonitis but no specific pathogen histologically. The authors concluded that autopsy represented the best way of obtaining accurate etiological data on the causes for pneumonia.

One potential problem with either histological or microbiological diagnosis is that finding an organism (especially in a mixed infection) does not mean it is the direct cause of disease or death. For instance, a major cause of death in influenza pandemics is secondary pyogenic bacterial pneumonia [8]. However, a dual approach, where the histological pattern of disease can be linked to the microbiological results, would strengthen the attribution of causality. No study has yet systematically compared the histological findings with antemortem or postmortem microbiology.

An etiological diagnosis of pneumonia can be made using histology, where pathogens are indicated by characteristic tissue changes such as granulomatous inflammation in tuberculosis or viral inclusion bodies; specific organisms can be identified using morphology and histochemical or immunohistochemical staining. In addition, autopsy can reveal lung conditions that clinically simulate acute infectious pneumonia, such as malaria, lymphocytic interstitial pneumonia (LIP), noninfectious pneumonia (due to aspiration) or acute heart failure. Infections may produce "final common pathway" appearances in the lung, such as consolidative pneumonia, interstitial inflammation, or diffuse alveolar damage, which are not specific to a particular pathogen. However, the diagnostic sensitivity of autopsy tissue can be increased by the use of special stains (such as Gram, Giemsa, Ziehl-Neelsen or silver stains), immunohistochemistry or molecular pathology techniques to identify pathogens.

The diagnosis of pulmonary tuberculosis is important, because it is treatable, contagious and may contribute to a fatal outcome, but it can also be difficult, especially in HIV-infected children [9–12]. Typically, tuberculosis is diagnosed antemortem on the basis of clinical and radiological findings, but these features are often shared with other opportunistic infections, reducing their specificity in diagnosis. The Zambia autopsy study demonstrated a high prevalence of pulmonary tuberculosis in both HIV-positive and HIV-negative patients [7]. However, in another study of 93 HIV-infected children in South Africa, Rennert et al confirmed the diagnosis of tuberculosis from lung tissue in only 4% of the patients [13]. Among patients who had been on empiric treatment for tuberculosis before death, some 18% had no postmortem evidence of active disease in the lung. This disparity may have been due to effective treatment or could indicate clinical misdiagnoses because other

pathogens, specifically cytomegalovirus and *P. jirovecii*, were found at autopsy. Histological evaluation of lung tissue will improve diagnosis and provide quality assurance of antemortem diagnosis, as well as establish the prevalence of tuberculosis in different populations.

Validation of Antemortem Microbiological Diagnosis

Data from autopsies can help to validate new and existing antemortem diagnostic methods. Some lung infections, particularly those caused by fungi or viruses, are difficult to diagnose even given optimal antemortem diagnostics due to a lack of specific clinical features or the fastidious nature of their growth in culture. A study of invasive pulmonary aspergillosis in Switzerland found that 38% of cases in non-neutropenic patients (n = 67) were recognized only at autopsy [14]. Not only does autopsy increase the sensitivity of a study to detect the underlying infection, but the increased specificity of postmortem examination also leads to a more accurate estimate of the contribution of major pathogens to pneumonia etiology.

Tissue samples also have the advantage of allowing ancillary diagnostic tests. A study on influenza A H1N1 deaths in the US showed that testing autopsy lung tissue using PCR and immunochemistry assays identified many bacterial lung infections missed by standard clinical methods [15]. Immunohistochemistry for specific common bacterial pathogens has been used on lung tissues to identify *S. pneumoniae* [16], *H. influenzae* or *Staphylococcus aureus*, and viruses such as respiratory syncytial virus and influenza [17]. In situ or extraction PCR techniques from frozen or formalin fixed, paraffin-embedded tissues can also be used for ancillary molecular diagnostic tests that will identify viral and fungal pathogens [18, 19]. Molecular analyses of clinical samples using a variety of techniques are increasingly used in diagnostic practice [20]. Pathogen identification by 16s rRNA sequencing [21] or viral diagnostics using multiplex PCR on postmortem samples can also be used to establish a cause of death or validate antemortem diagnosis. However, such methods are beyond the scope of routine laboratories, and are not yet validated against standard microbiological tests. The PERCH project offers a unique opportunity for comparative antemortem and postmortem diagnostic correlation in a clinically well-characterized and geographically diverse patient group.

Forming a Bioresource for Future Research

Samples from an autopsy study form a valuable potential resource for further investigation of both the host and pathogen, which may influence disease susceptibility or outcome in individual cases. Using host mRNA transcripts extracted from lung tissue for microarray analysis or sequencing allows investigation of host responses and pathogenic mechanisms within the lung, as has been validated for transcriptomic studies of lung tumors and interstitial lung disease [22–24]. High throughput genomic, proteomic, microRNA or siRNA screening technologies have been proposed as a way of using systems biology to increase our understanding of the interaction between pathogens and the host response [25, 26].

Postmortem culture isolates may contain a mixed bacterial flora, some of which may be difficult to grow using standard culture methods or may be overgrown by other bacteria. 16S rRNA pathogen sequencing may allow the detection of more fastidious organisms, and in combination with control samples from the unaffected lung, allow differentiation from background postmortem contaminants [21]. The opportunity to biobank pathogens from postmortem cultures, in addition to antemortem isolates, should aid the rapid sequencing of entire genomes from multiple strains of a pathogen. This will aid vaccine design and the study of transmission, antibiotic resistance and virulence factors in individual pathogens, such as the development of multidrug resistance in *S. pneumoniae*, or immune escape following the introduction of the pneumococcal vaccine [27].

Novel Pathogen Discovery

Another potential use of postmortem samples is the search for novel pathogens [28]. It is difficult to attribute causation in pathogen discovery projects that do not have supporting histopathology studies. New pathogenic agents from autopsy samples can be thoroughly described and correlated clinicopathologically, as was done with hantavirus infection or influenza A H1N1 [29–31], and the likelihood of their role in the etiology and pathogenesis of pneumonia can be determined. The PERCH project aims to collect postmortem tissue biopsies from clinically well-defined, fatal pneumonia cases in a number of different centers and link them to high quality clinical, radiological and antemortem diagnostic information, which will represent a unique and valuable resource for future research into pneumonia etiology.

DRAWBACKS TO AUTOPSY STUDIES

There are several potential problems with autopsy studies. First is the inherent bias in autopsy data due to the selection of fatal cases. Surviving cases may have a different, less pathogenic etiology. Second, patterns of consent ensure that pathology is only studied in a proportion of fatal cases, which may introduce bias if a particular pathogen is common in a group where autopsy was unacceptable for religious or cultural reasons. Additional problems include microbiological contamination of postmortem tissue and lack of detection sensitivity due to the fastidiousness of a pathogen or choice of diagnostic test.

ETHICAL ISSUES IN AUTOPSY TRIALS

Giving consent for an autopsy is difficult for a grieving relative. For some families, the desire to know the cause of death or the altruistic recognition of the value of studies for future patients may outweigh any objections to autopsy. Factors adversely influencing consent include any reluctance of the staff to ask parents for consent, religious or cultural objections, concerns about mutilation of the body, interference with burial arrangements, the perception that the procedure is of no benefit to the patient or a lack of interest in the study rationale [32, 33]. Initial and careful consultation with key stakeholders in the potential research setting should help identify the range and depth of sensitivities, and if and how to proceed. This will depend on the study planned; for instance, the process of gaining consent for a lung aspirate during life, which is less invasive and may provide diagnostic information of value in treating that individual, would be very different as compared to asking for a postmortem sample, and would require a different ethical approval and consent process.

It is preferable to obtain the relatives' consent only after the death of a patient, as preemptive consent can give rise to perceived issues of conflict of interest. However, this timeline introduces the risk of consent refusal at a time of great emotional stress. Obtaining consent requires sensitivity and training, and should be done by local clinicians or nurses who are familiar with the aims of the study in order to reduce language difficulties and explain the study rationale clearly. Ideally, they would be trained counselors who would be able to adequately deal with their own emotions and those of the parents, as well as answer all questions and establish a personal link for parents so that they could come back and talk in the future if they wish. This would involve prestudy training that utilizes protocols, enacts scenarios, and involves mentoring by senior staff, with the goal of gaining experience in asking for consent in a research study setting, to avoid coercion and exacerbating distress. The results of an individual autopsy should be made available to parents, and the results of the study should be disseminated to health professionals and the local community, in order to validate the reasons for doing it and to demonstrate how this information may change the management of future patients with pneumonia. In general, close engagement with the local community is key for the consenting process, because the centers that are capable of performing autopsy studies are often involved in multiple projects, and the introduction of a new, unfamiliar autopsy-based project could threaten participation in other studies.

METHODS FOR AUTOPSY EXAMINATION OF PNEUMONIA ETIOLOGY

Given the constraints of open autopsy examination, less invasive methods of postmortem examination of the lung would be extremely helpful, and would enable a trained clinical staff member who is not an expert pathologist to perform the biopsies. We considered two such approaches: postmortem lung aspirates and percutaneous needle lung biopsy. These offer a simpler and less invasive way to obtain lung samples for microbiology and histology.

There are several potential problems with the use of biopsy tissues derived from autopsy studies, including sampling error, sample preservation and contamination. Obtaining tissue directly from the diseased area is easier in open autopsy. Percutaneous sampling methods risk missing areas of pathology, so it is important to correlate antemortem clinical examinations and radiographs when obtaining a tissue sample of the affected area. Several pathogens, such as *S. pneumoniae*, are highly temperature sensitive and are destroyed by refrigeration (sometimes used to preserve bodies before autopsy), whereas desiccation of a specimen prior to microbiological testing can destroy viral pathogens. Close cooperation is required between the pathologist and microbiologist to process post mortem microbiological samples as rapidly as possible. This presents problems if consent for sampling is delayed and if laboratories are distant from the site of the biopsy.

A criticism commonly leveled at postmortem microbiological testing is the issue of contamination. In the immediate perimortem period, widespread cellular degradation and breakdown in physiological barriers begins (such as in the large bowel), which is associated with the translocation of a mixed flora of intestinal bacteria into the bloodstream and contamination of tissues. Resuscitation attempts may exacerbate contamination from the upper respiratory tract. Sampling lung tissue quickly after death would reduce the risk of postmortem bacterial invasion, but would not prevent agonal changes. Contaminating organisms can multiply during the interval between death and sampling, obscuring a true pathogen.

Postmortem microbiological studies of children who died from Sudden Unexplained Death in Infancy have shown that the translocation of bacteria across mucosal membranes does not lead to an increased probability of a positive postmortem bacterial culture, nor of polymicrobial cultures. Indeed, positivity rates decrease with a postmortem interval of 2 days, probably due to death of the organisms [34]. However, a proportion of postmortem microbiological diagnoses will be uninterpretable due to contamination, and success requires sterile technique [35] and choosing the appropriate culture method (such as with fungal pathogens or *M. tuberculosis*) that may not be a first line microbiological test. The ability of an autopsy examination to distinguish between a positive microbiological result representing contamination or a truly causative organism will increase if a result can be compared with antemortem samples, or concurrent histology.

Needle Lung Aspirates

Much of the data on pneumonia etiology upon which our current understanding of the field is based are derived from clinical studies utilizing lung aspirates on living patients. Previous studies have proven the increased sensitivity of needle lung aspirates in living patients [36], and have described their use for postmortem microbiological testing [37]. A needle lung aspirate would offer the simplest way of gaining access to local diseased tissue, without scarring or the need for specialist training or facilities. However, this approach would not provide tissue samples that could be histologically analyzed.

Percutaneous Needle Biopsy

This offers several potential advantages to open autopsy. It allows tissue collection for microbiological and histopathological diagnosis with ancillary approaches as described above. Taking a biopsy confined to the lung may also allay some concerns regarding consent, as it is rapid, will not delay burial arrangements, is minimally invasive and leaves little mark on the body. The principal disadvantage of this approach is sampling error. Studies of needle biopsy as an alternative to open autopsy have reported variable success, and depending on the organ sampled, the success rate can be as low as 50% of the total biopsies. Sampling error can lead to disparities between the resultant tissue diagnoses compared with an open autopsy [38–40]. If multiple biopsy sites are used, then the sampling of other tissues such as the spleen or body fluids such as CSF or urine can be performed, which may increase the value of a microbiological diagnosis on the lung biopsy tissue, without requiring open autopsy.

Open Autopsy

Examples of successful postmortem studies from countries such as Zambia [7] and the Ivory Coast [41] confirm the practical feasibility of doing open autopsy studies on pneumonia. However, their principal disadvantage is low consent rates; for example, only 25% of families approached in Lusaka consented to open autopsy following the death of their child [7]. They also depend on finding experienced pathologists at the study sites who can examine autopsy material adequately, with appropriate laboratory, microbiological and clinical support.

FORMULATING THE DESIGN OF A POSTMORTEM PERCUTANEOUS NEEDLE BIOPSY STUDY

The inclusion of an autopsy study within PERCH highlighted the dilemma of antemortem studies that are inherently limited and full autopsy studies that are impracticable in many of the study sites. We therefore propose that a postmortem percutaneous needle biopsy study should be included in the PERCH project. A biopsy approach offers greater practical utility in study centers where resources are limited and consent rates make it unrealistic to perform an open autopsy study. Some of the potential advantages and disadvantages of this plan are shown in Supplementary Table 2.

To offset the issues of sampling error inherent in a biopsy approach, we will increase the anatomical representativeness by taking multiple biopsies from both lungs through the same entry site on each side. If antemortem clinical or radiological findings indicate lobar pneumonia, then we will biopsy the diseased area preferentially, but will collect control, uninfected tissue from the contralateral lung as a baseline to interpret polymicrobial contaminating growth in subsequent cultures. However, in diffuse or multifocal disease, we will take biopsies from all lobes to minimize sampling error. If no antemortem localization of disease is available, then the case will be sampled with the protocol outlined in Supplementary Figure 1.

We plan to use the resulting biopsies for histological and microbiological testing and preserve other biopsies in specific fixatives to allow further ancillary diagnostic tests (Supplementary Figure 2), including mRNA or DNA extraction for molecular pathology studies and pathogen 16s rRNA typing. Frozen tissue will also be preserved, as this is superior to formalin-fixed tissues for subsequent immunohistochemistry and molecular pathology [18].

CONCLUSIONS

The diagnosis of the cause of fatal pneumonia is important in identifying new pathogens and guiding treatment and prevention strategies in the postvaccine era. The inclusion of postmortem examinations can enhance the fulfillment of these objectives in studies of pneumonia etiology. Antemortem testing of blood and upper respiratory tract samples can be difficult and lacks sensitivity and specificity. Examination of diseased lung tissue postmortem offers the chance to establish a diagnosis if it is unknown, or confirm an uncertain antemortem diagnosis. Histological examination can identify specific pathogens, can corroborate a microbiological diagnosis, and may often reveal unexpected infective etiologies. It is likely that new pathogens will be discovered from the pathology tissue, leading to research into host responses that will justify the difficulties involved in performing autopsies in challenging environments. Comparative studies are needed to measure the effectiveness of less invasive procedures such as needle aspiration and biopsy with the "gold standard" of open autopsy. A percutaneous needle lung biopsy postmortem study based within the PERCH project has been designed to improve the diagnosis of etiology in severe pneumonia and provide evidence to influence treatment and prevention programs in the future.

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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