

Biomarkers for Infectious Disease Diagnostics
in the Developing World:
Diagnosis of Bacterial and Severe Acute Lower Respiratory
Tract Infection

Laura Penny, Paul Neuwald, and Mickey Urdea

June 2006

Halteres Associates, LLC
5858 Horton Street, Suite 550
Emeryville, CA 94608
510-420-6733
www.halteresassociates.com

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Acute Lower Respiratory Tract Infection

1. Introduction to Diagnostic Needs for Acute Lower Respiratory Tract Infection in Resource-Limited Settings

Respiratory tract infections occur in both the upper respiratory tract (nose, sinus, pharynx, and larynx) or in the lower respiratory tract (below the vocal chords (larynx)). Although upper respiratory tract infections (URI) are the most common illnesses in early childhood, it is the lower respiratory tract diseases that account for the majority of severe or chronic cases.

The major acute lower respiratory diseases include pneumonia, measles, pertussis, and bronchiolitis, while the major chronic diseases include pulmonary tuberculosis (PTB) and noninfectious diseases including asthma and bronchiectasis. Pneumonia is a condition in which the alveoli of the lungs become inflamed and filled with fluid. Pneumonia is caused primarily by bacterial and viral acute lower respiratory infections (ALRI), though fungal or parasitic infections, as well as environmental insults and medical conditions, cause a minor percentage of cases as well. The HIV epidemic continues to change the epidemiology of respiratory illnesses, and HIV-associated acute and chronic respiratory diseases have emerged as a major factor in parts of the developing world.¹

ALRIs, and their associated pneumonia, remain a major cause of childhood morbidity and mortality, particularly in the developing world. Unfortunately, the differences in the definitions of pneumonia that are used by different groups, and the challenges in diagnosing pneumonia in areas with underdeveloped health care systems, make it very hard to accurately estimate the disease burden in many parts of the world.² Recent estimates, from 2000 to 2003, from the World Health Organization (WHO) regarding the striking proportion of all childhood deaths that are attributed to pneumonia were 21% in the WHO Africa region, 21% in the WHO Eastern Mediterranean region, and 19% in the WHO South East Asia Region.³ These region-wide estimates do not reveal the smaller localized pockets with much higher incidence, such as 35.8% in Tanzania.⁴ Despite all the methodological challenges involved with making these estimates, the existing evidence indicates that pneumonia is one of the leading causes of death for children in all countries where there is still significant childhood mortality.^{2,4,5}

Even in areas of the world with well-developed health care systems, ALRI is often treated without determining its etiology. In the resource-limited settings that serve much of the world's population, there is no access to useful diagnostic tools that could guide the selection the most appropriate course of therapy for ALRIs, and this has a number of serious negative consequences. In most cases, children who are examined in a health facility and have symptoms indicative of an acute respiratory infection are offered antimicrobials, without any investigation into the etiology of the infection.⁶ Upper respiratory tract infections do not generally require treatment with antibiotics, but because they are not always distinguishable from ALRI, inappropriate use of antibiotics occurs frequently. In addition, because there is no good way to distinguish viral ALRI from bacterial ALRI, the viral cases end up being treated with antibiotics as well. Not only are these cases a waste of precious health care resources, whether they are publicly funded or paid out of pocket by the families, but they also increase the global health burden of drug resistant bacterial infections, which has reached high levels in some areas, and continues to grow.^{7,8,9}

Some studies suggest the ratio between URI and ALRI is as high as 6 to 1, and therefore the over-treatment that results from use of antibiotics in URIs could be extremely large.¹⁰ Other diseases that can have a similar clinical presentation are tuberculosis and malaria, which are not often successfully distinguished from typical URI and ALRI. Yet another consequence of the lack of tools to determine the etiology of ALRI in resource-limited settings is that it is extremely difficult to develop intervention programs (such as vaccination programs), and to determine their cost-effectiveness, because there is so little data on the incidence of particular infective agents.

In resource-limited settings, the first decisions that must be made in the management of a respiratory tract infection are 1) whether to treat with antibiotics, and 2) whether the infection is severe enough to attempt to transport the patient to a hospital or clinic where supportive treatment such as oxygen, fluids, and intravenous antibiotics could be administered. These decisions are often made by a local health care worker, who may have very limited training, in consultation with the family of the patient.

2. Distinguishing Bacterial and Fungal from Viral ALRI in Resource-Limited Settings: Status of Currently Available Biomarkers

Data regarding the contributions of specific etiologic pathogens to ALRI in the resource-limited areas of the world is very incomplete and difficult to obtain. It seems reasonable to assume that the pathogens that are identified, and their incidence in different studies, will vary for biological reasons (such as microbiological floral differences in regions of the world, the population being investigated, e.g., HIV- vs. HIV+ groups, or patients that receive treatment in an outlying area versus those that make it to a hospital, and based upon the season of the year^{11,12}) as well as for technical reasons (such as sample type collected,⁶ and detection method used,⁵ which are not likely to perform equally well for the detection of all infectious agents, or in all laboratories¹²). Another source of incompleteness in the data is the fact that bacterial culture, the main method used for the identification of bacteria in regional hospitals, is well-known to be a very insensitive way to detect bacteria, with published studies reporting that bacteria were successfully cultured from ~ 50% to as low as 0% of the samples studied.^{5,13,14}

The percent of pneumonia caused by particular infective agents in the developing world is not well defined. There are relatively few published studies that have looked for a wide range of both bacteria and viruses. In many of these studies, an infective agent was not identified in 50% or more of the individuals for whom there was a high suspicion of ALRI based on clinical and/or radiographic findings.^{12,15,16} Some reports have indicated that the most important bacterial causes of pneumonia in developing countries are *Streptococcus pneumoniae* and *Haemophilus influenzae* (mainly type B or Hib),^{17,6,18} and that respiratory syncytial virus (RSV) is the most important viral cause.¹⁹ However, other studies have indicated that a wide range of other infectious agents play significant roles as well, including the bacteria *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli*, *Chlamydia*, *Mycoplasma*, *Klebsiella*,^{6,11,20,5,15,12} and the viruses influenza A and B, parainfluenza viruses, measles, and adenoviruses.²¹ Studies that looked for both bacterial and viral strains often reported that ~ 5 to 20% of individuals were multiply infected, either with more than one bacterial pathogen, or both a bacterial and a viral pathogen.

Pneumocystis carinii is an opportunistic fungal pathogen that causes pneumonia in immuno-compromised individuals. In areas of the developing world where HIV infection is high, *Pneumocystis carinii*

pneumonia (PCP) has been reported at rates of 10 to 50% in HIV+ children.^{22,23} PCP is treated with antimicrobials, and therefore it is important to identify this pathogen, along with bacterial species, in the selection of appropriate therapy for ALRI.

Because of the technical challenges in identifying the causative agent(s), and the great differences in the incidences of particular infective agents from around the world, incidence data is still very incomplete. What is clear is that any biomarker(s) that is(are) used to identify bacterial and fungal pneumonia will need to detect the presence of at least half a dozen pathogen species.

Traditionally, two broad classes of fluid specimen types have been used in pneumonia diagnosis. The first class is respiratory tract specimens, including sputum, bronchoalveolar lavage (BAL), trans-thoracic needle aspirate (TNA), nasopharyngeal aspirates or swabs, and saliva. The second class includes fluid specimen types that are not respiratory specific, such as blood and its fractions, and urine. In this second class of specimen types, it is generally assumed that the biomarkers that are being detected are those that are present in that specimen type due to the normal pathways the body uses to dispose of pathogens and their degradation products, or they have “leaked” into the specimen types. It is known that bacteria and their degradation products do enter the blood stream from the lung. For instance, there is evidence which suggests that pneumolysin, a multifunction cytotoxin produced by *Streptococcus pneumoniae*, plays a role in the penetration of bacteria from alveoli into the interstitium of the lung, and the dissemination of pneumococci into the bloodstream.²⁴ Other evidence indicates that *Chlamydia pneumoniae* infects cells of the immune system, such as granulocytes and alveolar macrophages, which then transmigrate through the mucosal barrier in the lung and give the bacteria access to the systemic circulation.²⁵ Therefore there are a variety of ways that bacteria enter the bloodstream. The mechanisms that each species uses to enter the bloodstream, and their viability in the bloodstream or other extra-pulmonary sites, are all likely to affect the quantity of each bacterial species, and their degradation products, that are found in the different fractions of blood. In addition, it has been well documented that bacterial degradation products, particularly the stable capsular polysaccharides, can be detected in the urine of many patients with bacterial infections, presumably as a normal part of the process of elimination of these molecules.^{26,27,13} Therefore, biomarkers for bacterial pneumonia are known to exist in both respiratory and non-respiratory specimen types.

In this section, we will consider the currently available biomarkers and methods used to distinguish bacterial and fungal ALRI from viral ALRI. There are several important issues that are relevant to most or all of the currently available approaches. The evaluation of all these methods is hindered by the lack of a useful diagnostic gold standard for identifying the etiology of ALRI. Another major challenge for many of the approaches is the fact that certain specimen types that might be used to diagnose ALRI can be contaminated by biomarkers that originate from the upper respiratory tract, or otitis, which is an issue that is discussed in detail in the following sections.

Because the main clinical decision to be informed – whether to treat with antibiotics or not – is determined by the presence of bacterial or fungal infection, regardless of whether or not there is a viral infection, this manuscript will focus only on the issues related to the detection of bacteria and fungal causes of ALRI, and not on methods to detect the presence of viruses. If in the future there are treatments

available for viral pneumonia, then a new version of a diagnostic would need to be developed that could detect the presence of viral species that play an important role in the developing world.

2A. Clinical Findings and Radiographic Biomarkers

Though the WHO Integrated Management of Childhood Illness (IMCI) guidelines for using clinical signs to identify ALRI are considered to perform reasonably well, the clinical findings that occur in bacterial, fungal, and viral ALRIs are not sufficiently different to be useful in distinguishing the etiology of an infection. There are some reports in the literature which indicate that radiographic findings can provide some information regarding the etiology of pneumonia. These reports suggest that there is a fairly strong association between dense, homogenous infiltrates or pleural fluid and a bacterial etiology, and an association between central and peri-bronchial infiltrates and viral etiology.^{6,28} However, other reports indicate that radiographic findings are not sufficiently sensitive or specific,^{29,30} and the utility of this method is hampered by the significant variation in interpretation between radiologists, and the biological variation in the infections. Aside from these issues, radiographic facilities are not generally available in the outlying areas of developing countries, and the cost of X-ray film and consumables prevents the use of routine radiography in the diagnosis of childhood pneumonia in resource-limited settings.

2B. Visualization of Bacteria as Biomarkers of Infection

Bacteria can be detected by either microscopic examination of respiratory specimens, or by first culturing any organisms in the specimen. Bacterial species can then be identified using various stains, morphological features, or by the visualization of colonies in culture based methods.

Culture is the most well-established way of distinguishing bacterial from viral pneumonia in developing countries. A variety of sample types can be used to culture bacteria. The culture of bacteria from the blood of patients suspected of having pneumonia, particularly children, is often attempted at sites that have the facilities. Though the diagnostic value of a positive blood culture is high, the sensitivity is very low, ranging from 0 – 50%, even for resource-rich sites.^{31,32} Culture from sputum or trans thoracic needle aspirate (TNA) samples is superior in some studies, but since sputum cannot be reliably obtained from children, cultures from sputum lack specificity,³³ and the resources required for TNA cannot be reasonably expected to exist in low-resource settings.⁶ Even in well resourced laboratories, cultures from “gold standard” methods such as TNA or bronchoalveolar lavage are able to identify the etiology of pneumonia in typically only 50 – 60% of cases.^{34,35} Cultures from nasopharyngeal swab and throat specimens generally correlate poorly with the microbial flora of the lung, because certain bacterial species, such as *Streptococcus pneumoniae*, are part of the normal microflora of the nose and throat.^{36,37,38} While the presence of a few types of bacteria, such as *Mycoplasma pneumoniae*, in the upper respiratory tract does correlate well with disease in the lower respiratory tract, this is not true for other species such as *S. pneumoniae*, because a significant proportion of healthy children are nasopharyngeal “carriers” of the bacteria.¹² Regardless of the advantages or disadvantages, the resources required for culture-based approaches do not exist in resource-limited settings, and the turnaround time, ranging from days to weeks depending on the organism, is not practical for these settings.

2C. Pathogen-Specific Biomarkers Detected Using Immunodiagnostic Methods

Pathogen-specific biomarkers that could be used to distinguish bacterial and fungal pneumonia from viral pneumonia include antigens produced by the pathogens, and antibodies produced by the patient in response to the infection. Antigens offer advantages as biomarkers because their detection is often not dependent on the viability of the organism (like culture-based methods), and therefore they can still be detected for some time after the patient has begun taking antibiotics. Some antigens are quite stable chemically and can be detected upon elimination from the body, such as in the urine. This can become a disadvantage, though, for antigens that persist for weeks in the urine after an active infection has cleared.

It appears that the researchers developing diagnostic tests have, in general, done a reasonable job of ensuring that the binders they select as components for their tests have a useful degree of specificity for binding only to the antigens of the species that they aim to detect, and not to antigens of closely related but non-pathogenic species. This ability to discriminate between related bacteria will be called “species specificity” in this document, which is different from what will be called the “diagnostic specificity,” in the context of this document. The diagnostic specificity is the ability of the test to correctly identify individuals who do not have ALRI. The diagnostic specificity of many molecular tests for the etiology of pneumonia is affected by nasopharyngeal colonization, and remains one of the major challenges for determining the etiology of ALRI. At least two important pathogens, *Streptococcus pneumoniae* and *Haemophilus influenzae*, that cause pneumonia can also benignly colonize the upper respiratory tract (sometimes called nasopharyngeal “carriage”). It has been shown nasopharyngeal carriage of these pathogens can result in a high degree of false positive results, because bacterial antigens from the colonizing bacteria are found in upper respiratory tract specimens, the blood, and/or in the urine. This ultimately leads to a low diagnostic specificity, at least with respect to the organisms for which there is a high level of colonization in the population. Therefore, the general downfall of bacterial antigens as diagnostic biomarkers for ALRI is that they do a relatively poor job of distinguishing colonization of the upper respiratory tract from ALRI.

The detection of antibodies produced by the patient in response to infection is another approach that has been taken in number of currently available tests. This approach has a number of disadvantages. First, it often takes time to develop sufficient levels of antibodies to be detected by these methods. Because a significant number of children who contract an ALRI die within 72 hours, a rapid test is required. Antibodies as biomarkers often provide the greatest utility, in terms of sensitivity, in the convalescence phase of the disease, rather than the acute phase (at least that is the case in resource-rich sites). Second, many children in resource-limited settings contract several respiratory infections per year, and the presence of antibodies to an organism may indicate a past infection, as well as current exposure without ALRI, or past vaccination, rather than a current ALRI. Therefore a diagnosis is considered confirmed when using antibodies as biomarkers only when the antibody titer rises over time. The requirements for paired serum samples, taken over several days, and a quantitative test, make this approach impractical for resource-limited settings.

The immunologically-detected biomarkers, methods, and sample types that can be used to identify bacterial and fungal pneumonia are summarized in Table 1, with emphasis on the performance characteristics that have been obtained in pediatric populations (where available).

Table 1. Biomarkers, Methods, and Samples Used in Immunodiagnostic Methods

Infective agent	Biomarker	Sample Type	Method or Format	Examples of Manufacturers	Sensitivity & Specificity	TAT	Resource Level
<i>S. pneumoniae</i> , <i>H. influenzae</i> , <i>M. pneumoniae</i> , <i>others</i>	Many bacterial antigens	Respiratory specimens, often sputum, & cultures	Fluorescence microscopy + FITC labeled antibodies	DakoCytomation	Low sensitivity, high specificity	Hours	High
<i>S. pneumoniae</i> , <i>H. influenzae</i> , <i>M. pneumoniae</i> , <i>others</i>	Many bacterial antigens	Respiratory specimens, serum, urine, other fluids	Counter-immunoelectrophoresis (CIE)	DakoCytomation	Moderate to low sensitivity and specificity – depends on many factors ^{39,40}	Hours	High
<i>S. pneumoniae</i>	Many biomarkers, for both host antibodies, bacterial antigens, & immune complexes	Blood, serum (may require samples taken over time), urine	ELISA	Many labs assemble ELISAs from components purchased from various suppliers	May perform well in adults, ⁴¹ but low sensitivity, low specificity reported for some species in children ^{6,42,43,44,45}	Hours	High
<i>H. influenzae</i>	Polyribose phosphate (PRP)	Serum, urine, others	ELISA	Assembled by researchers from components	High sensitivity and specificity in children (small numbers) ⁴⁰	Hours	High
<i>S. pneumoniae</i> <i>H. influenzae</i>	83 capsular polysaccharides, Pneumococcal capsular polysaccharide antigens (PCPA), C-polysaccharide, or pneumolysin	Concentrated urine, urine, serum	Latex agglutination	Slidex Meningite Kit 5 (bioMérieux) Slidex Meningite Strepto B kit	Conflicting data. For Streptococcus, some reports of moderate sensitivity (70 – 77%) and specificity (90%) in children compared to “reference diagnosis” and using concentrated urine. ^{13,12} For Haemophilus: 80-92% sensitivity in children ⁴⁰	Minutes or days (if specimen processing is used)	Medium-low. Higher if urine is processed

Infective agent	Biomarker	Sample Type	Method or Format	Examples of Manufacturers	Sensitivity & Specificity	TAT	Resource Level
<i>S. pneumoniae</i>	Various antigens, some specific to serotype groups	Respiratory samples	Latex agglutination	Slidex Kit, bioMérieux	Conflicting data: Low sensitivity (30%) & high specificity (80%) in UR specimens from children, ^{45, 46} other studies report high sensitivity & low specificity in UR specimens ^{38,44,6}	Min - Hours	Medium-low
<i>H. influenzae</i>	Polyribosylribitol phosphate (PRP)	Serum, urine	Latex agglutination	Bactigen, Wampole Laboratories	Moderate - high sensitivity and specificity reported in children, but small numbers ^{39,40}	Min - Hours	Moderate
<i>M. pneumoniae</i>	Host antibodies to <i>M. pneumoniae</i> . (PlateLIA antigen production enriches for P1 adhesin & associated proteins)	Paired serum samples (acute-phase and convalescent-phase)	ELISA	PlateLIA <i>M. Pneumoniae</i> IgM/IgG enzyme immunoassay, Sanofi Pasteur (at least 12 on the market)	Sensitivity ranging from 35 – 77%, specificity ranging from 49 – 100% in adults, ⁴⁷ compared to PCR-based detection. IgM test had a sensitivity of 79% & specificity of 98% in acute-phase serum from children in Finland with radiographically defined pneumonia who were positive for <i>M. pneumoniae</i> by at least 2 of 5 methods used for detection ⁴⁸	Hours	High
<i>S. pneumoniae</i>	C-polysaccharide	Urine or concentrated urine	Lateral flow device	Binax Now (Binax, Portland, ME)	Sensitivity can be high in adults (80 - 100%) especially with concentrated urine. Specificity is generally very low in children, ^{49,50,51,52} but inconsistent results ⁵³	Min - Hours if urine is concentrated	Moderate (no sample processing); Higher with sample processing
<i>Pneumocystis carinii</i>	82 kDa component, surface glycoprotein gpA	Respiratory fluid specimens	Fluorescence microscopy + FITC labeled antibodies	Antibodies provided by various sources (e.g. DAKO)	High sensitivity and specificity (but very small numbers) ^{54,55}	Hours	High

The intervention point under consideration – whether to treat an ALRI with antibiotics or not – should be informed by information regarding whether any bacteria or fungi are causing the ALRI, and the presence or absence of viruses does not assist in making this decision. Therefore, a diagnostic test to inform this decision using immunologically-detected biomarkers should be designed to detect one or more antigens from each of the important pathogens. The antigen biomarkers and their binders would need to provide a high sensitivity for all the important bacterial and fungal pathogens, as well as a sufficient degree of binding selectivity (species specificity) such that they do not detect related but non-pathogenic species. Ideally, one practical specimen type could be used to detect all of the bacterial species that cause pneumonia, and the test could distinguish species by the treatment that is required (i.e., the test output identifies “treatment groups,” rather than identifying species individually). However, the latter might be a capability that is added in a second generation product.

Respiratory fluid specimens are unlikely to be practical for such a test for two reasons. First, lower respiratory track specimens are impractical to obtain in resource-limited settings, especially from children. In addition, because two of the main bacterial species that cause pneumonia, *Streptococcus pneumoniae* and *Haemophilus influenzae*, often colonize in the upper respiratory tract of individuals without pneumonia,^{56,57} the more easily-obtainable upper respiratory tract specimens (e.g., swabs, nasopharyngeal aspirates, saliva) are likely to contain colonizing bacteria, and therefore contribute to an unacceptably low diagnostic specificity of a test. Therefore, the issue becomes whether these biomarkers can perform sufficiently well using the non-respiratory sample types that are more practical, such as blood from a finger prick specimen, or urine.

For some infectious diseases, IgM antibodies to pathogen-specific antibodies are biomarkers that provide useful diagnostic performance. While the presence of IgM antibodies against certain species, such as *M. pneumoniae*, has shown promising performance in children in some studies,^{48,58} pathogen-specific IgM antibodies are unlikely to be a useful marker for other species, such as *Streptococcus pneumoniae*, because of the relatively large fractions of healthy children, and children without pneumonia, with significant levels of anti-pneumococcal IgM antibodies. For instance, Brussow et al. (1992) report that, in a group of 1,301 Ecuadorian children, 6% of infants (< 6 months old), 28% of children 6 to 11 months old, 49% of children 12 to 17 months old, and 58% of children 18 to 23 months old had anti-*S. pneumoniae* IgM, as measured by an ELISA assay.⁵⁹ In addition, a smaller set of German children had a similar age-related prevalence of pneumococcus-specific serum IgM antibodies.

The limited evidence that is available suggests that host antibodies, as a biomarker class, are unlikely to ever provide sufficient sensitivity in resource-limited settings for several reasons, including low sensitivity that results from the low levels of antibody for some species in the acute phase of infection, as well as low specificity for some species resulting from the presence of antibodies due to past infections, chronic environmental exposure, or colonization (which results in the impractical requirement to test two samples over a period of several days). Therefore, further efforts to improve tests for host antibody biomarkers are unlikely to result in a practical test that provides adequate performance.

Though some pathogen antigen biomarkers have shown adequate performance in respiratory specimens from adults in the developed world, the performance of this class of biomarkers is much less certain when

using blood or urine specimens from children, particularly children in the developing world.⁶ Though a few studies report promising sensitivities and specificities for antigen biomarkers of pneumonia caused by certain species of bacteria,¹³ there is relatively little data on the performance of biomarkers in this class from studies which have examined blood or urine specimens from a large number of children who are served by a health outpost-type of setting. For instance, the few studies on the detection of *Haemophilus influenzae* antigens in urine or blood had very small numbers of pediatric patients with pneumonia and the potential influence of colonization on test performance has not been sufficiently evaluated. The study by Marcon et al. (1984) includes only 2 pediatric patients with pneumonia.³⁹ The study by Wang et al. (2002) enrolled 154 children with probable pneumonia, but the study does not delineate which samples (blood and nasopharyngeal aspirate) were positive for particular organisms by the detection methods employed (culture vs. detection of host antibodies vs. detection of pathogen antigens).¹⁵ In addition, this study did not include a negative control arm of pediatric patients without pneumonia, and it is unclear if nasopharyngeal aspirates were even tested for the bacterial pathogens, which are two additional lines of experimentation that might have allowed the role of colonization to be evaluated.

The studies that are listed in Table 1 were performed, in general, on samples from patients whose pneumonia is well-characterized by clinical, radiological, and other laboratory methods that would not be available “in the field.” The performance could be much worse in populations from developing countries, where there may be a larger number of instances where the diagnosis of pneumonia is less certain, or where there are much higher rates of colonization, and possibly chronic exposure to many pathogens in the environment.^{60,6,57} It is probably fair to say that it is still unknown whether the pathogen-antigen biomarkers that are in use today for the important bacterial pathogens COULD provide adequate sensitivity in specimen types that are practical for resource-limited settings (e.g. blood and possibly urine). The challenges posed by the lack of a gold standard method for determining the etiology of pneumonia make it extremely difficult to evaluate new methods.

Perhaps the exception to this, where a reasonable body of data already exists, is the antigen biomarkers for *Streptococcus pneumoniae*, for which there are a number of studies on blood or urine specimens from children. The available evidence suggests that *Streptococcus pneumoniae* antigens are unlikely to ever provide sufficient diagnostic specificity in young children because *S. pneumoniae* is part of normal flora of nasopharynx. The specificity of these tests is greatly reduced when using any sample that must pass through the upper respiratory tract (e.g. sputum), or a blood- or urine-derived specimen, which contain bacterial antigens regardless of the site of infection.^{52,51,53,49} Dominguez et al. reported that the BINAX assay (for *S. pneumoniae*) detects higher levels of pneumococcal antigen in the urine of younger children (two years old) who are nasopharyngeal carriers of *S. pneumoniae* than in older children.⁴⁹ Though the reasons for this are unclear, they postulate that this could be explained by differences in the immunological status of children in these age groups, such as their level of IgA. In children under two, the concentration of IgA is very low or absent.⁶¹ The production of secretory IgA begins and escalates in older children, and it is this secretory IgA that intercepts antigens at the mucosal surface level, which results in their luminal degradation and clearance, rather than elimination through the urine.⁴⁹ It may be these biological differences in young children that limit the specificity of antigen-based diagnostic tests that use non-specific fluids (blood, urine) in this age group. In any case, the sample types that might reduce this

diagnostic specificity problem, such as transthoracic needle aspirates, are not practical for resource-limited settings. Few published studies have tried to detect antigens from non-colonizing species, such as *M. pneumoniae*, *C. pneumoniae*, and *P. carinii*, in a specimen type that is practical for resource-limited settings, such as blood or urine. These species are more typically detected using upper respiratory specimen types. It remains to be determined if antigens from these species could also be detected in more practical specimen types for resource-limited settings (e.g. blood from a finger stick or urine).

In summary, when considering the goal of developing a single diagnostic test that could detect pathogen antigens for all the important pneumonia-causing bacterial and fungal species in one specimen, the available data indicates that pathogen antigen biomarkers have not provided adequate performance in the practical specimen types from children in resource-limited settings.

2D. General (Non-Specific) Host Response Biomarkers

A number of proteins found in the blood, which are elevated in response to inflammation and/or infection, have been investigated as possible biomarkers of bacterial ALRI. van der Meer et al. report in their recent meta analysis that C-reactive protein (CRP) levels were neither sufficiently sensitive to rule out, nor sufficiently specific to rule in, a bacterial etiology of a lower respiratory tract infection, and that the use of CRP levels to guide antibiotic prescription in ALRI is not consistently supported by the present evidence.^{62,63} Sensitivities ranging from 8% to 99%, and specificities ranging from 27% to 95%, were reported, and not surprisingly these performance characteristics were inversely related within the individual studies. Other biomarkers, such as blood white cell count (WBC), IL-6, and erythrocyte sedimentation rate (ESR), have been examined for their ability to distinguish bacterial and viral etiologies. There is little evidence that these biomarkers, either alone or in combination, are sufficiently sensitive and specific to be used to distinguish bacterial from viral ALRI in children.^{64,65,66}

Serum procalcitonin (PCT) levels have been evaluated in several studies, and a meta analysis of its diagnostic performance (through 2002) is presented by Simon et al. 2004.⁶³ Procalcitonin is the prohormone of calcitonin, which is normally secreted by the C cells of the thyroid in response to hypercalcemia. In “normal” individuals, little or no PCT is detected. In inflammatory conditions, it is thought that PCT is produced by the liver and peripheral blood mononuclear cells, and may be modulated by lipopolysaccharides (found in bacterial capsules) and sepsis-related cytokines. PCT secretion begins within four hours after stimulation, peaks at eight hours, and is cleared after the infection controlled.⁶³ The commonly used test for PCT is an immunoluminometric assay, LUMitest PCT (BRAHMS Diagnostic, Berlin). It requires a resource rich laboratory setting, 20 µL of plasma, can be completed in 2 hours, costs about \$10, and the detection limit is 0.08 µg/L, with low inter- and intra-assay variability.⁶³ One publication specifically evaluated PCT levels as a way to distinguish bacterial from viral pneumonia in children, where they generated the following data.⁶⁵

Procalcitonin Cut-Off	Sensitivity	Specificity	Pos. Pred. Value	Neg. Pred. Value
> 0.5 µg/L	95%	60%	80.3%	88.4%
> 1.0 µg/L	86%	87.5%	90.2%	80%
> 2.0 µg/L	62.7%	96%	96.4%	60%

This study was conducted on a population of 88 children in France who were admitted to the hospital for severe community acquired febrile pneumonia. Attempts were made to identify the causative agent via blood culture, and serology testing for *M. pneumoniae*, *C. pneumoniae*, and the principal respiratory viruses. Sputum or pharyngeal aspirates were examined by microscopy, and bacteria were considered to be the causative agent if more than 25 polymorphonuclear cells and fewer than 10 squamous epithelial cells per mL were detected by microscopy, and if cultures contained a predominant microorganism with more than 10⁶ CFU/mL in the sample.

A number of points should be kept in mind when assessing the data from this study. First, an infective agent could not be identified in 16 patients, who were excluded from the calculations of test performance for obvious reasons, but it leaves the question open regarding how this would change the test performance under more realistic conditions. Second, only immunocompetent children without chronic diseases and who had not received antibiotics in the previous 10 days were enrolled. This type of population is unlikely to be encountered in resource-limited settings. Third, the study does not have an arm in which healthy children, children with nasopharyngeal colonization, or children with other infections, were evaluated. Therefore, though this initial data from a relatively small population in the developed world is interesting, it remains to be determined how such a test would perform in populations that are served by resource-limited settings.

2E. Nucleic Acid Biomarkers

Nucleic acid biomarkers have the potential advantages that they can be very sensitive, they can detect all pathogens regardless of their ability to be cultured, their detection is less affected by prior antimicrobial therapy (compared to culture-based methods), and they can be implemented in a way that provides a relatively rapid result. Some reports suggest that a panel of nucleic acid based tests has the potential to identify the etiology of a much larger fraction of pneumonia cases than do the other currently used techniques (76% for the panel of PCR tests vs. 50% for conventional tests), at least in samples from adults that are analyzed in a state-of-the-art clinical laboratory.⁶⁷ Implementing robust multiplex nucleic acid amplification diagnostic assays has traditionally been challenging, but there are several research groups, such as the commercial entities IBIS⁶⁸ and GHC Technologies⁶⁹ that have been successful. The platforms and assays developed by these groups have the potential to provide extremely sensitive, robust multiplex detection of a broad range of pathogens, but their performance has not yet been evaluated for the detection of pneumonia-causing pathogens in clinical studies. In addition, the platforms these groups have developed are very expensive, require a very high level of resources, and have not yet been adapted to diagnostic laboratories even in resource-rich sites.

The use of nucleic acid-based biomarkers to determine the etiology of pneumonia was recently reviewed by Murdoch et al., and this approach has been explored in all specimen types, including respiratory specimens, blood and its fractions, and other types such as pleural fluid. In general, researchers in this area appear to have done an adequate job in selecting the nucleic acid sequences to ensure that they provide sufficient discrimination from other related bacteria that are non-pathogenic (i.e., species specificity, as discussed in the previous section).^{40,70,71} Despite the large number of published studies, a relatively limited number of genes have been used as biomarkers to detect the organisms that have been reported to be important causes of pneumonia in the developing world. For example, of the 13 studies on detection of *Streptococcus pneumoniae* reviewed by Scott et al. (as of 2003), only six genes were targeted,⁷² and for the 34 studies on the detection of *Mycoplasma pneumoniae* reviewed by Loens et al. (as of 2003), only four genes are targeted.⁷³ These genes are summarized in Table 2.

Table 2. Non-Viral Infectious Organisms that Cause Pneumonia and the Genes Used as Targets in Nucleic Acid Diagnostic Approaches

Organism	Genes	References
<i>Streptococcus pneumoniae</i>	autolysin (lytA) gene	⁷⁴ ,16
	pneumolysin gene	⁷⁵ , ⁷⁶ , ⁷⁷ , ⁷⁸ , 31, 14, ⁷⁹
	penicillin binding protein 2B (PBP 2B) gene	32
	16sRNA gene	80
	surface adhesion A (PsaA) gene	72
<i>Mycoplasma pneumoniae</i>	Pan-mycobacteria test for 16S rRNA gene	⁸¹ , ⁸² , 31
	P1 adhesin gene	⁸² , 47, ⁷⁰ , 16, ⁸³ , ⁷¹
	ATPase operon gene	84
	elongation factor Tu (tuf) gene	85
<i>Chlamydia pneumoniae</i>	omp2 gene	16,31
	16s rRNA	⁸⁶ , ⁷³
	major outer membrane protein (MOMP)	87
<i>Haemophilus influenzae</i>	omp2 gene	31
	bexA gene	88
	16S rRNA gene	16
<i>Pneumocystis carinii</i>	16S rRNA gene	54
	5S rRNA	Reviewed by ⁸⁹
	DHFR	Reviewed by ⁸⁹
	mtLSU rRNA	Reviewed by ⁸⁹
	ITS	Reviewed by ⁸⁹
	MSG	Reviewed by ⁸⁹

When considering the goal of developing a diagnostic test that could distinguish bacterial and fungal pneumonia from viral pneumonia, nucleic acid biomarkers could be used if the test were designed to detect sequences from each of the major bacterial and fungal pathogens that cause pneumonia. The assay format would need to provide a high sensitivity for all the important pathogens, as well as a sufficiently high degree of species-specificity such that related but non-pneumonia-causing species would not be detected. Ideally, one practical sample type could be used to detect all of the species that cause pneumonia. Several studies have already reported the development of multiplex assays designed to detect nucleic acid biomarkers for several bacterial species that cause pneumonia, though their performance, for the most part, has not been evaluated using respiratory specimens.^{80,90,91,92,16}

In the studies that aim to measure the performance of nucleic acid biomarkers, the measurements of sensitivity are confounded by the lack of a useful “gold standard” diagnostic technique, and the measurements of diagnostic specificity are compromised by the fact that not all studies have looked at the rate of false positives in individuals who do not have pneumonia.

Some studies which use more sensitive nucleic acid amplification methods have reported better sensitivities than other studies in which more traditional diagnostic methods (e.g., culture and serology) have been used to detect bacterial pathogens that do not colonize the upper respiratory tract, such as *Mycoplasma* and *Legionella*.^{47,67,16} The generally high sensitivity of this approach becomes a disadvantage, however, when detecting the species capable of colonization, where the increased sensitivity results in a decreased specificity. As discussed in the previous section, respiratory specimens are unlikely to be useful in the diagnostic approach. Because of the ability of *S. pneumoniae* and *H. influenzae* to colonize the upper respiratory tract of individuals without pneumonia,^{56,57} the more easily obtainable upper respiratory tract specimens (e.g., swabs, nasopharyngeal aspirates) are likely to contribute to an unacceptably low diagnostic specificity of a nucleic acid based test. Sputum samples are considered unreliable, due to their potential to be contaminated during their passage through the upper respiratory tract, and subsequent inability to differentiate colonization from infection.⁹³ They may also be contaminated by organisms causing otitis. Transthoracic needle aspirates do not suffer from this issue, but the challenges of insuring that they are obtained from the focus of infection may limit their sensitivity, and in any case, neither of these lower respiratory tract fluid specimen types are practical to obtain from children and HIV infected individuals in resource-limited settings.

Relatively few studies have investigated whether the detection of nucleic acid based biomarkers in blood or urine specimens, rather than respiratory specimens, might be able to circumvent the problem of false positives in individuals who are merely colonized. Therefore, the question remains as to whether the existing nucleic acid biomarkers can perform sufficiently well in children using the sample types that are practical, such as blood from a finger prick, or urine. The performance of nucleic acid biomarkers for a variety of species in studies that have examined blood or urine specimens is presented in Table 3, with a focus on performance in pediatric populations, where the data exists. Cells filled in blue indicate aspects of the studies that do not match the most relevant populations or specimen types, but these studies were included because more relevant data (e.g., on children, or in the practical specimen types) is not available.

Table 3. Performance of Nucleic Acid Biomarkers of Pneumonia Etiology Using Blood or Urine

Study	Organism	Sample type	Target	Format & Detection	Performance
Mayoral et al. 2005 ⁷⁶	<i>S. pneumoniae</i>	Sera from children in Argentina	pneumolysin gene	(unable to obtain paper)	(unable to obtain paper)
Michelow et al. 2002 ⁷⁹	<i>S. pneumoniae</i>	Blood, buffy coat, or plasma from 154 children admitted to hospital for LRI in Dallas, 42 children without respiratory infection	pneumolysin gene	Single step, agarose gel visualization	Sensitivity 92 – 100% compared to culture (11 of 12), sensitivity of 71% (94 of 154) compared to diagnosis via PCR OR serological test; 5% (2 of 42) control subjects were positive
Isaacman et al. 1998 ³²	<i>S. pneumoniae</i>	Blood from 459 children with suspected pneumococcal bacteremia, and 103 apparently healthy children in Pittsburgh	penicillin binding protein 2B (PBP 2B) gene	Single step, detection via gel electrophoresis & hybridization of ³² P-labeled probe	Sensitivity 57% compared to blood culture; 16% of healthy children tested positive
Dagan et al. 1998 ¹⁴	<i>S. pneumoniae</i>	Blood, CSF from 281 total subjects (261 children) with various infections, in Israel	pneumolysin gene	Single step, agarose gel visualization	High sensitivity, but low specificity – 17% of healthy controls were positive (33% of healthy 2 year olds)
Shoma et al. 2001 ⁸⁸	<i>H. influenzae</i>	Sera from 180 children with pneumonia in India	bexA gene	(unable to obtain paper)	94% sensitivity, 97% specificity compared to blood culture
Daxboeck et al. 2005 ⁸⁴	<i>Mycoplasma pneumoniae</i>	Sera from 29 adults with verified Mycoplasma-caused pneumonia in Austria	Not specified	Compared conventional PCR (single step, agarose gel visualization) with real-time PCR	Conventional PCR negative in all cases, real-time PCR positive in 52% of cases
Loens et al. 2006 ⁸⁶	<i>Chlamydia pneumoniae</i>	Respiratory specimens	16S rRNA	real-time NASBA	Diagnostic-sensitivity and diagnostic-specificity not evaluated
Apfalter et al. 2001 ⁹⁴	<i>Chlamydia pneumoniae</i>	Compared respiratory and blood (PBMC) specimens from 58 adult ALRI patients & 57 healthy adults	16S rRNA	Nested PCR (unknown visualization – can't get article)	Low diagnostic sensitivity, unclear specificity; <i>C. pneumoniae</i> DNA not detected in healthy adults
Rabodonirina et al. 1999 ⁸⁹	<i>Pneumocystis carinii</i>	Blood samples from 84 HIV infected (adults?) and 35 normal individuals	5S rRNA	Nested PCR detected by agarose gel visualization	<i>P. carinii</i> DNA detected in 19 HIV positive patients, but low sensitivity

It is difficult to draw conclusions about the utility of any of these nucleic acid biomarkers for a variety of reasons. Few studies have utilized blood specimens, and even fewer have enrolled children. A number do not use amplification technologies that are considered to be very sensitive, such as single-step PCRs with detection via ethidium bromide staining of an agarose gel. Though some studies have compared the effect of using different specimen types, few compare how different specimen processing methods (e.g., whole blood vs. serum; different protocols, equipment, reagents, etc.), and different assay methods (e.g., single step PCR, nested PCR, real time PCR, and other non-PCR-based amplification methods; different detection platforms) affect the performance. The wide range of study designs and analytical approaches are overlaid on the confounding factor of the lack of a good gold standard method, and the differences in “reference standards” that have been employed (e.g., blood culture, sputum culture, lung aspirate culture, sputum stain, clinical and radiographic findings, immunodiagnostic methods, as well as different combinations of these). In addition, most of the studies enrolled relatively small numbers of subjects, and very few have attempted to systematically evaluate how different specimen processing methods, assay methods, or the selection of the biomarker itself might be affecting the sensitivities and specificities that are generated.⁹⁵ All the studies were conducted on hospital in-patients, in relatively well-resourced urban centers of their respective countries, and it is therefore unclear how results from rural populations in the developing world would differ. The studies that focus on detecting multiple organisms often lack a control “arm” which includes individuals who are not suspected of having pneumonia. It is therefore fair to say that there are no widely accepted sensitivity or specificity numbers that can be obtained with nucleic acid biomarkers, especially as they apply to a resource-limited setting.

Ultimately, there may be biological limitations in the specificity that can be achieved when detecting *Streptococcus pneumoniae* and *Haemophilus influenzae*, and possibly other organisms where it is unclear if colonization occurs or not.⁸⁹ Dagan et al. (1998) reported that 17% of serum samples from 194 healthy children in Israel tested positive for *S. pneumoniae* by PCR-mediated amplification of the pneumolysin gene.¹⁴ They also noted a marked effect of the age of the individual on the rate of positivity among healthy controls, with the highest rate (33%) being in the 2-year-old children, which is also the age group with the highest rate of nasopharyngeal carriage, and the lowest rate was found among infants less than 2 months of age (13%) and in adults age 18 to 50 (0%), which are the age groups with the lowest rate of nasopharyngeal carriage. Though they found their PCR-based assay to be sensitive, its high rate of positivity in healthy children, which was related to nasopharyngeal carriage, suggest that this approach will not provide sufficient performance in children.

3. Identification of Severe Pneumonia Infection Requiring Hospitalization in Resource-Limited Settings: Status of Currently Available Biomarkers

Much of the morbidity and mortality resulting from acute lower respiratory infections is due to hypoxemia, which is the most dangerous manifestation of severe pneumonia in children.⁹⁶ Hypoxemia puts the patient at a four to six times greater risk of death.^{97,98,99} In resource-rich areas, oxyhemoglobin saturation (SaO₂) is used as a measure of the severity of pneumonia, but this method of assessing severity is generally not available in resource-limited settings, due to the acquisition and maintenance costs of pulse oximeters.¹⁹ The very high mortality rates that result from pneumonia in resource-limited settings might be reduced if

there were a simple way to determine which children have severe pneumonia, and therefore ought to be referred to a healthcare center with more extensive treatment resources.

3A. Clinical Symptoms as Biomarkers of Severity

Though there are classic symptoms that suggest a child has severe pneumonia, such as cyanosis, respiratory rate of greater than 70 per minute in an infant or greater than 50 per minute in an older child, difficulty breathing, or grunting,¹⁰⁰ these signs are not always present, and may vary among populations.¹⁰¹ It is therefore difficult to know if a child should be referred. The decision to recommend referral from a health outpost to a hospital is often a very significant one, because it may involve walking for several days with a sick child to reach the hospital, and pose a significant financial burden as well for the family.

It appears that there are no published studies that measure the ability of clinical symptoms, as determined in a health outpost setting, to correctly identify children that should be referred to a hospital. The most relevant published work has been performed on children that have already arrived at a hospital in a developing country, after a referral decision has already been made. The clinical decisions that were being made in these studies were either whether the case of pneumonia was severe enough to admit the patient to the hospital, or whether the case of pneumonia was severe enough to require the administration of oxygen.

Many studies have attempted to generate algorithms that predict hypoxemia using clinical symptoms. There is conflicting evidence regarding whether (and which) clinical symptoms are useful predictors of hypoxemia. Cyanosis is regarded as a highly specific clinical predictor of hypoxemia, but because it is an infrequent, late-developing, and a subtle sign, it lacks sensitivity and can be difficult to detect.^{102, 103, 96} Its use is complicated by inter-observer error, and its accuracy is affected by skin pigmentation, high altitude, high hematocrit, and the presence of anemia.^{104,101,97} Head nodding (caused by using the accessory muscles of respiration) is easily recognizable and is another fairly specific symptom in children with suspected ALRI that are under five years of age. But like cyanosis, its high specificity generally comes with a lower sensitivity (e.g., sensitivity of 57%, and specificity 85% reported in Usen et al. 1999).^{97,98} Chest in-drawing is considered a highly specific symptom, but also lacks sensitivity.¹⁰² Raised respiratory rate, grunting, and nasal flaring have also been evaluated in some studies. However, disagreement remains regarding their ability to predict hypoxemia.^{98,101}

The general approach of developing an algorithm that combines multiple clinical signs, rather than any one sign, has been taken in a number of studies to predict hypoxemia in children with ALRI.⁹⁶ Though these algorithms have better performance than the use of single criteria, in general researchers have found that adding in symptoms can increase sensitivity but inevitably results in a loss in specificity. For instance, Duke et al. (2006) developed two algorithms for predicting hypoxemia in children from one month to five years of age with ALRI in a hospital in the highlands of Papua New Guinea. Their algorithm 1 (respiratory rate of greater than 60 or cyanosis or not feeding) had a sensitivity of 82% and a specificity of 49%, and their algorithm 2 (respiratory rate of greater than 60 or cyanosis or reduced activity) had sensitivity of 83% and a specificity of only 51%, for predicting hypoxemia.¹⁰⁵ Another algorithm for predicting hypoxemia in children from two to 33 months of age in a regional hospital in The Gambia (including inability to cry, head nodding, or a respiratory rate of greater than or equal to 70 breaths per minute) was found to have a

sensitivity of 70% and specificity of 79%.⁹⁷ Weber et al. found eight independent symptoms that were predictors of hypoxemia, but recommended a simple model which they thought provided the best sensitivity combined with a reasonable specificity for the settings in which these evaluations would be performed.⁹⁶ In their model, children with cyanosis or head nodding or not crying were predicted to be hypoxemic (defined as oxygen saturation of <90%). This algorithm had a sensitivity of 59% and a specificity of 93 to 94%. Adding other symptoms in to the algorithm increased the sensitivity but decreased the specificity. If the WHO algorithm (cyanosis, inability to drink, severe chest in-drawing, or a respiratory rate of over 70 breaths per minute) were applied to the population studied in this Weber et al., they would have predicted hypoxemia with a sensitivity of 62% and a specificity of 76 to 82% (depending on which control group was used). If the respiratory rate symptom is left out of the WHO algorithm, the specificity would increase to 96 to 95%, but the sensitivity would be lowered to 52%. If the algorithm reported by Onyango et al. (respiratory rate greater than 70 or grunting or severe chest in-drawing for children under 1 year, and respiratory rate of greater than 60 for older children), this would have given a sensitivity of 71% and a specificity of 47 to 61% (depending on the control group used) for identifying hypoxia in this population.¹⁰⁴ In general, therefore, when considering clinical symptoms as biomarkers of hypoxia (as a surrogate for severe pneumonia), there are no generally agreed-upon individual clinical signs or combination of clinical signs that provides sufficiently high specificity and sensitivity.⁹⁷

The acceptability of these trade-offs between sensitivity and specificity depends, of course, on how the information will be used. A lower positive predictive value is considered unacceptable in resource-limited settings when it will inform a decision to administer oxygen, because it will result in a waste of very expensive and limited oxygen on children who do not require it. Weber et al. note that in the setting in which their study was conducted, at a major hospital in The Gambia, a decrease in specificity from 94% to 76% would result in a quadrupling of the false positive rate (i.e., the number of children who receive oxygen but do not actually required it). This is unacceptable in countries where oxygen is expensive to purchase and transport. Yet, a lower positive predictive power might be more acceptable if it were used to inform the decision to refer a patient to a hospital or not. However, the data regarding the utility of clinical signs as a referral tool from health outposts to a hospital has not yet been collected.

3B. Oxyhemoglobin Saturation (SaO₂) as a Biomarker of Severity

The use of oxyhemoglobin saturation (SaO₂) as a marker of pneumonia severity is based on the assumption that hypoxemia is an appropriate surrogate marker for pneumonia severity. The most reliable ways to detect hypoxemia are an arterial blood gas analysis, or the measurement of arterial oxyhemoglobin saturation (SaO₂) by pulse oximetry.

Pulse oximetry is a non-invasive and accurate method of measuring arterial oxygen saturation, and is a useful predictor of hypoxemia.^{106,101} The most common pulse oximeters measure hemoglobin saturation. Typically they have a pair of small light-emitting diodes against a photodiode on a probe that is clipped to a translucent part of the patient's body, such as a fingertip or an earlobe. One LED emits red light with a wavelength of 660 nm, and the other emits infrared light with a wavelength of 910 nm. Absorption of each wavelength differs significantly between oxyhemoglobin and its deoxygenated form, and therefore the oxy/deoxyhemoglobin ratio can be calculated from the difference between the absorption of the red and

infrared light. A pulse oximeter generally converts its measurement into a percent of the normal saturation level. Acceptable normal ranges are from 95 to 100%. For a patient breathing room air, near sea level, an estimate of arterial partial pressure of oxygen (pO₂) can be made from the pulse oximeter's SaO₂ reading.

Studies from the developing world regarding the ability of SaO₂ measurements to predict which pneumonia patients have a higher risk for a poorer outcome are presented in Table 4.

Table 4. Evaluations of SaO₂ as a Predictor of Poor Outcome in Pneumonia in the Developing World

Publication	Location	Population	Definitions	Findings
Junge et al. 2006 ¹⁰⁷	Tertiary hospital, Gambia	4,047 children admitted for all causes.	Severe hypoxemia defined as <90%	Hypoxemia predicted poor outcome (based upon the abstract – unable to obtain the paper)
Djelantik et al. 2003 ⁹⁹	Major hospital on Lombok Island, Indonesia	4,351 children admitted for pneumonia (1999-2001)		Children with oxygen saturation ≤85%, or <4 months of age, were 5.6 times more likely to die than children with neither of these factors. Children less than 4 months old, mortality was increased at an oxygen saturation of <88% compared with <80% for older children. Laboratory, physical, or radiological findings did not contribute substantially to mortality prediction.
Onyango et al. 1993 ¹⁰⁴	Referral hospital in Nairobi, Kenya	256 infants and children under 3 years of age with symptoms of respiratory infection (pneumonia & bronchiolitis)	Hypoxemia defined as oxygen saturation <90%	Short term mortality was 4.3 times greater in hypoxemic patients than non-hypoxemic patients. Hypoxemia on admission predicted short-term mortality with 90% sensitivity and 34% specificity.

These studies have shown that the fatality rate (the most commonly measured severity-related outcome in the developing world) attributed to pneumonia is inversely related to the SaO₂,¹⁰⁴ and that the severity of hypoxia predicts poorer outcomes among children reaching a hospital in developing parts of the world.^{99,107} Therefore, the available evidence indicates that SaO₂ is a useful biomarker for pneumonia severity and for which children ought to receive oxygen. It appears that SaO₂ has not yet been evaluated as a biomarker to be used to inform the decision whether to refer a pneumonia patient from a health outpost setting to a hospital.

3C. Respiratory Acidosis as a Biomarker of Pneumonia Severity

Respiratory acidosis develops when the lungs do not expel carbon dioxide adequately, which can occur in diseases that severely affect the lungs, such as severe pneumonia, emphysema, chronic bronchitis, pulmonary edema, and asthma. The diagnosis of acidosis generally requires the measurement of blood pH in a sample of arterial blood, usually taken from the radial artery in the wrist. Arterial blood is used because venous blood contains high levels of bicarbonate and thus is not an accurate measure of blood pH. To determine

the cause of the acidosis, the levels of carbon dioxide and bicarbonate in the blood are measured as well. Point of care blood gas analyzers are now on the market in developed countries which can function on battery power.¹⁰⁸ However, because of the cost of the acquisition and maintenance of the equipment (\$1,000 to \$3,000) and consumables (an individual-use cartridge is \$ 4.00 in the developed world), and the challenges of obtaining an arterial blood gas sample, the measurement of arterial blood gasses is not a practical approach for determining the severity of pneumonia in resource-limited settings. SaO₂ can be more cheaply and non-invasively measured with a pulse oximeter, and in most cases SaO₂ is a practical and sufficient surrogate for partial pressure of arterial oxygen (paO₂).¹⁰⁹

3D. Non-specific Host Responses (Measured Using Immunodiagnostic Methods) as Biomarkers of Severity

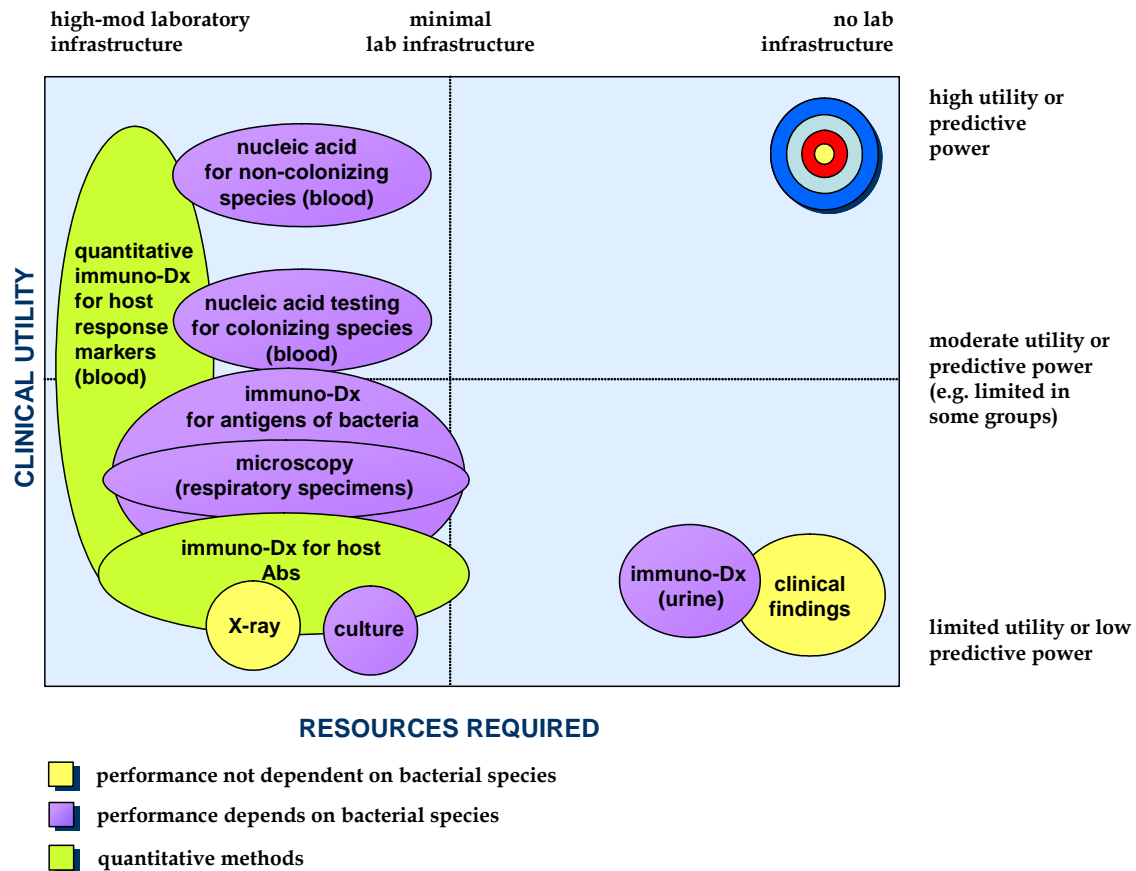
Several non-specific host response markers have been investigated for their potential to identify severe pneumonia. The most extensively studied molecule is C reactive protein (CRP). Levels of CRP have been reported to correlate with severity in adults in a developed country,¹¹⁰ and levels of CRP of greater than or equal to 100 have been reported to be independently associated with a higher risk of death in elderly patients in the developed world with severe lower respiratory tract infections.¹¹¹ Only one study has examined CRP levels in a pediatric population in the developing world, which suggested that CRP levels in infants less than 3 months of age had some value as a predictor of invasive bacterial infection. A more thorough evaluation of blood CRP levels as an indicator of pneumonia severity in pediatric populations with ALRI remains to be conducted.

Fibrin fragment D levels were investigated in adults admitted to a hospital in Israel with pneumonia, and though a correlation was observed between the Pneumonia Patient Outcome Research Team (PORT) score and other measures, no sensitivity or specificity was reported.¹¹² This biomarker has not been evaluated in children or in resource-limited settings.

Summary of the Status of Currently Available Biomarkers

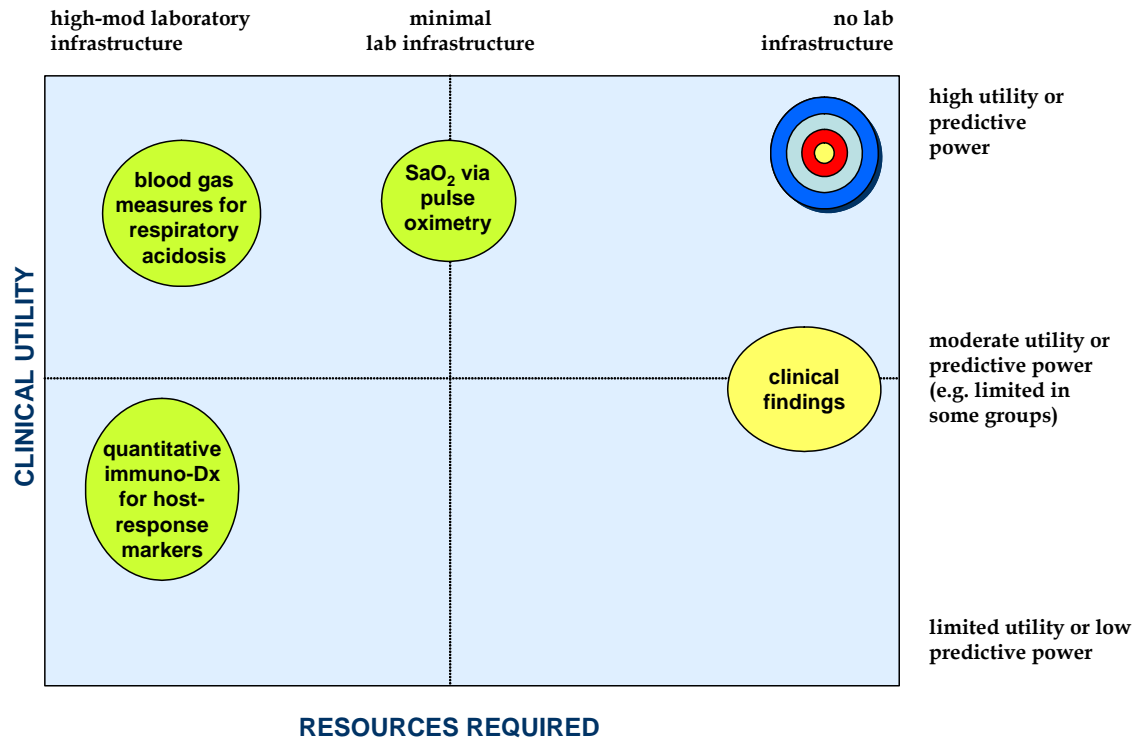
The relative merits of the major diagnostic approaches discussed above are presented diagrammatically in Figure 1 and Figure 2. In these figures, the ideal approach would be in the upper right quadrant, because of its high clinical utility or predictive power in resource-limited settings, and the low level of resources required for successful implementation. The figures identify the approaches that are easy to perform, but are limited by the biology of the infectious agents causing pneumonia, or that of the human host. Such approaches appear in the lower right quadrants. In addition, approaches with high predictive power, but that currently require significant resources to perform, are seen in the upper left quadrants.

Figure 1. Map of Currently Available Diagnostic Approaches for Identifying Bacterial and Fungal ALRI



There are many different immunodiagnostic formats, and some of these require fewer resources than others, which is why some of the ovals cover a large horizontal area. It was generally assumed that quantitative immunodiagnostic methods would be more challenging to perform (i.e. require more resources and training) than qualitative immunodiagnostic methods. The vertical placement of a number of approaches, such as the measurement of host-response markers, or urine dipsticks for pathogen antigens, are only estimates, because there is little data on the actual performance of these methods in children in developing countries or resource-limited settings.

Figure 2. Map of Currently Available Approaches for Identifying Severe Pneumonia Cases in Resource-Limited Settings that Should be Referred to a Hospital



The vertical placement of these approaches is based on estimates, because there is no data on the utility of these methods as a way of identifying severe cases of pneumonia that should be referred to a hospital. The vertical placement is an extrapolation from the utility of these methods for other clinical decisions, including predicting which children with ALRI in developing countries require oxygen, or determining which children with ALRI ought to be admitted to the hospital.

4. Current Deficiencies ALRI Diagnostics in Resource-Limited Settings

The deficiencies of the current approaches are summarized in Table 5. Cells that are filled in blue are characteristics that limit the utility of the approach in resource-limited settings. Cells filled in yellow are characteristics that also limit the utility of the approach, but that may be more easily overcome than the limitations shown in blue.

Table 5. Summary of Current Deficiencies in Diagnostic Tests for Bacterial ALRI

Clinical Decision	Test	TAT	Specimens	Sensitivity & Specificity	Resources
Distinguishing bacterial or fungal pneumonia from non-bacterial pneumonia, to determine if antibiotics should be administered	clinical signs	minutes	None	sens: poor spec: poor	low
	radiography	minutes	None	sens: poor spec: poor	high
	visualization of bacteria (primarily via culture, sometimes via microscopy)	can be weeks, some species not culturable	respiratory or blood	sens: poor spec: good	culture: high
	immuno-diagnostic detection of bacterial antigens	mins - hrs	lower respiratory	sens: good spec: good	high (to obtain specimen)
	immuno-diagnostic detection of bacterial antigens	mins - hrs	upper respiratory	sens: moderate - poor spec: poor in children for colonizing species	moderate ^b
	immuno-diagnostic detection of bacterial antigens	mins - hrs	blood ^a	sens: moderate – poor spec: poor in children for colonizing species	moderate ^b
	BINAX Now dipstick for <i>Strep pneumoniae</i>	minutes	urine	sens: moderate(?) spec: poor in children	low
	immuno-diagnostic detection of host antibodies	can be weeks	blood (often 2 samples over time required)	sens: moderate spec: good	moderate (time)
	quantitative immuno-diagnostic for host responses	hours	blood ^a	sens: poor spec: poor	high
	nucleic amplification based methods	hours	lower respiratory	sens: good spec: good	high ^b
	nucleic amplification based methods	hours	upper respiratory	sens: good spec: poor for colonizing species	high ^b
	nucleic amplification based methods	hours	blood ^a	sens: varies by species spec: poor for colonizing species	high ^b
Evaluation of severity to determine if patient should be referred to a hospital	clinical signs	minutes	none	depends on algorithm: gain in one inevitably lowers the other	low
	pulse oximetry	minutes	none	sens: high (likely) spec: high (likely)	high ^c
	blood gas analysis for respiratory acidosis	minutes	arterial blood	sens: high (likely) spec: high (likely)	high
	quantitative immuno-diagnostic for host responses	hours	blood ^a	sens: moderate? spec: unknown?	high

a. Could be overcome if finger prick sample could be used

b. Could be overcome if field-usable platforms (e.g. lateral flow devices) were developed

c. Could be overcome if inexpensive and robust instruments were developed

When considering the goal of developing a diagnostic test that could distinguish bacterial and fungal pneumonia from other causes, the ideal test would detect biomarkers of all of the important pathogens that cause pneumonia in the developing world. Some health-economic criteria need to be developed to define “important,” which could then be re-evaluated periodically to determine if there is a significant enough shift to make it worthwhile to adjust the species that are detected (especially as vaccination programs are implemented in various regions). The assay format would need to provide high sensitivity for all the pathogens, as well as a sufficiently high degree of species-specificity such that related but non-pneumonia-causing species would not be detected. In addition, the test should have sufficient diagnostic specificity to provide a meaningful reduction in the inappropriate use of antibiotics. Ideally, one practical sample type could be used to detect all of the pathogens of interest, or their effects on the host. Unfortunately, this idealized test does not exist even in resource-rich settings. Even in the best research studies that attempt to broadly identify the etiologies of community acquired pneumonia within a population, combining ALL of the available approaches, the etiological agent(s) are identified in only 50 to 60% of cases.

In summary, there are four major categories of deficiencies with the currently available biomarkers and approaches for identifying bacterial and fungal ALRI in resource-limited sites. First, the data that would indicate which pathogens are important to detect in the test has many gaps. Information on the epidemiology of many pathogens, from many regions of the world, as well as their health economic impact, would be the best way to decide which species ought to be detected in the first- and subsequent-generation products. Second, for the pathogens that are likely to be selected as “important” for the test to detect, there are biomarkers whose utility is limited by the biology of either the pathogen or the host, and therefore these challenges will be difficult to overcome. These include clinical signs, radiography, culture & microscopy, and immuno-diagnostic methods that detect host antibodies or non-specific host response markers. Third, there are biomarkers whose utility is limited when using the specimen types that are practical for resource-limited settings (e.g. upper respiratory, urine, and possibly blood) by the biology of either the pathogen or the host, primarily due to the lack of diagnostic specificity likely to be obtainable for colonizing species in children in the developing world. These include the immunodiagnostic detection of pathogen antigens, and nucleic acid amplification methods. Finally, no unified multiplex diagnostic product exists which is able to detect most of the important pathogens using one specimen type, one biomarker type, and one low-resource analytical method. Therefore, there is currently no combination of biomarkers, specimen type, and diagnostic approach that provides adequate performance in resource-limited settings.

The issue of identifying severe cases of pneumonia that should be referred to a hospital is somewhat less complex because none of the available methods has been evaluated as a “referral tool.” Though there is very little data on the use of clinical signs as a referral tool, their evaluation as a tool for triaging oxygen use suggests that a combination of both high sensitivity and specificity is unlikely to be achievable due to limitations in host (patient) biology. Three available approaches that might be used to identify severe pneumonia are currently limited by the high level of resources required for their implementation (oxygen saturation, blood gas measurements, and non-specific host responses). Oxyhemoglobin saturation (SaO₂), as measured by pulse oximetry, is likely to provide useful sensitivities and specificities as a measure of severity, but again there is no data on its use as a “referral tool” in resource-limited settings, and therefore it is currently unknown what the useful cut-off values would be.

The categories of deficiencies with the currently available biomarkers and diagnostic approaches for the ALRI clinical decision points considered in the manuscript are presented in Table 6.

Table 6. Categories of Deficiencies for ALRI Biomarkers and Diagnostic Approaches

Clinical Decision	Deficiencies	Biomarkers and Approaches
Distinguishing bacterial or fungal pneumonia from non-bacterial pneumonia, to determine if antibiotics should be administered	Data necessary to determine the “important” species to detect has many gaps	Applies to all currently available biomarker types and analytical approaches
	Biomarkers whose utility is limited by the biology of pathogen or host (patient)	<ul style="list-style-type: none"> • Clinical signs • Radiography • Culture & microscopy • Immuno-diagnostic detection of host antibodies • Immuno-diagnostic detection of non-specific host response biomarkers
	Biomarkers that might provide useful performance, but not in the specimen types that are practical for resource-limited settings	<ul style="list-style-type: none"> • Immuno-diagnostic detection of pathogen antigens • Nucleic acid amplification methods.
	Lack of a multiplex assay that uses one specimen type, one type of biomarker, and one analytical assay	Applies to all currently available biomarker types and analytical approaches
Evaluation of severity to determine if patient should be referred to a hospital	Level of resources required (primarily cost) is too high for resource-limited settings	<ul style="list-style-type: none"> • SaO₂ measured by pulse oximetry • Blood gases measured via point of care device
	Potential biomarkers that have not been evaluated for this intervention point	<ul style="list-style-type: none"> • SaO₂ measured by pulse oximetry • Blood gases measured via point of care device • Immuno-diagnostic detection of non-specific host response biomarkers

8B

5. Opportunities to Improve the Clinical Performance of Existing Biomarkers

There are a variety of improvements that could be made to existing test technologies that would improve the performance obtained from the currently available biomarkers, which are outlined in the next two subsections for each of the intervention points selected for ALRI.

5A. Opportunities to Improve the Performance of Biomarkers to Identify Bacterial and Fungal ALRI

An important improvement to existing approaches would be to combine the detection of all the pathogens of interest into one assay. This raises the issue of which pathogens should be included in such a test. Although there are many gaps in the data regarding the most prevalent species, this data is unlikely to ever be generated until the very diagnostic product under discussion is developed. A practical and expeditious approach might be to develop a first generation product for 5 or 6 bacterial species and *Pneumocystis*

carinii, and then add other species in later-generation products, as the gaps in the data are filled for various regions of the world. In addition, it is unnecessary to include the detection of viral species if the clinical decision to be informed by the test is whether or not to prescribe antibiotics. A patient who tests positive for bacterial or fungal pneumonia should receive treatment, whether or not there is also a co-infecting virus. If at some point in time, treatments for viral ALRI become available, then the product should be re-designed to detect these pathogens as well.

The two diagnostic approaches that are the closest to providing useful sensitivities and specificities (in resource-rich settings) for distinguishing bacterial and fungal pneumonia are the detection of pathogen antigens and the detection of nucleic acid biomarkers in lower respiratory specimen types, such as transthoracic needle aspirates or bronchoalveolar lavage specimens. These are the specimen types that are unlikely to be contaminated by pathogens that can colonize the upper respiratory tract. However, for either of these approaches to work in resource-limited settings, several significant improvements would need to be made.

First, a method for obtaining a liquid lower respiratory specimen that is feasible in resource-limited settings would need to be developed. The method of obtaining the specimen would need to ensure that the specimen is not contaminated by organisms that have colonized the upper respiratory tract or are derived from other locations. It is currently difficult to imagine how this improvement could be achieved for a liquid respiratory specimen. The possibility of achieving this end via the use of exhaled breath condensate specimens is described in the next section.

Then, there would need to be improvements made in the diagnostic platform in order to make it feasible to perform in resource-limited settings. In order to detect pathogen antigens using immunodiagnostic methods, the currently available plate-based methods would need to be converted into a point-of-care device, such as a lateral flow device, that provides multiplex detection of the important pathogens, state-of-the-art techniques for improving the sensitivity would need to be applied as well. For a review of the recent technological advances in the design and manufacture of lateral flow devices, please refer to the report previously presented on technology used in diagnostic products.¹¹³

Alternatively, a totally novel approach might be taken, such as the approach under development by GHC Technologies (La Jolla, California).⁶⁹ This company is developing a method to trap very small numbers of bacteria, even a single bacterium, using air filters, and then determining the species by labeling them with a panel of quantum dot coated antibodies and imaging. This might be a useful approach for identifying bacteria in an exhaled breath condensate (see discussion of this specimen type in the next section).

In order to use an approach that detected nucleic acid biomarkers, it is likely that gains in both sensitivity and specificity could be achieved with the known nucleic acid biomarkers by systematically evaluating the specific amplicons, amplification approach, and detection technology, because relatively few studies report the effects of altering these different variables.⁷⁴ The effects of amplifying multiple amplicons in the same reaction should also be examined, given the challenges that diagnostics manufacturers have faced in implementing multiplex PCR-based diagnostics.¹¹⁴ Ieven et al. reported a higher sensitivity for a gene that was present in multiple copies in the genome,¹¹⁵ and Von Kuppeveld et al. reported higher sensitivity when

rRNA, which is present in many more copies than rRNA genes, was targeted.¹¹⁶ Daxboeck reported much higher sensitivity using real-time PCR (TaqMan assay analyzed on an ABI 7700 instrument) compared to “conventional” PCR with a single step amplification and agarose gel detection.⁸⁴ Whatmore et al. suggests that the pneumolysin gene, a common target of amplification to detect *Streptococcus pneumoniae*, does not provide the required species-specificity, and that targets should be more carefully selected.¹¹⁷ More sensitive assays which use molecular beacons for detection have been developed for *Mycoplasma pneumonia* and *Chlamydia pneumonia*, among others, but their performance has not been reported in blood from children with pneumonia and healthy controls.^{70,67} The effects of using different sample preparation methods remains to be more systematically examined, especially in light of the fact that the high sensitivity of Taq polymerase to porphyrin inhibitors (generated during the breakdown of hemoglobin) has the potential to generate false negatives. Several authors have acknowledged that a significant fraction of their false-negative results were attributable to problems in specimen processing or DNA extraction,⁷² which could often be overcome by simply diluting the extracted DNA. This common finding suggests that many researchers are using methods that are either inadequate or inconsistent for purifying DNA, and the lower sensitivity reported in some studies should be interpreted with caution.

In order to use nucleic acid biomarkers, a multiplex assay would need to be developed for a platform that is close to being usable in resource-limited settings (which does not truly exist today), such as the Cepheid GeneXpert platform or other platforms which have not yet been commercialized. Ideally, the platform would use a sample preparation method that consistently obtains very pure nucleic acids, a problem that has plagued many published reports on nucleic acid-based methods. In addition, the platform would utilize a detection method that is more sensitive than the agarose gel based detection methods that are reported in many of the published studies. At the same time, engineering and manufacturing improvements would need to be made to the platform to reduce component costs, reduce manufacturing costs, reduce energy consumption, improve the robustness, improve the ability of local (perhaps less-specialized) technicians to make repairs in the country in which the test is being used, and to improve the ability of the reagents to withstand stability challenges throughout the supply chain.

5B. Opportunities to Improve the Performance of Biomarkers for the Severity of Pneumonia

If evaluation studies indicate that oxyhemoglobin saturation (SaO₂) will have useful performance characteristics as a referral tool (see next section), the main improvement necessary to achieve the implementation of this diagnostic approach will be in reducing the cost of acquiring, maintaining, and powering a pulse oximeter. Engineering and manufacturing improvements could focus on reducing component costs, reducing manufacturing costs, reducing energy consumption, improving robustness, and improving the ability of local (perhaps less-specialized) technicians to make repairs within the country in which the instrument is being used.

6. Evaluation of Known Molecules That Have Not Yet Been Clinically Validated

There are many known molecules whose performance has not been validated in the relevant populations, particularly pediatric populations in resourced-limited settings. Significant avenues, such as evaluating previously un-explored combinations of markers, using a more strategic selection of nucleic acid markers, as well as evaluating newly discovered molecules, are outlined below.

6A. Evaluation of Known Molecules as Biomarkers for Bacterial and Fungal Pneumonia

The currently-known pathogen-derived molecules have not been shown to be useful in distinguishing upper respiratory colonization from lower-respiratory infection (pneumonia) in samples such as blood or urine. It is conceivable that some as-yet unexplored combination of pathogen specific markers and host-response marker(s) might provide useful diagnostic accuracy. For instance, there appears to be no information in the literature on the combined predictive power of detecting pathogen antigens in blood with quantifying general host response markers, such as CRP or PCT. It remains to be evaluated whether such a combination could provide improved accuracy in identifying bacterial and fungal pneumonia and distinguishing it from colonization.

As previously mentioned in Section 2D, serum PCT levels remain to be evaluated in populations served by resource-limited testing centers.

There is also at least one known, non-specific host response molecule whose diagnostic utility is under evaluation, namely the triggering receptor expressed on myeloid cells-1 (TREM-1). This member of the immunoglobulin superfamily is expressed on the surface of neutrophils and monocytes. TREM-1 is up-regulated in these cells types in the presence of microbial products, once the neutrophils and monocytes have infiltrated tissues that are infected with bacteria, and is also up-regulated in alveolar macrophages. *In vitro* studies demonstrate that the expression of TREM-1 is strongly up-regulated by extracellular bacteria and their cell wall components, and by fungi, but not by mycobacteria. TREM-1 is also shed by the membrane of activated phagocytes and can be found in this soluble form, s(TREM-1), in body fluids. Though most of the information on the diagnostic potential of this molecule is related to sepsis, a few studies have evaluated its potential to diagnose bacterial pneumonia. Though a number of authors suggest that it is induced specifically by bacteria, it appears that there are no published studies that have examined its levels in viral pneumonia, so this remains to be clarified. In an evaluation of 148 hospitalized adults in France, serum levels of s(TREM-1) were not found to differ, but s(TREM-1) levels in bronchoalveolar lavage (BAL) fluid were found to be significantly higher in individuals with bacterial pneumonia than those without pneumonia. At a cut-off of 5 pg/ml or greater in BAL fluid, the s(TREM-1) level had a sensitivity of 98% and a specificity of 90% for diagnosing pneumonia.¹¹⁸ Another study on 66 adults in Italy found variable but generally very high levels of s(TREM-1) in bronchoalveolar lavage specimens of patients “likely to have pneumonia caused by extracellular bacteria” compared to patients with tuberculosis or patients with non-infectious interstitial lung diseases.¹¹⁹ Additional information is required to determine this molecule’s usefulness in distinguishing bacterial from viral pneumonia in resource-limited sites, such as its levels in children in the developing world (with and without co-morbidities such as TB, HIV, or diarrheal diseases that are common), its level in nasopharyngeal carriers of bacteria, and its levels in respiratory specimens that are more easily obtainable, such as throat or nasal swabs or saliva.

Nucleic acid biomarkers for non-colonizing organisms, such as *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Pneumocystis carinii* have not been evaluated systematically in blood samples from large numbers of children in developing areas of the world (see Table 3). Some of the publications that focus on describing these methods do not report their diagnostic sensitivity or specificity.

6B. Evaluation of Known Molecules as Biomarkers for Pneumonia Severity

The published studies that have evaluated SaO₂, as measured by pulse oximetry, have evaluated its utility as a way to triage the use of oxygen, but not as a method to determine which patients presenting in resource-limited settings have severe enough pneumonia that they ought to be referred to a health care facility with greater resources, such as those required for the administration of oxygen, or intravenous antibiotics. Therefore, SaO₂ remains to be validated as a referral tool. These validation studies would ideally measure the impact on morbidity and mortality, as well as the cost effectiveness of the approach, in a number of regions of the world, and at a number of altitudes, which could alter the selection of a threshold, or even the ultimate utility, of SaO₂ as a referral tool. These studies should be designed to determine a threshold that maximizes the reductions in morbidity and mortality in a cost-effective manner for a given distance to, and resources of, the referral center, and perhaps even a threshold below which the prognosis is sufficiently poor that referral is unlikely to reduce morbidity and mortality.

It is also possible that some combination of general host-response markers (or a combination of general host response markers with pulse oximetry) might provide useful predictive power as a referral tool. Little is known about the levels of general host response proteins, such as CRP, PCT, or TREM-1, in individuals, particularly children, who live in areas with limited resources, and how the levels compare between healthy individuals, individuals with non-pneumonia infections, as well as individuals with mild, moderate, and severe pneumonia. Studies that evaluate these variables still remain to be conducted, to determine if (a combination of) any of these biomarkers holds sufficient promise to warrant a clinical validation study.

Levels of nitric oxide (NO*), or its by-products which are easier to measure, such as nitrate (NO₃⁻) have been investigated in exhaled gas or exhaled breath condensates, as a marker of pneumonia,^{120,121,122} but there is currently no evidence that they have been evaluated as markers to distinguish bacterial from viral pneumonia, or as markers of pneumonia severity.

Several studies have evaluated the levels of hepatocyte growth factor in either serum or exhaled breath condensates (see below) as a marker for response to therapy in pneumonia.^{123,124} Hepatocyte growth factor (HGF) is a protein produced by mesenchymal cells in many organs in response to injuries, and a major source of HGF is the lung. Serum and EBC levels of HGF were shown to be significantly higher in adult European patients with pneumonia than in normal individuals. It remains to be determined whether HGF has any value in distinguishing evaluated bacterial and fungal from viral pneumonia.

7. Approaches for the Discovery of Novel Biomarkers for ALRI

In addition to improvements that could be made to existing diagnostic test methods through technology improvements, or the incorporation of currently known biomarkers into these tests, there are also opportunities to discover new biomarkers that might be better suited for deployment in resource-limited settings. Several of these approaches use exhaled breath - an underexploited specimen type that is very relevant to ALRI, as well as an underexploited biomarker type - volatile organics. These and other discovery approaches are reviewed in the following section.

7A. Novel Biomarkers for Bacterial and Fungal Pathogens in Fluid Specimen Types

As reviewed extensively above, several bacterial species that are important causes of pneumonia are also commonly found to colonize the upper respiratory tract. Because of this, a diagnostic test that uses the presence of bacterial antigens or DNA in a fluid specimen type that is practical for resource-limited settings to diagnose all the major pneumonia-causing strains of bacteria will suffer from unacceptably low diagnostic specificity. It is possible that there are other nucleic acid or antigen biomarkers that are unique to pneumonia infection (versus colonization) that could be detected in practical sample types, such as a finger-prick specimen (or less likely, urine or saliva), but discovery studies would need to be appropriately designed to discover them. It is possible that the pathogens capable of colonization express a different spectrum of RNAs or proteins in the upper respiratory tract versus the lower respiratory tract perhaps due to differing states of anaerobic conditions, and these spectra might therefore be used to distinguish these states if they can be preserved during collection and processing. It is likely that the spectra will differ quantitatively, rather than qualitatively, and therefore the discovery experiments should employ quantitative methods. Because of the broad coverage possible with whole-transcriptome based discovery methods, it would be interesting to determine whether an RNA profile could be identified that would have a useful predictive power. If one is found, it could then be determined if any of the differences in RNA levels might be detectable as differences in the level or presence of proteins. Though such a correlation is not particularly likely to be found (perhaps only 10% to 40% of differences in particular mRNA levels correlate with a similar change in protein levels),¹¹⁴ it would allow the biomarker to be detected using an immunodiagnostic-based test, which is likely to be more feasible to implement for resource-limited settings than a test that detected RNA biomarker(s).

7B. Novel Host Response Biomarkers in Fluid Samples

Identifying a host response that is unique to bacterial and fungal pneumonia is attractive because it has the potential to overcome the poor diagnostic specificity caused by colonizing bacteria that results when detecting pathogen antigens or pathogen DNA. There are a number of proteins and polypeptides that are effectors of the innate immunity system and are active in the lungs {reviewed by Rogan et al. 2006}.¹²⁵ Though many of these substances appear to be produced at the site of infection, (so a lower respiratory tract specimen would be required) it is possible that some of them, or changes in the circulating cells that produce them (often neutrophils), could be detected in a more easily accessible sample such as a blood finger prick specimen, or in an exhaled breath condensate (see below). Because the innate immune responses occur much more rapidly than those of the adaptive immune system, changes in these components may occur very early in the infection, which would be advantageous for a biomarker of ALRI. Several substances involved in innate immunity, such as bactericidal permeability-increasing protein (BPI), chemokine ligand 20 (CCL20), and the human beta defensins (HBD), appear to have primarily anti-bacterial roles (as far as it is known today).¹²⁵ It is possible that these substances are specifically induced (or induced at higher levels) in bacterially-caused ALRI, compared to virally caused ALRI. Another class of substances that might be worth investigating are those that function as chemotactants (e.g. attracting cells such as neutrophils to the site of infection), because these proteins are secreted and changes in their levels may be detectable in the circulation.¹²⁶ It is possible that a pattern of these substances, perhaps in combination with biomarkers such as PCT level, might be specific for bacterial and fungal pneumonia.

7C. Novel Non-Volatile Biomarkers in Exhaled Breath Condensate Samples

Exhaled breath condensate (EBC) can be obtained by cooling exhaled air under conditions of spontaneous breathing. Respiratory droplets can be collected by methods other than condensation on cold surfaces, such as filtration, impaction, and electrostatic precipitation on devices that are at body temperature.¹²⁷ The liquid that is collected contains water that was generated as a gas from the lung surfaces (which cannot act as a vehicle for electrolytes and other non-volatile solutes), as well as droplets of epithelial lining fluid (ELF) that are generated from the membrane surfaces of the cells that line the lungs (which do contain non-volatile molecules). These droplets are thought to be formed at a variety of sites in the lungs, including the airways, upper respiratory tract, and even the upper gastrointestinal tract. The contents of the liquid condensate can then be analyzed using conventional techniques, such as an ELISA. Because the relatively small volume of the droplets is diluted by a very large amount of condensed water vapor, the solutes in the EBC samples are generally present in extremely low concentrations. A variety of potential biomarkers have been detected in exhaled breath condensates, including leukotrienes, IL-6, endothelin, vibronectin, IL-4, and hepatocyte growth factor, which are derived from the ELF droplets in the sample.¹²⁸ Effros et al. (2006) report that substances from the alveoli and bronchi are present in EBC samples, along with substances from the larynx, pharynx, upper gastrointestinal tract (e.g. HCl, pepsin), nose (e.g. inflammatory mediators), and mouth (e.g. NH₃, amylase).¹²⁷ Unfortunately, it appears that researchers have not yet explored this technique as a potential method to detect pathogen-derived biomarkers. EBC collection is easy and non-invasive, and is currently used in resource-rich settings to study non-volatile molecules in exhaled air. It is currently envisioned to be a non-invasive alternative to induced sputum samples, but is still under development and the utility of the biomarkers that can be detected using this approach is still being explored, primarily for diseases such as asthma and chronic obstructive pulmonary disease. Because both sputum and EBC specimens can be contaminated by passage through the upper respiratory tract, the pursuit of this specimen type as a way around the colonization issue should be approached with caution. EBC could be examined for both host-response and pathogen-derived biomarkers, either via empirical approaches (e.g., measuring levels of host response substances listed above), or hypothesis-free approaches such as mass spectrometry.

There are still technical challenges to be overcome when using EBC specimens. The analytes of interest may adhere to the surface of the collecting device, which may affect the ability to detect the analyte. Another challenge is the extreme and variable dilution of biomarkers in the condensate, compared to that found in epithelial lining fluid, (reported to be from 1/12,000 to 1/20,000) which results in the requirement for very sensitive detection methods.¹²⁸

7C. Novel Volatile Biomarkers in Breath Samples

The potential of volatile substances in breath samples to be used to identify cases of bacterial and fungal pneumonia is particularly interesting. Volatile organic compounds (VOCs) found in the breath are mainly blood borne and therefore provide a non-invasive way to monitor biological processes that can be detected in the blood.¹²⁹ Researchers have just begun to explore the possibility of using this biomarker type to diagnose pneumonia. The analytical approaches that have been undertaken include hypothesis-generated

evaluations of specific molecules, and hypothesis-free evaluations that may or may not identify the specific substances that serve as biomarkers.

The hypothesis-driven studies have included evaluations of NO as a predictor of ventilator-associated pneumonia in adults in the developed world. Adrie et al. (2001) reported that end-expiratory exhaled NO concentrations, measured using a fast response chemiluminescence analyzer, were significantly higher in patients with acute pneumonia, and that a cut-off of 5 parts per billion had a sensitivity of 88% and specificity of 76% for diagnosing acute pneumonia. It appears that NO has not been evaluated as a method to distinguish bacterial and fungal from viral pneumonia.¹²⁰

The hypothesis-free studies have involved an analysis of the volatile compounds in the breath using either electronic nose or mass spectrometry instruments. Devices characterized as electronic noses (e-nose) utilize a variety of technologies, though perhaps the most prevalent utilizes an array of non-specific chemical sensors that bind volatile chemicals in the vapor headspace over a sample. The most common types of sensors are metal oxide sensors, conducting polymers, and piezoelectric-based sensors. The sensors typically have a partial specificity, in that they respond to certain classes of chemicals, such as alcohols and aldehydes, rather than to single compounds. The interaction of volatile compounds with the sensor surface results in changes in the physical properties of the sensor, such as its resistance, conductivity, and frequency, which are then measured.¹³⁰ Therefore the nature and relative ratio of the molecules in the headspace determines the response pattern of the sensor array. The device is used to sample a set of “case” samples (e.g., known cases of bacterial and fungal pneumonia) and control samples (e.g., viral pneumonia, other infections, and healthy individuals), and pattern-matching algorithms are used to identify a pattern that segregates the cases from the controls. The device can then use the pattern to classify unknown samples.

Several recent studies by Hanson et al. (2005) have investigated the use of a commercially available e-nose with carbon black/polymer composite sensors to diagnose ventilator-associated pneumonia in adults in the United States, with moderate success.^{131, 132} In this study, the e-nose may have “smelled” pathogen metabolites, gases emitted during the immune response, or a combination of these classes of substances. This research group has also shown that several major respiratory pathogens, such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa*, deposited on swabs taken from *in vitro* cultures, could be distinguished by an e-nose, suggesting that it might be possible to find a distinguishing pattern of volatile organics that identifies individual pathogens in the breath of infected patients. E-noses have also been explored as a means to detect other respiratory pathogens, such as *Mycobacterium tuberculosis* in human sputum¹³³ and *Mycobacterium bovis* infection using serum samples from badgers and cattle.¹³⁰ The study on *M. bovis* detection determined that there was also sufficient information present in the analysis to discriminate between the experimentally delivered infectious doses, as well as the time since infection.¹³⁰ Unfortunately, it appears that e-nose approaches have not been specifically evaluated for their ability to distinguish bacterial and fungal from viral pneumonia, in either breath or blood specimens, in children or adults from the developing world. In addition, the degree to which upper respiratory colonization might affect the diagnostic specificity has not been evaluated. It might be possible to use an existing instrument in the field in the initial evaluation

studies, as at least one commercially available e-nose model is portable and battery powered. The Cyranose 320, developed by Cyranose Sciences but now sold by Smiths Detection, is a hand-held, battery-powered device designed for field-use. One drawback to the e-nose approach is that molecular identity of



the molecules that make up the pattern cannot be determined, because the device only recognizes the pattern.

The Cyranose 320⁶⁰

Another hypothesis-free approach to discovering biomarkers in the breath utilizes mass spectrometry detection methods, which can often identify the particular volatile substances that are unique to a disease etiology. Ruzsanyi et al. reported the use of multi-capillary chromatography columns coupled to an ion mobility spectrometer to analyze breath samples from individuals with a variety of respiratory diseases, including bacterial and fungal infections. In this study, they compared the spectra obtained from a relatively small number of healthy individuals (18) and those with various lung diseases (22 individuals), and their results indicated the presence of specific peaks that were unique to *S. pneumoniae* and candida infection, compared to emphysema or general inflammation.¹³⁴ Other work recently published by Phillips et al. (2006) identified 12 principle components in the volatile organic compounds in the breath of tuberculosis patients, such as 1-methyl-naphthalene and 1,4-dimethyl-cyclohexane that served as a fingerprint for *Mycobacteria tuberculosis* infection. This pattern identified sputum-positive patients with 83% sensitivity and 100% specificity.¹³⁵ These promising initial results would need to be followed up with more extensive studies, which would determine if there were a pattern of volatile breath biomarkers that would distinguish patients with bacterial, viral, and *Pneumocystis carinii* pneumonia, as well as distinguish them from individuals who are merely colonized, or who had other non-respiratory infections. It is also an intriguing possibility that such an analytical approach might identify biomarkers of pneumonia severity, as well as allow the discrimination of *M. tuberculosis* infection from other respiratory pathogens. The deployment of a single diagnostic platform that can detect many diseases has obvious economic and logistic advantages.

It is possible that once the important principle components of the volatile organics in the breath are identified via mass spectrometry approaches, then an e-nose instrument (of which at least one is already field-deployable) could be specifically designed to detect and discriminate the important molecules.

8. Clinical Sample and Study Design Issues for Biomarker Discovery and Validation

There are a variety of open research questions that could be addressed much more rapidly by using samples from children that are readily accessible to researchers (e.g., in the U.S. and Europe). For example, it seems reasonable to perform the initial evaluations of the recently described host-response substances (as markers that could identify bacterial pneumonia, such as TREM-1) in children in developed countries. In addition, it seems reasonable to perform the initial discovery experiments to determine if volatile organics can successfully distinguish bacterial and fungal from viral pneumonia using samples from children that are easy to obtain. The next round of evaluations could be conducted more rapidly if banked samples (most likely blood) existed from populations of children from the developed world on whom an extensive set of tests were run to identify the etiology of their pneumonia. An important set of control samples to obtain from/for a bank would be children with nasopharyngeal carriage of the colonizing species (but without pneumonia), as well as children with other common infections. Because it is unlikely that the appropriate breath samples have been banked, the initial discovery and then validation studies would almost certainly require the use of prospectively collected samples. These evaluation studies on breath samples should also be designed to identify potential severity biomarkers, which might have more predictive power than pulse oximetry. If these initial studies, using samples that are easy to access, appear promising, then the greater effort and expense could be undertaken to validate the performance of the biomarkers in prospectively collected samples from children in resource-limited settings.

There are, however, other research questions that must be addressed using samples from children served by resource-limited settings. The challenge for such a sample bank is the lack of any gold standard method for identifying the etiology of pneumonia. In order to have as complete a set as possible, patients from whom samples are banked should have all the available methods used to determine the etiology of their pneumonia, including culture, immunodiagnostic, and nucleic-acid based diagnostics of respiratory and blood samples (including nasopharyngeal sample culture, to identify carriers), in order to identify the etiology in as many instances as possible. This could be quite an expensive undertaking. A bank might contain a complete set of specimen types from relatively few patients (representing infections from all the prominent pathogens), which could be used in discovery experiments. The bank might also contain just the practical specimen types (such as blood, breath, and possibly urine) from a relatively large number of children and adults, which could be used in validation studies.

Many of the studies in the literature were designed to address diagnostic issues other than the specific intervention points of interest in this manuscript, and therefore there are many gaps in the data. Many of the studies that used a panel of organism-specific tests to determine the etiological agent in known pneumonia cases did not include control arms with either normal individuals, individuals with other infections, or individuals with nasopharyngeal colonization, and therefore the study design did not allow the performance characteristics to be fully elucidated. Future studies should be designed to rectify this situation.

Studies designed to evaluate pulse oximetry as a referral tool have yet to be conducted. These studies must be implemented in the health outpost setting, ideally in a number of populations and at various altitudes. Such studies should be designed to evaluate whether there is a threshold that can be used in a referral

decision that would improve morbidity and mortality outcomes, and should include a health-economic component to estimate the costs involved in obtaining any benefits that are observed.

9. Discussion and Recommendations for the Improvement of Diagnostics for Acute Lower Respiratory Infections

The following recommendations are presented for consideration, in light of the deficiencies of current diagnostic test methods for deployment in resource-limited settings, the opportunities for improving the deployment of existing biomarkers, and the approaches that might be used for discovering novel biomarkers that are more appropriate for use in resource-limited settings.

9A. What clinical information and user specifications are required for the design and development of the diagnostic products needed for ALRI?

For distinguishing bacterial and fungal from viral ALRI, and therefore identifying which patients should be treated with antibiotics, an assay is needed that detects the presence (or specific host effects) of all the important non-viral pathogens (so that individuals doubly infected with a bacterium and a virus, for instance, are included in those individuals to whom treatment would be administered). The assay should favor a high specificity to reduce the instances in which antibiotics are used inappropriately (primarily in viral pneumonia). Preferably the test would detect all the pathogens, the specific host response, or both, in a single specimen type that is practical to collect and process in a resource-limited setting.

For identifying cases of severe ALRI which should be referred to a hospital, a test that requires minimal resources is needed. It is likely that a specimen will not be needed to perform this test.

9B. What biomarkers, sample types, and technologies are most appropriate for ALRI?

The most likely candidates for practical specimen types are finger prick whole blood, saliva, or a breath specimen (exhaled volatiles). Liquid respiratory specimens are either not practical to collect reliably (particularly in children) or are unlikely to ever provide the diagnostic specificity that is required.

The available evidence suggests that there may not be sufficient levels of some of the pathogen specific-biomarkers of interest in the circulation of the majority of patients with ALRI, and in other instances, the pathogen-specific biomarkers in blood do not provide the necessary diagnostic specificity. Therefore the likelihood of achieving the necessary performance using a blood sample to detect all the most prevalent pathogens of interest via antigens or nucleic acids seems low. It therefore appears more fruitful, in terms of likelihood of achieving the necessary sensitivity and specificity, to discover a set of host response biomarkers in blood or saliva that could provide sufficient performance in children. The host response marker could be either a protein or RNA marker. There are significant biological and technical risks, but the rewards appear to outweigh the risks.

Another option, which seems to hold a great deal of promise, is an approach that utilizes breath specimens. Of the two breath specimen types, testing of volatile organics seems to be the most likely approach to succeed within the next few years, based upon the small amount of existing data which suggests that volatile molecules in the breath that are specific for bacteria and fungal pneumonia reported to date..

Exhaled breath condensates appear to be more challenging to work with and, apart from the technical hurdles that this approach still must overcome, there is no evidence today that a biomarker with useful predictive power has already been discovered in this specimen type.

The future diagnostic tests for bacterial and fungal ALRI in resource-limited settings are depicted in Figure 3, and the future tests for identifying severe ALRI are depicted in Figure 4. In these figures, the current approaches are shown as light gray spheres and the future approaches are shown in color. The position of each sphere on the graph illustrates the resource requirements (x-axis) of the test method and the clinical utility of the test in a resource-limited setting (y-axis). In these figures, therefore, an ideal biomarker and test method will be in the upper right quadrant, with a high predictive power and low resource requirements.

Figure 3. Future Approaches for Distinguishing Bacterial and Fungal from Viral ALRI and Their Utility in Resource-Limited Settings.

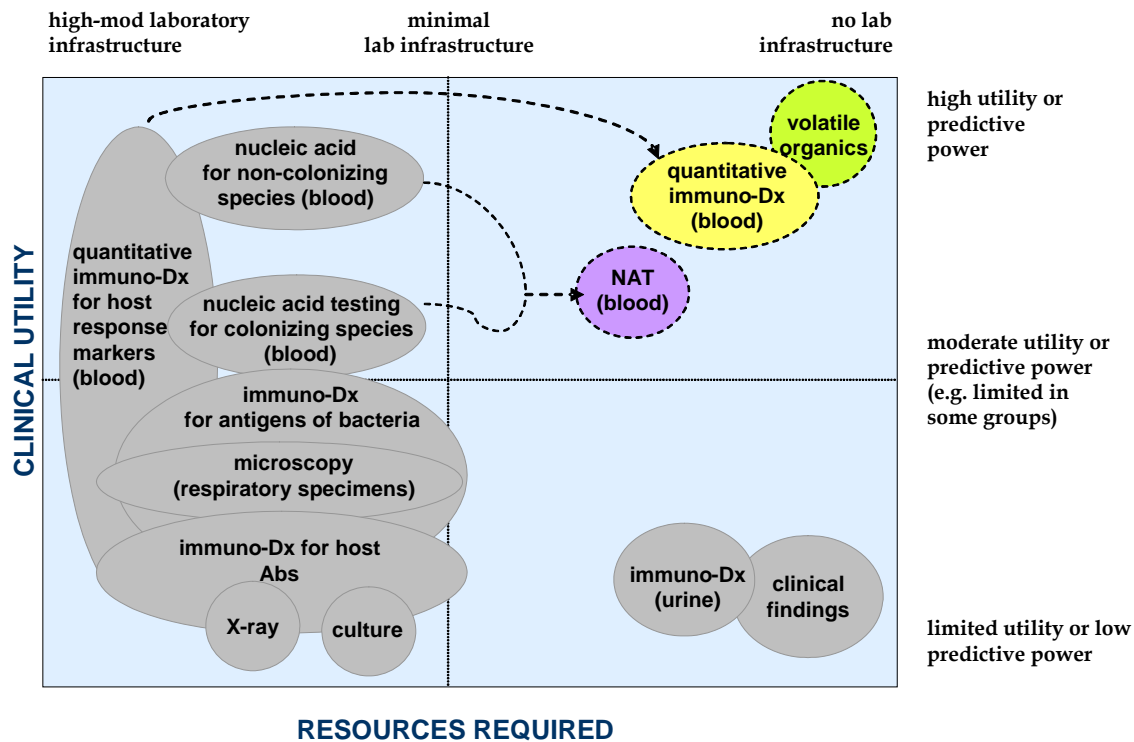
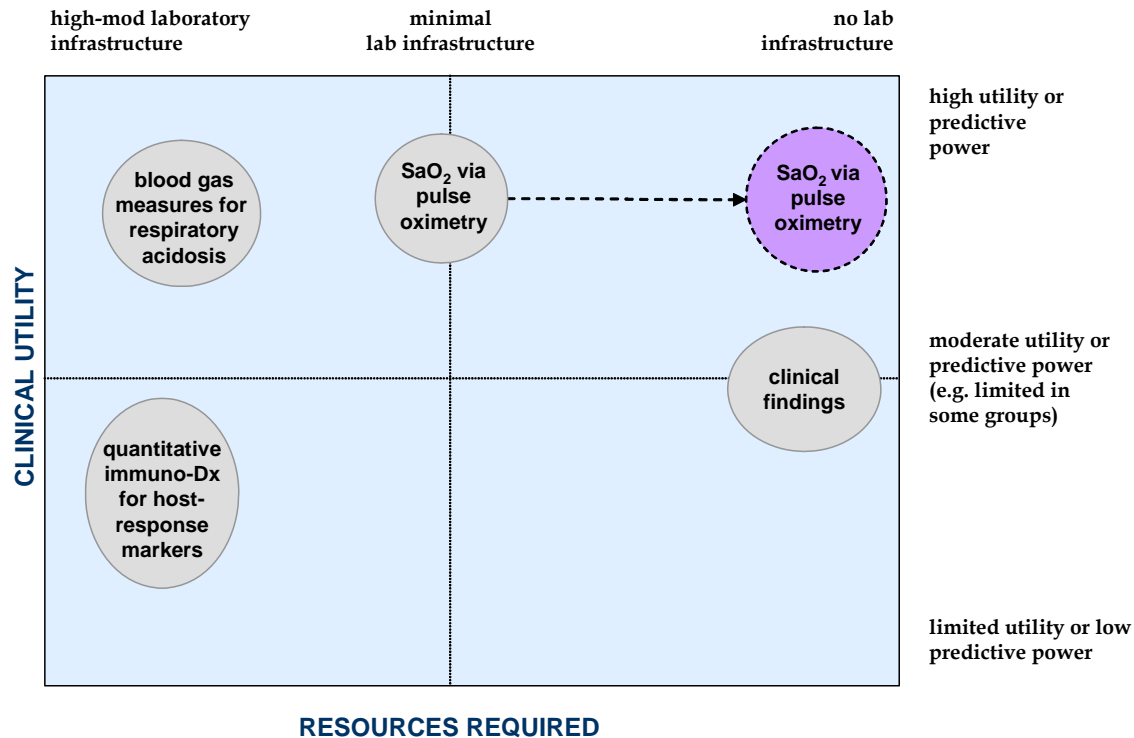


Figure 4. Future Approaches for Identifying Severe Cases of ALRI and Their Utility in Resource-limited Settings.



9C. Recommended Course of Action and Resources Required

There are several potential sets of activities that would be required to develop the future diagnostic tests for bacterial and severe ALRI, which are presented in the following sections.

Potential Path Forward 1: Pathogen-Specific Biomarkers that Distinguish Colonization from ALRI

In order to identify and develop RNA or protein biomarkers that are differentially expressed in bacteria that are merely colonizing an individual compared to causing ALRI, an extensive series of discovery and evaluation experiments would need to be conducted. An initial set of discovery experiments might be carried out using upper respiratory tract and lower respiratory tract specimens from children with *S. pneumoniae* and *H. influenzae* in the developed world. If a useful pattern is identified, it would then be necessary to repeat the experiments using samples from children who are served by resource-limited settings to determine if the identified pattern has sufficient predictive power in these settings. This follow-on set of experiments is likely to require the prospective collection of samples, because it is unlikely that sample banks (blood) already exist in which the RNA has been appropriately preserved. Because of the anticipated challenges in working with RNA as a biomarker type in resource-limited settings, it would be useful to determine if the differences in RNA levels using research methods could be detected as differences in protein levels using more practical immunodiagnostic methods. This might make it more feasible to develop a commercialization platform that is feasible for resource-limited settings. If a pattern was identified with useful predictive power, this method of detecting the colonizing species would still

need to be combined in a multiplex detection method utilizing all the other important bacterial and fungal species. Unfortunately, reports to date do not support the linear correlation between RNA and protein expression levels.

The biological risk is high, even though *S. pneumonia* and *H. influenzae* antigens, DNA, and live bacteria can be detected in blood, because it is not at all certain that a differential pattern of RNA expression or protein levels actually exists in colonizing vs. infecting bacteria, and that this pattern would be maintained in bacteria that have made their way into the blood stream. In addition, it is by no means certain that these biomarker classes occur in the blood stream of the vast majority of patients with pneumonia. The technology risk is high because it is likely that a very sensitive, multiplex and possibly quantitative technology would be needed to detect this pattern in the minute quantities of biomarkers that are found in the blood. In addition, if the biomarker type to be detected is RNA, this might pose an even greater challenge than other types of biomarkers that are more stable.

Protein biomarker discovery instead of RNA biomarkers is also possible. However, the availability of high quality protein biomarker discovery platforms is lacking. We have separately recommended improvements in basic technology for protein biomarker discovery which would open the door for an ALRI discovery effort. However, the probable concentration of organism-specific proteins would still render the biological risk as very high.

Because of the high degree of technological risk and biological risk in these approaches, we do not recommend that they be pursued at this time.

Potential Path Forward 2: General Host-Response Biomarkers

The initial biomarker discovery studies would aim to determine whether any known host-response proteins are differentially expressed, and whether any specific RNA or protein pattern in the blood cells could be used to distinguish bacterial, fungal, and viral pneumonia in children from the developed world. Additionally, it would be desirable to distinguish children with upper respiratory tract colonization from children with non-pneumonia infections and healthy children. If the results were promising, this experimental approach could be repeated on samples from children in the developing world. If banked samples exist (at this time we are unaware of such sample banks), RNA biomarker discovery experiments could be conducted immediately. If samples must be collected, sample collection and transport methods will be required. Protein markers can be investigated based upon RNA expression (a low likelihood) or determined based upon newer protein discovery platforms that we advocate. If any of the evaluated protein or RNA molecule combinations were found to have useful predictive power, then an appropriate assay for the biomarker types would need to be developed in a format that is appropriate for resource-limited settings. Because the assay would probably require a quantitative readout, it is reasonable to expect a longer development time for both the assay and the development of a field-usable reader than for individual markers. This biomarker evaluation approach has a moderate degree of both biological and technical risk. The biological risk is moderate because it is likely that there are known components of the innate immune system that respond differently to bacteria and virus (e.g. TREM-1), but this remains to be confirmed, especially in pediatric populations in the developing world. However, many aspects of the induction of the

innate immune response occur at the site of infection, and it is not clear that these changes can be detected in the circulation (e.g. in a finger prick specimen). The technological risk is moderate because a quantitative and multiplex measurement of host proteins or RNAs is likely to be required, and would ultimately need to be implemented in a format that is practical for resource-limited sites

Potential Path Forward 3: Volatile Organic Biomarkers in Breath Detected Using Electronic Noses

The discovery and validation of volatile organic biomarkers in breath samples may be one of the most promising paths forward. Given that the analytical technology already exists for discovery (e.g., an e-nose, or multi-capillary-column-ion-mobility-mass spectrometry)¹³⁴ it should be possible to complete initial biomarker discovery proof-of-concept experiments in the U.S. or Europe fairly rapidly in a lab that already has the capability. Then validation studies would need to be performed in populations that are served by resource-limited settings, which would almost certainly require prospectively-collected samples to be analyzed (unless sample banks exist that we are unaware of). If the biomarkers were validated, then it is likely that an engineering program would need to be initiated to improve the affordability and robustness of the existing analytical instrumentation. Although the program is likely to be more lengthy and costly than other possibilities, the potential advantages in the ease of use, no requirement for specimen preparation, and rapid turn around are extraordinarily attractive for resource-limited settings.

This experimental program has moderate degree of both biological and technical risk. The biological risk is moderate because it is not at all clear that ALRI caused by the important bacterial and fungal species could be distinguished from viral ALRI or other lung conditions, even though a number of researchers have already generated preliminary data which indicates that particular infective organisms can be identified by volatile organic compounds in breath samples. The effect of upper respiratory tract colonization on the predictive power of this approach remains to be determined. The technology risk is moderate because it is still unclear that the existing e-nose instruments have an adequate set of sensors that would be required for the discrimination necessary. There are already commercially available, portable MCC-IMS and e-nose instruments that could be used in the evaluation studies, and which might be migrated to a truly field-deployable commercialization platform.

Potential Path Forward 4: SaO₂ Measured Using Pulse Oximetry as a Biomarker of Severity

Evaluations of oxyhemoglobin saturation (SaO₂) as a referral tool in resource-limited settings could begin immediately, with existing pulse oximeter models. If SaO₂ is successfully validated as a useful biomarker for referral to a hospital, an engineering program could be initiated to improve the robustness and affordability of a pulse oximeter that is designed for use in resource-limited settings. Such an instrument might be available for distribution relatively quickly, and the cost-benefits of waiting for this more robust and affordable instrument could be weighed against the option of distributing an existing model immediately.

Summary of Recommendations

In summary, we recommend that the gaps in knowledge about the relevant microbiological epidemiology be filled, and that completely new approaches be taken to develop a diagnostic test that identifies bacterial and fungal versus viral pneumonia given the low likelihood that current approaches will ever provide the necessary performance in resource-limited settings. The biological limitations on the predictive power of the known pathogen-specific biomarkers, and the logistic limitations on specimen types, are hurdles that will be extremely challenging for existing approaches to overcome, and we do not recommend pursuing the path of developing a diagnostic based upon the pathogen-specific biomarkers that are now known. We recommend that efforts be made to determine if a host response biomarker, either protein or RNA, with sufficient diagnostic power can be identified. When considering the new approaches that might be taken, the intriguing area of breath analysis is worth pursuing. For a test that could be used as a tool to identify severe ALRI that should be referred to a hospital, a promising biomarker (SaO₂) and measurement approach (pulse oximetry) already exist, but the validation studies remain to be conducted. In addition, we recommend the initiation of engineering programs to reduce the resources required to implement this approach.

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