

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,*}, Gerard A.J. Morris^{1,*}, Rafal Tokarz², Bernard E. Ebruke¹, Eunice M. Machuka¹, Readon C. Ideh¹, Osaretin Chimah¹, Ousman Secka¹, John Townend¹, Michel Dione¹, Claire Oluwalana¹, Malick Njie³, Mariatou Jallow³, Philip C. Hill⁴, Martin Antonio¹, Brian Greenwood⁵, Thomas Briese², Kim Mulholland⁵, Tumani Corrah¹, W. Ian Lipkin², Richard A. Adegbola^{1,6}

¹Medical Research Council Unit, Fajara, The Gambia

²Center for Infection and Immunity, Columbia University, New York, USA

³Ministry of Health and Social Welfare, Republic of The Gambia

⁴Centre for International Health, University of Otago, Dunedin, New Zealand

⁵London School of Hygiene & Tropical Medicine, London, UK

⁶GlaxoSmithKline Vaccines, Wavre, Belgium

Corresponding author: Dr Stephen R. C. Howie, Medical Research Council Unit, The Gambia; PO Box 273, Banjul, The Gambia, West Africa. Email: showie@mrc.gm, Ph/Fax: +220 4494438

*Stephen R.C.Howie and Gerard A.J. Morris contributed equally to this manuscript

Alternative contact: Prof Richard A Adegbola, Rue Fleming 20, 1300 Wavre, Belgium; tel:+32474880406 email: radegbola@hotmail.com

Abstract

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

Introduction

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 etiology 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 an HIV prevalence of less than 2%. A study of the etiology of severe childhood pneumonia was undertaken in the coastal area of The Gambia (Appendix 1). At the time of the study (2007-2009) there was high coverage with conjugate Hib vaccine but no routine usage of pneumococcal conjugate vaccine, 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

(RVTH), and the major health centers at Fajikunda, Serekunda, and Brikama. Children with a cough of ≥ 2 weeks, or severe anemia ($Hb < 6\text{g/dL}$) or confirmed wheeze were excluded. Radiological pneumonia was defined using WHO criteria ('endpoint pneumonia' and '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 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, no contraindications, and written informed consent had been obtained. Pleural aspiration was undertaken in those with pleural effusions.

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

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

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), one participant undergoing two aspirates at different time points (Appendix 4). HIV testing was done in 33/55 (60%) participants, two of whom were positive for HIV 1. The characteristics of those who underwent lung or pleural aspiration were similar to the radiological pneumonia group from which they were drawn (Appendix 5).

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

S. pneumoniae was detected by one or more molecular assays (Appendix 6) in 48/53 (91%) samples and in two or more assays in 36/53 (68%). Molecular pneumococcal serotyping (40 serotypes, Appendix 7) showed the following prevalences: serotype-1 22%, serotype-4 18%, serotype-14 18%, serotype-5 16%, serotype -6A/B/C 4%, serotype -9V/A 2%. Ten and 13-valent conjugate pneumococcal vaccines both include serotypes that account for 76-80% of serotypes identified in the samples in this study.

H. influenzae was detected in 12/53 (23%) samples. Four of the 12 had sufficient deoxyribonucleic acid (DNA) for full MLST typing, 3 being non-typeable *H. influenzae* (NTHi), while one 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, one being in a culture-positive case confirmed as being non-type b on latex agglutination. *Staphylococcus aureus* was detected in 3/53 (6%) samples, all of which were pleural fluid specimens. One or more other bacteria were identified to genus or species level in 8/53 (15%) samples. Viruses, led by Respiratory Syncytial Virus (RSV), adenovirus and bocavirus, were detected by one or more methods in 10/53 (19%) samples.

Half (28/53, 53%) of samples had more than one organism detected to genus or species level, predominantly two or more bacterial species. Co-detection of *S. pneumoniae* and *H. influenzae* occurred in 11/53 (21%) samples, *H. influenzae* having the higher bacterial load in 4/11. In the three specimens positive for *S. aureus* low loads of *S. pneumoniae* were detected in two 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 one sample (98%), in contrast to the use of culture alone, which yielded an organism in 38% of specimens. *S. pneumoniae* and *H. influenzae*, predominantly NTHi, were detected together in around 1 in 5 cases in this Hib vaccinated population. Current pneumococcal conjugate vaccines 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 *H. influenzae* type b (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 conjugate pneumococcal vaccines have not yet been introduced. The multi-country 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 two laboratories analysed 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 emphasise the importance of bacteria, prominently *S. pneumoniae*, as a cause of severe pneumonia, and the potential for current conjugate pneumococcal vaccines 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 using more generally available specimen types, and this will require the additional development of robust biomarkers of pathogen-specific disease.

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Conflict of Interest

RAA is an employee of GlaxoSmithKline Vaccines in Belgium and received previous grant awards for studies of bacterial diseases whilst working as an employee of the MRC Unit, The

Gambia. This does not alter the authors' adherence to CID's policies on sharing data and materials. No other conflicts of interest, real or perceived, are declared.

Author contributions

SH: Proposed and led the study; designed epidemiological and clinical aspects; contributed to interpretation; completed first draft of paper, finalised paper. GM: Led Gambian-based molecular analysis design, and conduct; contributed to interpretation; contributed to first draft of paper. RT: Undertook laboratory analyses at Columbia University and contributed to interpretation. BE, RI, OC, CO: collected lung and pleural aspirates and clinical data from participants. RA: Contributed to the conception of the study, led conventional microbiological aspects and contributed to study oversight and interpretation of data. MA, OS, EM, MD: contributed to laboratory design, conduct or interpretation. JT: contributed to data analysis and interpretation; WIL, TB oversaw and contributed to analyses at Columbia University and contributed to interpretation; KM, BG, PH, TC: contributed to design and interpretation. MJ and MN contributed to the design and conduct of the study. All authors contributed to critical review of the manuscript.

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Table. Organisms identified to at least genus level detected in 53 lung aspirate and pleural aspirate specimens examined by both culture and molecular assays obtained from 52 children with severe pneumonia. Table references are cited in parentheses.

Organism	N	(%)
Common recognised community-acquired pneumonia pathogens (1,2)		
<i>S. pneumoniae</i>	48	91
<i>H. influenzae</i>	12	23
<i>S. aureus</i>	3	6
Klebsiella species	2	4
<i>Streptococcus</i> species (non- <i>pneumoniae</i>)	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		
<i>Acinetobacter</i> species (3)	3	6
<i>Enterobacter</i> species (3)	2	4
<i>Salmonella</i> species (4)	2	4
<i>Streptococcus pseudopneumoniae</i> (5)	1	2
<i>Bacteroides</i> species (6)	1	2
<i>Prevotella</i> species (6)	1	2
Co-detection		
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 co-detection	21	40
Bacterial-Viral co-detection	10	19
<i>S. pneumoniae</i> and <i>H. influenzae</i>	11	19
<i>S. pneumoniae</i> and <i>S. aureus</i>	1	2
<i>S. aureus</i> and <i>H. influenzae</i>	1	2

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