Laboratory Methods for Determining Pneumonia Etiology in Children

David R. Murdoch,1,2 Katherine L. O’Brien,3 Amanda J. Driscoll,3 Ruth A. Karron,4 Niranjan Bhat,3,5 The Pneumonia Methods Working Group,a and the PERCH Core Teamb

1Department of Pathology, University of Otago, and 2Microbiology Unit, Canterbury Health Laboratories, Christchurch, New Zealand; 3International Vaccine Access Center, 4Center for Immunization Research, Department of International Health, Johns Hopkins Bloomberg School of Public Health, and 5Division of Infectious Diseases, Department of Pediatrics, Johns Hopkins School of Medicine, Baltimore, Maryland

Laboratory diagnostics are a core component of any pneumonia etiology study. Recent advances in diagnostic technology have introduced newer methods that have greatly improved the ability to identify respiratory pathogens. However, determining the microbial etiology of pneumonia remains a challenge, especially in children. This is largely because of the inconsistent use of assays between studies, difficulties in specimen collection, and problems in interpreting the presence of pathogens as being causally related to the pneumonia event. The laboratory testing strategy for the Pneumonia Etiology Research for Child Health (PERCH) study aims to incorporate a broad range of diagnostic testing that will be standardized across the 7 participating sites. We describe the current status of laboratory diagnostics for pneumonia and the PERCH approach for specimen testing. Pneumonia diagnostics are evolving, and it is also a priority of PERCH to collect and archive specimens for future testing by promising diagnostic methods that are currently under development.

Laboratory diagnostics are the cornerstone of any pneumonia etiology study. In developing the microbial testing strategy for the Pneumonia Etiology Research for Child Health (PERCH) study, we sought to incorporate traditional and state-of-the art diagnostics to target a broad range of recognized and putative pathogens, including bacteria, viruses, mycobacteria, and fungi. The intention was to perform as much testing as possible at each of the 7 study sites and to make considerable efforts to ensure across-site standardization of testing methods. The aim was to be relatively assumption-free in applying testing methods to potentially identify previously unrecognized roles for known pathogens. Pneumonia diagnostics are evolving, and it is a priority of PERCH to collect and archive specimens that would be available for future testing with promising diagnostic methods that are currently under development.

In this article, we review the challenges faced on the pathway to achieving these aspirations for the PERCH diagnostic testing strategy. We first review what is known about the state of laboratory diagnostics for determining the etiology of pneumonia, with a particular emphasis on childhood pneumonia and the complexities of assigning causation. We then discuss the PERCH diagnostic testing strategy and the decisions encountered in developing the final approach, taking into consideration logistic, technical, and financial constraints.

LABORATORY DIAGNOSTIC TESTING METHODS FOR PNEUMONIA

The routine laboratory evaluation of patients with pneumonia continues to rely on methods that have been used for decades: microscopy and culture of respiratory tract specimens, blood cultures, detection of antigens in
Microscopy and Culture

Microscopy and culture of sputum or other lower respiratory tract specimens (eg, bronchoalveolar lavage fluid) and blood cultures have historically been the main diagnostic tools for identifying the microbial etiology of pneumonia. The identification of respiratory pathogens in high-quality specimens collected directly from the site of infection or from a normally sterile site (eg, blood) provides good evidence of likely causative microorganisms.

Lower respiratory specimens are typically cultured on standard microbiological media, such as the combination of blood, chocolate, and MacConkey agars, which will isolate most common bacterial pneumonia pathogens. Some bacteria require special media (eg, Legionella species; buffered charcoal yeast extract-based media) or cell cultures (eg, Chlamydia pneumoniae), or cannot be readily cultured in a diagnostic laboratory (eg, Mycoplasma pneumoniae).

The process of specimen collection has a huge impact on microscopy and culture results and their interpretation. Lower respiratory specimens can be contaminated by upper respiratory secretions during the collection process, or the specimen collected may comprise mainly upper respiratory tract secretions. This is especially true for sputum (both expectorated and induced), and it can lead to the incorrect conclusion that an upper airway colonizer is a pneumonia pathogen. As a result, sputum specimens should be checked for quality before processing, confirming that they have been obtained from the lower respiratory tract. This typically involves assessing the number of squamous epithelial cells (SECs) and polymorphonuclear cells (PMNs) in a Gram stain smear of the specimen. The presence of <10 SECs and >25 PMNs per low-power field (magnification, ×100) [2], or ≥10 leukocytes for each SEC [3], is regarded as being indicative of a high-quality expectorated sputum specimen in adults. Sputa, which contain relatively low numbers of PMNs and high numbers of SECs, are likely to represent oropharyngeal contamination. Despite the ongoing debate about the utility of sputum culture, there is evidence that prompt examination of a high-quality sputum specimen collected before antibiotic treatment has reasonably high sensitivity and specificity for pneumococcal pneumonia in adults [3]. However, the application of these criteria to sputum specimens (including induced sputum) from children is still undetermined.

Direct immunofluorescence microscopy and isolation in cell cultures have been the standard diagnostic approach for the detection of viral pathogens in respiratory specimens. However, increasingly these methods are being replaced by more sensitive, rapid, and/or less labor-intensive nucleic acid detection methods. The rapid detection of Pneumocystis jirovecii infection still relies on microscopy of respiratory tract samples using special stains [4].

Blood cultures are an important diagnostic tool for pneumonia, but only a minority of patients with pneumonia have documented bloodstream infections. Among patients admitted to hospital with community-acquired pneumonia, 7%–13% of adults [5–8] and 1%–5% of children [9–14] have documented bacteremia, with a higher prevalence among those with more severe disease.

Antigen Detection

Assays to detect microbial antigens in body fluids have long been used for the diagnosis of respiratory infections, and these methods are the diagnostic tools most easily applied as point-of-care tests. However, the development of clinically useful assays is reliant on the discovery of suitable antigens that are present in detectable quantities in clinical specimens. To date, commercial assays have only been developed for a limited range of pathogens, particularly the detection of selected bacterial pathogens in urine and the detection of viruses in respiratory specimens.

Among bacterial respiratory pathogens, assays for Streptococcus pneumoniae and Legionella pneumophila are the most developed. An immunochromatographic test that detects the C-polysaccharide cell wall antigen in urine (BinaxNOW) has
been an important advance in the diagnosis of pneumococcal disease [15]. This test has a sensitivity of 70%–80% and a specificity of >90% compared with conventional diagnostic methods for detection of pneumococcal pneumonia in adults. Unfortunately, the BinaxNOW test cannot be used reliably in children because it also detects pneumococcal carriage. In a variety of studies from developed and developing countries, 22%–67% of healthy children who were documented pneumococcal carriers have positive BinaxNOW results [16–20]. Recently, the BinaxNOW test has proven to be useful in rapidly detecting \textit{S. pneumoniae} in pleural fluid [21, 22] and in improving the sensitivity of blood cultures for \textit{S. pneumoniae} [23], although the latter application has not been fully validated.

Detection of soluble \textit{Legionella} antigen in urine is an established and valuable tool for the diagnosis of Legionnaires’ disease, although current commercial assays can only reliably detect infection caused by \textit{L. pneumophila} serogroup 1 [24]. Some assays claim to detect other legionellae [25], although performance is suboptimal.

Detection of respiratory viral antigens in respiratory secretions has become an important diagnostic tool [26–28], with commercial rapid diagnostic tests widely used for the detection of influenza or respiratory syncytial virus (RSV) directly in respiratory specimens. The sensitivity of rapid tests for the detection of seasonal influenza in clinical specimens varies widely, ranging from 10% to 96% [29, 30] depending on virus type or subtype, timing of specimen collection, specimen type, patient age, and the test comparator [31, 32]. Specificity for influenza is 90%–100%. Commercial RSV rapid diagnostic tests have sensitivities of 71%–95% and specificities of 80%–100% compared with culture [33–35].

**Nucleic Acid Detection**

Nucleic acid detection tests, such as PCR, have many features that make them attractive tools for diagnosing the etiology of respiratory tract infections [36]. These tests can detect very low levels of nucleic acid from potentially all respiratory pathogens, do not depend on the viability of the target microbe, can provide results within a clinically relevant time frame, are probably less affected by prior antibiotic administration than culture-based methods, and have the potential to provide supplemental information such as the presence of antibiotic resistance genes. Nucleic acid detection assays have been particularly useful for diagnosing infections that are difficult or impossible to rapidly diagnose by other methods.

Although nucleic acid detection tests have been widely applied to the diagnosis of respiratory tract infections, only recently have commercial assays become available. These assays have particularly contributed to understanding the role of respiratory viruses, \textit{Legionella} species, and \textit{C. pneumoniae} in acute respiratory infections [1]. Nucleic acid detection tests can also be used for determining the microbial etiology of pneumonia [37]. The recent development of an integrated sample processing and nucleic acid amplification test for \textit{Mycobacterium tuberculosis} and rifampicin resistance has the potential to greatly improve the diagnosis of pulmonary tuberculosis [38].

An inherent problem with all nucleic acid detection tests, which are likely to have higher clinical sensitivity than most traditional diagnostic tools, is the lack of a suitable comparator gold standard. Consequently, a positive nucleic acid detection test result cannot necessarily be dismissed as being falsely positive simply because a less sensitive comparison test is negative.

Very high analytical sensitivity (lower limit of detection; the smallest amount of target that can be accurately detected by the assay) is often used to promote nucleic acid detection assays, despite the fact that high analytical sensitivity does not guarantee high clinical sensitivity (the proportion of true positive patient samples that are correctly identified by the assay) [39]. Although it is desirable for an assay to have a high analytical sensitivity, the biological and diagnostic relevance beyond a certain level may be minimal.

**Antibody Detection**

Antibody detection assays exist for most respiratory tract pathogens and have provided useful epidemiological data. However, because of the need in most cases to test both acute and convalescent sera collected several weeks apart to document a ≥4-fold rise in reciprocal antibody titers, these assays have a limited impact on clinical decision making. Measurement of antibody titers in only acute serum samples is rarely sufficient for diagnostic purposes. Furthermore, most serological assays for respiratory pathogens have suboptimal sensitivity and specificity. In young infants, rises in pathogen-specific antibody may be masked or suppressed by the presence of maternal antibody. For a small number of respiratory infections, such as \textit{M. pneumoniae} infection, antibody detection remains an important diagnostic tool while improved alternative methods are being developed.

Despite limited use in the management of pneumonia, serological techniques continue to have a useful role in etiology studies. When used in comprehensive etiology studies, serology identifies infections that were not detected by other methods such as culture and PCR. For example, in 1 adult pneumonia study, 29 of 88 cases of viral pneumonia were diagnosed by serology alone [40]. Serology may be particularly useful for diagnosing respiratory viral infections that were the trigger for subsequent secondary bacterial pneumonias, because the viral pathogen may no longer be detectable in respiratory specimens. In addition, collection of acute and convalescent sera may be useful in novel pathogen discovery efforts because demonstration of seroconversion to a putative pneumonia pathogen supports the case for its pathogenicity.
**Ongoing Developments**

Most developmental work in respiratory pathogen diagnostics has focused on improving existing methods, particularly antigen and nucleic acid detection assays. More user-friendly formats that minimize the number of manual steps are becoming commercially available, some of which could potentially be used as point-of-care tests. Until recently, nucleic acid detection assays were only available as in-house assays. Now, there are several commercially available multiplex assays that can simultaneously detect most commonly recognized respiratory pathogens [37]. Rapid detection of respiratory viruses has been the driver for development of these assays, and further clinical studies are needed to establish their utility for all pathogens in the context of pneumonia. The role of quantitative PCR needs to be clarified, because a cutoff of clinical significance may help differentiate between carriage and disease, or incidental upper respiratory tract infection (URT) and pneumonia [41].

New approaches for respiratory pathogen detection are desperately needed. Breath analysis is an exciting new area with enormous diagnostic potential. Alveolar breath contains many biomarkers derived from the blood by passive diffusion across the alveolar membrane [42], and breath testing is noninvasive, easily repeatable, and requires minimal specimen workup. The use of breath analysis for the investigation of respiratory infections has not yet been extensively evaluated. Potential biomarkers have been reported for some respiratory pathogens, including *Aspergillus fumigatus* [43, 44] and *M. tuberculosis* [45, 46], but it is still uncertain whether they will prove to be useful as clinical diagnostic tools for determining pneumonia etiology.

**DIFFICULTIES ASSIGNING PNEUMONIA CAUSATION**

Despite the availability of an array of diagnostic methods and recent technological advances, interpretation of diagnostic tests for determining the etiology of pneumonia can still be challenging [39]. Simply detecting a potential pathogen in the upper or lower respiratory tract from a patient with pneumonia does not necessarily mean that it is the cause of pneumonia. Some pneumonia pathogens can also colonize the upper airways of healthy individuals (eg, *S. pneumoniae*). Therefore, distinguishing colonization from infection is a major challenge when these organisms are detected in sputum specimens. Quality checks based on the relative numbers of SECs and PMNs seen in a sputum Gram stain smear may help in this regard by assessing upper airway contamination [2], especially if correlated with sputum culture results, but still require careful interpretation.

All of the major viruses that cause pneumonia are more commonly associated with nonpneumonic URTI, and virus shedding can occur for a long period of time after symptoms have disappeared. Some viral URTIs occur at the same time as pneumonia, including (1) immediately causal (primary viral pneumonia), (2) in the causal pathway (eg, influenza URTI followed by pneumococcal pneumonia), or (3) incidental. In a study setting, these can be distinguished at a population level by determining the background prevalence of nasopharyngeal viral infection in a control group. Consequently, pneumonia etiology studies that test for viruses in nasopharyngeal specimens should use control groups to assess the likelihood of false-positive results. In addition, the use of quantitative methods (such as quantitative PCR) may provide evidence that a particular organism is causing pneumonia (eg, by demonstrating a higher microbial load in lower compared with upper respiratory specimens, or in cases compared with controls). This is an area that requires further research.

The detection of multiple potential pathogens in a single patient can also present problems with assigning causality. Which is the true pathogen, or do all have a role in pneumonia pathogenesis? This situation is more likely to occur with use of multiple sensitive testing methods and multiplex PCR assays.

**LABORATORY TESTING STRATEGY FOR THE PERCH STUDY**

The laboratory testing strategy for the PERCH project is shown in Table 2. This strategy was assembled after an extensive review process that involved consultation with the study’s expert Pneumonia Methods Working Group and site investigators.

**Microscopy/Culture**

Standard microbiological methods will be used for culturing blood, induced sputum, pleural fluid, lung aspirates, and postmortem tissue. Blood cultures will be processed on automated blood culture systems with bottles incubated for at least 5 days, unless positive. Because we are cognizant of the potential difficulties with interpretation, induced sputum specimens will be screened by microscopic assessment of numbers of PMNs and SECs. However, we will not be rejecting specimens on this basis because no screening method has been rigorously evaluated in children with pneumonia. The collection of induced sputum is discussed in detail in accompanying publications [47, 48]. Antimicrobial susceptibility testing will be performed on clinically relevant bacterial isolates according to Clinical and Laboratory Standards Institute guidelines [49].

Although culture of *S. pneumoniae* from the nasopharynx may represent coincidental carriage, some pneumococcal serotypes (eg, serotype 1) are strongly associated with invasive disease and have a high positive predictive value for causation if isolated from cases with pneumonia. Furthermore, the negative predictive value of nasopharyngeal specimens for *S. pneumoniae*...
may also be high and allow for inferences about unlikely etiologies. Pneumococcal quantitative culture from the nasopharynx is likely to serve an increasingly important role in drawing inferences about causal associations with lower respiratory tract disease. For these reasons, nasopharyngeal swabs will be collected for isolation of *S. pneumoniae* using established methods [50]. The nasopharyngeal specimens will be stored for use in the future for quantitative, molecular, or other diagnostic approaches.

The role of *M. tuberculosis* and nontuberculous mycobacteria in severe childhood pneumonia was identified as a priority of PERCH. Consequently, the testing strategy was designed for maximal sensitivity rather than maximal rapidity of diagnosis. Our approach is anchored on standard microscopy and broth-based culture methods for detection and antimicrobial susceptibility testing of mycobacteria in induced sputa, lung aspirates, pleural fluid, gastric aspirates, and postmortem lung tissue.

**Nucleic Acid Detection**

A central element of the PERCH testing strategy is to deploy a multiplex PCR assay to test all respiratory specimens for major recognized pneumonia pathogens. The use of PCR is the most significant recent advance in pneumonia diagnostics and is the major distinction from previous multicenter pneumonia etiology studies. Considerable efforts were focused on choosing the assay for PERCH as described elsewhere [37], with emphasis placed on an assay that could be deployed at each study site. In the end, a multiplex real-time PCR assay targeting an extensive array of potential pathogens (Table 3) will be used to test all induced sputa, combined nasopharyngeal/oropharyngeal swabs, lung aspirates, pleural fluid, and postmortem lung tissue. Furthermore, standards of known concentration will be run to provide quantitative data for all detected pathogens. Although they have yet to be fully evaluated, we believe that these quantitative data may help provide evidence for causation by distinguishing colonization from infection.

Testing blood specimens by PCR has provided inconsistent results in previous pneumonia studies [36]. However, recent studies indicate that detection of *S. pneumoniae* in blood by PCR may be a useful adjunct to other methods for diagnosing

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>Testing Methods</th>
<th>Target Pathogens</th>
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<tbody>
<tr>
<td>Whole blood</td>
<td>Culture</td>
<td>Bacteria</td>
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<tr>
<td>Serum</td>
<td>Uniplex PCR</td>
<td><em>Streptococcus pneumoniae</em></td>
</tr>
<tr>
<td>Combined nasopharyngeal</td>
<td>Antibiotic detection</td>
<td>Selected respiratory pathogens</td>
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<tr>
<td>and oropharyngeal swabs</td>
<td>Antibiotic bioassay</td>
<td>N/A</td>
</tr>
<tr>
<td>Nasopharyngeal swab</td>
<td>Multiplex PCR</td>
<td>Bacteria, viruses, and <em>Pneumocystis jirovecii</em></td>
</tr>
<tr>
<td>Induced sputum*</td>
<td>Microscopy/culture, multiplex PCR, mycobacterial culture</td>
<td>Bacteria, mycobacteria, viruses, and <em>P. jirovecii</em></td>
</tr>
<tr>
<td>Lung aspirate*</td>
<td>Microscopy/culture, multiplex PCR, mycobacterial culture</td>
<td>Bacteria, mycobacteria, viruses, and <em>P. jirovecii</em></td>
</tr>
<tr>
<td>Pleural fluid*</td>
<td>Microscopy/culture, antigen detection, multiplex PCR, mycobacterial culture</td>
<td>Bacteria, mycobacteria, viruses, and <em>P. jirovecii</em></td>
</tr>
<tr>
<td>Postmortem lung tissue*</td>
<td>Microscopy/culture, multiplex PCR, mycobacterial culture</td>
<td>Bacteria, mycobacteria, viruses, and <em>P. jirovecii</em></td>
</tr>
<tr>
<td>Gastric aspirate*</td>
<td>Mycobacterial culture (if no induced sputum obtained)</td>
<td>Mycobacteria</td>
</tr>
<tr>
<td>Urine</td>
<td>Antibiotic bioassay</td>
<td>N/A</td>
</tr>
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Abbreviations: N/A, not applicable; PCR, polymerase chain reaction; PERCH, Pneumonia Etiology Research for Child Health study.

* Cases only.

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**Table 3. Target Pathogens for the PERCH Multiplex Polymerase Chain Reaction Assay**

<table>
<thead>
<tr>
<th>RNA Targets</th>
<th>DNA Targets</th>
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<tbody>
<tr>
<td>Influenza viruses A, B, and C</td>
<td>Adenoviruses</td>
</tr>
<tr>
<td>Respiratory syncytial virus A and B</td>
<td>Bocavirus</td>
</tr>
<tr>
<td>Parainfluenza viruses 1–4</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>Rhinoviruses</td>
<td><em>Streptococcus pneumoniae</em></td>
</tr>
<tr>
<td>Enteroviruses</td>
<td><em>Haemophilus influenzae</em> (all strains)</td>
</tr>
<tr>
<td>Coronavirus OC43, 229E, NL63, HKU1</td>
<td><em>H. influenzae</em> type b</td>
</tr>
<tr>
<td>Human metapneumovirus</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>Parechovirus</td>
<td>Mycoplasma pneumoniae</td>
</tr>
<tr>
<td>Legionella species</td>
<td>Chlamydia pneumoniae</td>
</tr>
<tr>
<td>Chlamydophila pneumoniae</td>
<td><em>Moraxella catarrhalis</em></td>
</tr>
<tr>
<td>Klebsiella species</td>
<td><em>Salmonella species</em></td>
</tr>
<tr>
<td>Salmonella species</td>
<td><em>Bordetella pertussis</em></td>
</tr>
<tr>
<td>Pneumocystis jirovecii</td>
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Abbreviation: PERCH, Pneumonia Etiology Research for Child Health study.
invasive pneumococcal disease [51, 52]. Consequently, PCR testing of blood in PERCH is restricted to *S. pneumoniae* alone as a uniplex assay. A standard quantitative real-time PCR assay targeting the *lyt* gene will be used for this purpose. Testing blood specimens for other pneumonia pathogens by PCR may be explored at a later time.

The decision to also use molecular diagnostic methods for detecting mycobacteria was deferred while awaiting assays that are more relevant to the pneumonia etiology objective of the study; archived specimens will be available for testing.

### Serology

Acute and convalescent sera will be collected from all cases. Given the limited volume of serum that will be collected from each child, the decision about which specific assays will be performed has been deferred.

### Antigen Detection

At present, few antigen detection assays have established roles in the context of childhood pneumonia. For PERCH, use will initially be restricted to the BinaxNOW test for detecting *S. pneumoniae* in pleural fluid and in blood cultures that are alarm-positive but culture-negative. Testing of urine by the BinaxNOW test will not be performed because of concerns about false-positive results due to pneumococcal carriage in children. *Legionella* urinary antigen testing will also be deferred; this will be reviewed based on positive *Legionella* results from the multiplex PCR testing of respiratory specimens. It is also anticipated that archived specimens from PERCH will be used to evaluate promising new antigen detection assays.

### Antibiotic Bioassay

As discussed in detail elsewhere [53], serum and urine specimens will be tested by a standard bioassay for antibiotic activity, thereby providing an objective assessment of prehospital antibiotic use. These data will be used in conjunction with other laboratory data, particularly to assess any potential impact of antibiotics on culture results.

### Control Specimens

The testing of specimens from control subjects will help with the interpretation of some test results, particularly the PCR testing of upper respiratory tract specimens. The specimens to be collected and tests to be performed are listed in [Table 2](#). Serum will also be used to establish some population-specific serological parameters. The positivity for specific pathogens in subjects with pneumonia and in control subjects will be incorporated into statistical modeling of pneumonia etiology. Upper respiratory tract specimens from control subjects will help establish whether a detected organism is causally associated with a disease state, at least on a population basis.

In summary, PERCH will collect up to 10 different specimens in an estimated 6300 pneumonia cases and up to 5 different specimens in 7000 controls. In addition, specimens will be stored for future testing when new, more powerful tests become available. Standardized methods will ensure comparability across sites. This makes PERCH one of the largest pneumonia etiology studies undertaken thus far.

### Notes

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<tr>
<th>Name</th>
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<tbody>
<tr>
<td>Robert B. Black, Zulfiqar A. Bhutta</td>
<td><em>Pneumonia Methods Working Group</em></td>
<td>Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland</td>
</tr>
<tr>
<td>Paul Martin, James P. Nataro, Franco M. Piazza, Shamim A. Qazi, and Heather J. Zar.</td>
<td><em>PERCH Core Team</em></td>
<td>Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland</td>
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