LONDON
SCHOOL of
HYGIENE
&TROPICAL
MEDICINE

Etiology of Severe Childhood Pneumonia in The Gambia, West Africa, Determined by Conventional and Molecular Microbiological Analyses of Lung and Pleural Aspirate Samples

Stephen R. C. Howie,[1,a] Gerard A. J. Morris,[1,a] Rafal Tokarz,[2] Bernard E. Ebruke,[1] Eunice M. Machuka,[1] Readon C. Ideh,[1] Osaretin Chimah,[1] Ousman Secka,[1] John Townend,[1] Michel Dione,[1] Claire Oluwalana,[1] Malick Njie,[3] Mariatou Jallow,[3] Philip C. Hill,[4] Martin Antonio,[1] Brian Greenwood,[5] Thomas Briese,[2] Kim Mulholland,[5] Tumani Corrah,[1] W. Ian Lipkin,[2] and Richard A. Adegbola[1,6]

[1]Medical Research Council Unit, Fajara, Republic of The Gambia; [2]Center for Infection and Immunity, Columbia University, New York, New York; [3]Ministry of Health and Social Welfare, Banjul, Republic of The Gambia; [4]Centre for International Health, University of Otago, Dunedin, New Zealand; [5]London School of Hygiene and Tropical Medicine, United Kingdom; and [6]GlaxoSmithKline Vaccines, Wavre, Belgium

(See the Editorial Commentary by Graham on pages 686–7.)

Molecular analyses of lung aspirates from Gambian children with severe pneumonia detected pathogens more frequently than did culture and showed a predominance of bacteria, principally *Streptococcus pneumoniae*, >75% being of serotypes covered by current pneumococcal conjugate vaccines. Multiple pathogens were detected frequently, notably *Haemophilus influenzae* (mostly nontypeable) together with *S. pneumoniae*.

Keywords. etiology; pneumonia; children; Gambia; lung aspirate.

Pneumonia remains the leading cause of death in children worldwide. A better understanding of the range of pneumonia pathogens is needed to reduce child mortality further [1], but this is hampered by limitations at the bedside and in the laboratory. Pneumonia etiologic studies have depended largely on blood culture, which is insensitive [2]. Direct percutaneous aspiration from the site of lung infection, rarely done despite its good safety record, yields the highest-quality clinical specimen, but even then bacterial culture identifies a pathogen in no more than half of cases [3].

Molecular methods have advantages over conventional methods for the detection and characterization of pathogens in clinical samples, and hold promise for improving diagnostic sensitivity [4]. We describe the application of both approaches to the detection of pathogens in lung and pleural aspirates obtained from children with severe pneumonia in a West African setting. The objective of the study was to elucidate the etiology of severe pneumonia in this group more comprehensively than has been possible previously.

METHODS

The Gambia is a West African country of 1.8 million people with a human immunodeficiency virus (HIV) infection prevalence of <2%. A study of the etiology of severe childhood pneumonia was undertaken in the coastal area of The Gambia (Supplementary Appendix 1). At the time of the study (2007–2009), there was high coverage with conjugate *Haemophilus influenzae* type b (Hib) vaccine but no routine usage of pneumococcal conjugate vaccine (PCV), which is the case currently in many African countries. Cases were children aged 2–59 months with severe pneumonia defined clinically by modified World Health Organization (WHO) criteria (cough or difficulty in breathing, plus any of the following: lower chest wall indrawing, nasal flaring, or an oxygen saturation of <90% on pulse oximetry). Participants were recruited from the Medical Research Council (MRC) hospital in Fajara; the Royal Victoria Teaching Hospital in Banjul; and the major health centers at Fajikunda, Serekunda, and Brikama. Children with a cough of ≥2 weeks, or severe anemia (hemoglobin level <6 g/dL) or confirmed wheeze were excluded. Radiological pneumonia was defined using WHO criteria (ie, endpoint consolidation or pleural effusion). HIV testing was done if informed consent was given after standard counseling. A standard WHO guideline–based antibiotic regimen was used. Written informed consent was obtained for participation in the study from parents or guardians. The study was approved by the Gambian government/MRC Joint Ethics Committee (SCC/EC1062).

Details of lung aspiration in our setting over a period of 25 years, during which there have been no associated serious

adverse events, have been described previously [5]. Participants underwent lung aspiration if they had accessible consolidation adjacent to the chest wall, had no contraindications, and written informed consent had been obtained. Pleural aspiration was undertaken in those with pleural effusions.

Culture, nonmolecular serotyping, HIV testing, singleplex polymerase chain reaction (PCR) for *lytA* and *cpsA* (for *Streptococcus pneumoniae*) and *glpQ* (for *H. influenzae*), 16S rRNA PCR, multilocus sequence typing (MLST), molecular serotyping, and multiplex fast-track 33 PCR, all using standard methods (Supplementary Appendix 2) [2, 6–13], were performed at the MRC Unit in The Gambia, and multiplex MassTag PCR [14] was performed at Columbia University. Multiplex pathogen targets are listed in Supplementary Appendix 3. Laboratory analyses were done blinded and independent of each other.

Summary results of organisms identified to genus or species level using 1 or more detection methods were compiled. Demographic and clinical data were double-entered into an SQL database (Microsoft Corporation) and verified, and laboratory data were entered into an Access (Microsoft Corporation) database, cross-checked, and verified. Statistical analyses were done using Stata software, version 11 (StataCorp).

RESULTS

Fifty-five children, representing 74% of the radiological severe pneumonias and 26% of all clinical severe pneumonia cases identified in the study period, underwent lung aspiration (n = 47) or pleural fluid aspiration (n = 9); 1 participant underwent 2 aspirates at different time points (Supplementary Appendix 4). HIV testing was done in 33 of 55 (60%) participants, 2 of whom were positive for HIV type 1. The characteristics of those who underwent lung or pleural aspiration were similar to the radiological pneumonia group from which they were drawn (Supplementary Appendix 5).

Pathogens were identified to genus or species level in 53 of 56 (95%) samples by 1 or more laboratory methods (Table 1). An organism was cultured from 21 of 56 (38%) specimens: *S. pneumoniae* in 14 (25%), *H. influenzae* (all non–type b on latex agglutination) in 3 (5%), and *Staphylococcus aureus* in 3 (5%). Ziehl-Neelsen staining was done in 37 of 56 (66%) lung aspirate samples (all negative); 35 of 37 (95%) underwent culture for *Mycobacterium tuberculosis*, and all were negative.

Streptococcus pneumoniae was detected by 1 or more molecular assays (Supplementary Appendix 6) in 48 of 53 (91%) samples and in 2 or more assays in 36 of 53 (68%) samples. Molecular pneumococcal serotyping (40 serotypes; Supplementary Appendix 7) showed the following prevalences: serotype 1, 22%; serotype 4, 18%; serotype 14, 18%; serotype 5, 16%;

Table 1. Organisms Identified to at Least Genus Level Detected in 53 Lung and Pleural Aspirate Specimens Examined by Both Culture and Molecular Assays Obtained From 52 Children With Severe Pneumonia

Organism	No.	(%)
Commonly recognized community-acquired pneumonia pathogens[a,b]		
Streptococcus pneumoniae	48	91
Haemophilus influenzae	12	23
Staphylococcus aureus	3	6
Klebsiella species	2	4
Streptococcus species (non-*pneumoniae*)	2	4
Bocavirus	2	4
Respiratory syncytial virus	2	4
Adenovirus	2	4
Enterovirus B	1	2
Coronavirus HKU1	1	2
Influenza C	1	2
Cytomegalovirus	1	2
Uncommonly reported community-acquired pneumonia pathogens		
Acinetobacter species[c]	3	6
Enterobacter species[c]	2	4
Salmonella species[d]	2	4
Streptococcus pseudopneumoniae[e]	1	2
Bacteroides species[f]	1	2
Prevotella species[f]	1	2
Codetection		
1 organism only	24	45
2 or more organisms	28	53
No organism	1	2
Bacterial organism(s) only	41	77
Viral organism(s) only	1	2
Bacterial–bacterial codetection	21	40
Bacterial–viral codetection	10	19
S. pneumoniae and *H. influenzae*	11	21
S. pneumoniae and *S. aureus*	1	2
S. aureus and *H. influenzae*	1	2

[a] McIntosh K. Community-acquired pneumonia in children. N Engl J Med **2002**; 346:429–37.

[b] Ruuskanen O, Lahti E, Jennings LC, Murdoch DR. Viral pneumonia. Lancet **2011**; 377:1264–75.

[c] Jones RN. Microbial etiologies of hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia. Clin Infect Dis **2010**; 51(suppl 1): S81–7.

[d] Genzen JR, Towle DM, Kravetz JD, Campbell SM. *Salmonella typhimurium* pulmonary infection in an immunocompetent patient. Conn Med **2008**; 72:139–42.

Mankhambo LA, Chiwaya KW, Phiri A, Graham SM. Lobar pneumonia caused by nontyphoidal *Salmonella* in a Malawian child. Pediatr Infect Dis **2006**; 25:1190–92.

[e] Keith ER, Podmore RG, Anderson TP, Murdoch DR. Characteristics of *Streptococcus pseudopneumoniae* isolated from purulent sputum samples. J Clin Microbiol **2006**; 44:923–7.

[f] Bartlett JG. Anaerobic bacterial infections of the lung and pleural space. Clin Infect Dis **1993**; 16(suppl 4):S248–55.

serotype 6A/B/C, 4%; and serotype 9V/A, 2%. Ten- and 13-valent PCVs both include serotypes that account for 76%–80% of serotypes identified in the samples in this study.

Haemophilus influenzae was detected in 12 of 53 (23%) samples. Four of the 12 had sufficient deoxyribonucleic acid (DNA) for full MLST typing, 3 being nontypeable *H. influenzae* (NTHi), whereas 1 in a child with HIV infection had a serotype b infection. Two of the 12 had sufficient loads for 5 or 6 of the standard 7 alleles of MLST to be defined, and these were suggestive of NTHi, 1 being in a culture-positive case confirmed as being non–type b by latex agglutination assay. *Staphylococcus aureus* was detected in 3 of 53 (6%) samples, all of which were pleural fluid specimens. One or more other bacteria were identified to genus or species level in 8 of 53 (15%) samples. Viruses, led by respiratory syncytial virus, adenovirus, and bocavirus, were detected by 1 or more methods in 10 of 53 (19%) samples.

Half (28/53 [53%]) of samples had >1 organism detected to genus or species level, predominantly 2 or more bacterial species. Codetection of *S. pneumoniae* and *H. influenzae* occurred in 11 of 53 (21%) samples, *H. influenzae* having the higher bacterial load in 4 of 11 samples. In the 3 specimens positive for *S. aureus*, low loads of *S. pneumoniae* were detected in 2 and of *H. influenzae* in the other.

DISCUSSION

This study showed that *S. pneumoniae* was the predominant pathogen in this group of children with radiologic pneumonias, followed by *H. influenzae*, *S. aureus*, a range of gram-negative bacteria, and a few viruses. Potentially causative pathogens were found in all but 1 sample (98%), in contrast to the use of culture alone, which yielded an organism in 38% of specimens. *Streptococcus pneumoniae* and *H. influenzae*, predominantly NTHi, were detected together in around 1 in 5 cases in this Hib-vaccinated population. Current PCVs include >75% of serotypes identified in these samples.

Gambian studies of pneumonia etiology in the pre-Hib vaccine era showed a predominance of *S. pneumoniae* followed by Hib, as have other low-income country studies, including a recent lung aspirate study from Malawi using PCR [15, 16]. The role of NTHi in pneumonia has been raised before [17] and has been controversial [15].

It is likely that this study's findings are relevant to similar patient groups in other developing-country settings, especially those where conjugate Hib vaccine is routinely used and where PCVs have not yet been introduced. The multicountry Pneumonia Etiology Research for Child Health (PERCH) study will provide important data in this respect [4]. The prominence of *S. pneumoniae* but low frequency of Hib in our study appears to contrast with recent Global Burden of Disease 2010 estimates [18].

The strength of this study is its detailed analysis of the best possible specimens for diagnosing the cause of pneumonia using sensitive methods from a well-defined patient group that is representative of radiologic pneumonias. Multiple assays have provided several lines of supporting evidence, and 2 laboratories analyzed the raw clinical specimens, strengthening the findings. The predominance of *S. pneumoniae* along with its serotype distribution is supported by a high level of concordance between independent laboratories [19]. The study's weaknesses are that the best possible specimens are still subject to sampling error, that the study is relatively small, and that the identification of potential pathogens does not in itself confirm that these are the causative agents, a fundamental challenge for the field. Nevertheless, if there are any clinical samples for which pathogen detection alone is sufficient to assign causation, then these are lung and pleural aspirates.

The findings of this study emphasize the importance of bacteria, prominently *S. pneumoniae*, as a cause of severe pneumonia, and the potential for current PCVs to reduce the burden of this disease. This study also confirms that molecular methods are able to detect potential pathogens far more readily than culture and have a role in defining the etiology of pneumonia. The challenge of accurately assigning causation to the pathogens detected remains, particularly when using more generally available specimen types, and this will require the additional development of robust biomarkers of pathogen-specific disease.

Supplementary Data

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

Notes

References

1. Murdoch DR, et al. Breathing new life into pneumonia diagnostics. J Clin Microbiol **2009**; 47:3405–8.
2. Adegbola RA, et al. The etiology of pneumonia in malnourished and well-nourished Gambian children. Pediatr Infect Dis J **1994**; 13:975–82.
3. Scott J, Hall A. The value and complications of percutaneous thoracic lung aspiration for etiologic diagnosis of community-acquired pneumonia. Chest **1999**; 116:1716–32.
4. Levine OS, et al. The Pneumonia Etiology Research for Child Health Project: a 21st century childhood pneumonia etiology study. Clin Infect Dis **2012**; 54(suppl 2):S93–101.
5. Ideh R, et al. Use of percutaneous transthoracic lung aspiration for the etiologic diagnosis of pneumonia: a 25 year experience from The Gambia. Int J Tuberc Lung Dis **2010**; 15:729–35.
6. Brito DA, Ramirez M, de Lencastre H. Serotyping *Streptococcus pneumoniae* by multiplex PCR. J Clin Microbiol **2003**; 41:2378–84.
7. Chakravorty S, et al. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods **2007**; 69:330–9.
8. Enright MC, Spratt BG. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology **1998**; 144(pt 11):3049–60.
9. Meats E, et al. Characterization of encapsulated and noncapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. J Clin Microbiol **2003**; 41:1623–36.
10. Morris G, et al. A novel *Campylobacter jejuni* sequence type from a culture-negative patient in The Gambia. PLoS One **2008**; 3:e1773. doi:10.1371/journal.pone.0001773.
11. Nadkarni M, et al. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology **2002**; 148:257–66.
12. Smith-Vaughan H, et al. Measuring nasal bacterial load and its association with otitis media. BMC Ear Nose Throat Disord **2006**; 10:10.
13. Tokarz R, et al. Longitudinal molecular microbial analysis of influenza-like illness in New York City, May 2009 through May 2010. Virol J **2011**; 8:288.
14. Briese T, et al. Diagnostic system for rapid and sensitive differential detection of pathogens. Emerg Infect Dis **2005**; 11:310–3.
15. Rudan I, et al. Epidemiology and etiology of childhood pneumonia. Bull World Health Organ **2008**; 86:408–16.
16. Carrol ED, et al. PCR improves diagnostic yield from lung aspiration in Malawian children with radiologically confirmed pneumonia. PLoS One **2011**; 6:e21042.
17. Shann F, et al. Aetiology of pneumonia in children in Goroka Hospital, Papua New Guinea. Lancet **1984**; 324:537–41.
18. Lozano R, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet **2012**; 380:2095–128.
19. Tokarz R, et al. Serotype analysis of *Streptococcus pneumoniae* in lung and nasopharyngeal aspirates from children in The Gambia by MassTag PCR. J Clin Microbiol **2013**; 51:995–7.